USE OF PEPTIDE-MHC II-SPECIFIC-CHIMERIC ANTIGEN RECEPTOR TREGS TO REGULATE AUTOIMMUNITY IN TYPE 1 DIABETES

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

Use of Peptide-MHC II-Specific-Chimeric Antigen Receptor Tregs To Regulate Autoimmunity in Type 1 Diabetes

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Abstract

In type 1 diabetes (T1D), autoreactive T cells mediate the destruction of insulin-producing islet beta cells, leading to an inability to regulate blood glucose. If the patient cannot control their disease, the prolonged insulin deficiency will cause chronic hyperglycaemia, which may result in blindness and organ failure. Autoreactive T cells initiate pathogenesis upon recognition of islet-derived peptides presented by major histocompatibility complexes (MHC). In healthy individuals, these autoimmune reactions are normally suppressed by regulatory T cells (Tregs), but people with T1D are thought to have dysfunctional Tregs.

Potential therapeutic strategies for T1D designed to minimize autoimmunity include pharmacological T cell inhibition and/or infusion of polyclonal Tregs. However, these strategies are non-specific and may have limited effects on beta-cell-directed autoimmunity. Research using the non-obese diabetic (NOD) mouse model has shown cell therapy with Tregs bearing a transgenic T cell receptor (TCR) specific towards an islet-derived antigen could be superior to prevent and/or reverse T1D. However, limitations of TCR-engineered Tregs include mispairing with the chains of the endogenous TCR and potential cross-reactivity with other peptides.

I sought to overcome these limitations by engineering Tregs utilizing chimeric antigen receptor (CAR) technology. Advantages of CARs include high-affinity antibody-based interactions and self-contained co-stimulation. I studied two CARs in the NOD mouse model: the 1B2 CAR (specific towards insulin $B_{10\text{-}23}$ complexed to MHC Class II I-A^{g7}) and the FS1 CAR (specific towards p63:I-A^{g7}). I validated CAR specificity by testing their binding capacity to peptide:I-A^{g7} tetramers. Proliferation and suppression assays were conducted to compare the function of CAR Tregs versus polyclonal Tregs. I found when IB2 or FS1 CAR Tregs were cultured with splenocytes pulsed with relevant peptide, they proliferated and upregulated

activation markers. FS1 CAR Tregs mediated enhanced suppression of T cell proliferation, and both 1B2 and FS1 CAR Tregs suppressed cytokine production more efficiently than polyclonal Tregs. Together, these proof-of-concept data show that T1D-peptide-MHC II-specific CARs can be used to re-direct the specificity of Tregs. These data set the stage for future testing in *in vivo* models of T1D and the development of similar therapeutic strategies for use in people with T1D.

Lay Summary

In type 1 diabetes (T1D), the insulin-producing beta cells are killed by the patient's own immune cells. If the patient cannot manage their disease, the insulin deficiency may result in blindness or organ failure. In healthy individuals, regulatory T cells (Tregs) suppress this autoimmune attack, but T1D patients may have dysfunctional Tregs. A potential therapy is to treat T1D by infusing additional Tregs. Thus, I engineered Tregs with enhanced specificity towards autoimmune cells as a potential treatment. I hypothesized that Tregs engineered to specifically suppress autoimmune responses will be more effective than unmodified Tregs. Antigen-specific Tregs cultured with target cells could proliferate, unlike unmodified Tregs. Antigen-specific Tregs were also more suppressive after culture with autoimmune cells. Overall, antigen-specific Tregs are a promising source of cells for Treg therapy, because their specificity makes them more effective than unmodified Tregs.

Preface

The work presented in this thesis has yet to be published. Due to the use of mouse models in this thesis, the study has been approved by the UBC Animal Care Committee with the animal care application ID A16-0149 and A20-0017. I analyzed all the flow cytometry data using FlowJo Software versions 10.1.6 and 10.7.1, and performed the subsequent statistical analyses using GraphPad Prism Software Version 7.

Generation of islet-peptide:I-A^{g7} MHC Class II CARs and CAR Tregs

CAR generation was done in collaboration with the Fife lab from the University of Minnesota, and the Verchere lab from the University of British Columbia. The Fife lab generated the monoclonal antibodies and provided the Levings lab with the DNA sequence. Dr. Paul Orban converted the antibody into scFvs compatible with CAR format, and Dr. Majid Mojibian and I worked to express the CAR on HEK 293T cells and confirmed expression by flow cytometry. With training from Dr. Mojibian, I was responsible for harvesting, isolating, and sorting Tregs from NOD mice. I also optimized the retrovirus transduction protocol to generate CAR Tregs and designed the flow cytometry panels required to phenotype the cells for CAR expression and Treg purity. To assess CAR specificity, the Fife lab provided monomers which I conjugated to fluorophores to generate the peptide:I-A^{g7} tetramers.

Performing Functional Assays to Test CAR Tregs *in vitro*

I optimized the *in vitro* functional assays and designed the flow cytometry panels to assess for antigen dependent cell activation, proliferation, and suppression. I was responsible for harvesting and isolating splenocytes from NOD mice, and the amino acid sequences required for generating the peptides used for splenocyte pulsing were provided by the Fife lab. I also harvested and isolated T cells from BDC2.5 mice for use as effector T cells in the suppression assays.

With training from May Wong, I measured the cytokine production in suppression assay supernatants to assess for CAR Treg mediated cytokine suppression.

Monitoring T1D development in NSG mice following CAR Tconv injection

With help from Dr. Mojibian and Christine Wardell, we worked together to develop the *in vivo* CAR Tconv model in NSG mice. Dr. Mojibian was responsible for cell injection, and Christine and I worked together to monitor mice body condition, blood glucose, and take blood for weekly phenotyping. We also optimized the flow cytometry panel used to phenotype the blood. At endpoint, Dr. Mojibian, Christine, and I worked together to euthanize the mice, collect the necessary organs for histology, and process the spleens and pancreas to phenotype by flow cytometry.

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List of Abbreviations

AIRE	Autoimmune Regulator
APC	Antigen Presenting Cell
BDC	Barbara Davis Center
CAR	Chimeric Antigen Receptor
CD	Cluster of Differentiation
CDI	Cell Division Index
Chga	Chromogranin A
CPD	Cell Proliferation Dye
cTEC	Cortical Thymic Epithelial Cell
CTLA-4	Cytotoxic T Lymphocyte-Associated Protein 4
DAISY	Diabetes Autoimmunity Study in the Young
DC	Dendritic Cell
EAE	Experimental Autoimmune Encephalomyelitis
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
Fc	Fragment Crystallizable
FoxP3	Forkhead Box Protein 3
GAD	Glutamic Acid Decarboxylase
GFP	Green Fluorescent Protein
GITR	Glucocorticoid-Induced Tumor Necrosis Factor Receptor
GVHD	Graft Versus Host Disease
HEK	Human Embryonic Kidney
HEL	Hen Egg Lysozyme
Her2	Human Epidermal Growth Factor Receptor 2
HLA	Human Leukocyte Antigen
HP	Hybrid Peptide

IA-2	Islet Tyrosine Phosphatase 2
IAA	Insulin Autoantibody
Idd	Insulin-Dependent Diabetes
IDO	Indoleamine 2,3-Dioxygenase
IFN-γ	Interferon Gamma
IL	Interleukin
INS	Insulin
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked
LAP	Latency Associated Peptide
mAb	Monoclonal Antibody
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MSCV	Murine Stem Cell Virus
mTEC	Medullary Thymic Epithelial Cell
NOD	Non-Obese Diabetic
NSG	Non-Obese Diabetic Severe Combined Immunodeficiency Gamma
PBMC	Peripheral Blood Mononuclear Cell
PE	Phycoerythrin
Plat-E	Platinum-E
Rad	Radians
RBC	Red Blood Cell
scFv	Single Chain Variable Fragment
T1D	Type 1 Diabetes
Tconv	Conventional T cell
TCR	T Cell Receptor
TEDDY	The Environmental Determinants of Diabetes in the Young
TGF-β	Transforming Growth Factor Beta

TIL	Tumour Infiltrating Lymphocyte
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TNFR2	Tumor Necrosis Factor Receptor 2
TNF-α	Tumor Necrosis Factor Alpha
Treg	Regulatory T cell
TSA	Tissue Specific Antigen
Znt8	Zinc Transporter 8

Acknowledgements

I would like to thank my supervisor Dr. Megan Levings for providing me with this amazing opportunity at the Levings Lab. I am truly thankful for her guidance and encouragement regarding not only my thesis project, but in my personal growth as a scientist. I also would like to thank my committee members, Dr. Bruce Verchere and Dr. Kevin Hay for their support and expertise. Special thanks are awarded to Dr. Majid Mojibian who taught me all the techniques and methods necessary for my project. From the mouse work to cell culture, I would not have been able to dive into my work so smoothly without the solid foundation you provided me.

This thesis would also not have been possible without the help of my University of Minnesota collaborators, Dr. Brian Fife and Dr. Justin Spanier. Thank you so much for all of your insights and tips to best tailor my experiments. Emma McIver, Christine Wardell, and Avery Lam, thank you for your support emotionally and physically throughout this entire process. I greatly treasure our friendship and the amount of coffees and snacks I owe you all are endless. Dr. Isaac Rosado Sanchez and Madeleine Speck, your guidance and mentorship have been invaluable and working so closely with you both has been a joy. Dr. Dominic Boardman, May Wong, Dr. Paul Orban, Dr. Manjurul Haque, Dr. Mahdis Monajemi, Dr. Kirsten Ward-Harstonge, Dr. Sabine Ivison, Rosa Garcia, Jana Gillies, Dr. Jessica Qing Huang, Katie MacDonald, and Chen Li, I would like to thank you all for your encouragement and kindness at the Levings Lab.

Finally, I would like to recognize my family for their boundless love and support. I am forever grateful for my parents and my sister.

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Chapter 1: Introduction

1.1 An Introduction to Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune disease affecting over 500,000 children worldwide [1]. The incidence rates of T1D in children are increasing, with over 90,000 children under the age of 15 diagnosed each year [2]. Canada is particularly affected, as the country has been ranked 6th for highest T1D incidence since 2019 [3].

T1D occurs when the insulin-producing beta cells of the pancreatic islets are destroyed by the patient's own immune system. Insulin is an important hormone for life as it facilitates lipid, carbohydrate, and protein metabolism by promoting cellular uptake of glucose from blood [4]. This autoimmune attack is primarily mediated by immune cells known as T cells: where $CD8^+$ T cells are responsible for the majority of beta cell destruction, and the $CD4^+$ T cells initiate and drive progression of the disease [1, 5-6]. If left untreated, the beta cell mass in the islets will continue to decline and ultimately result in hyperglycaemia resulting from insulin deficiency. Chronic hyperglycaemia, where blood glucose remains consistently high (> 7 mmol/L), can lead to many long-term and life-threatening complications, such as blindness, heart disease, and organ failure [1,4,6].

T1D is a multi-stage disease, with each stage defined by clinical symptoms and/or immune-related events. The first stage begins when the autoimmune responses towards the beta cells develop. During the initial stages of immune attack, the patient will not have any overt symptoms and will still present with normal blood glucose levels [7–9]. Patients at this stage will also have a fasting blood glucose of below 5.6 mmol/L. As the number of beta cells continue to decline, the patient will enter stage 2 where they will begin presenting with slightly elevated blood glucose levels [9], but will still appear healthy and without symptoms. During these pre-

diabetic stages, most patients are still unaware of their disease status. Symptomatic T1D (stage 3) occurs after the majority of functional beta cell mass has been lost [8,10,11], and patients will begin to experience weight loss, excessive thirst, and polyuria [9]. At this stage, their fasting blood glucose will exceed 7.0 mmol/L and patients will be diagnosed with clinical T1D.

Children with known familial histories of T1D can be screened for disease development by detecting the presence of islet autoantibodies. Antibodies are proteins produced by activated B cells and normally target and bind to foreign bodies, such as bacteria or viruses; however, in T1D, antibodies targeting islet-derived proteins become detectable in the blood [12]. The Diabetes Autoimmunity Study in the Young (DAISY) study found that children with particularly high genetic risk will likely develop their first islet autoantibody within their first two years of life [13]. Common islet autoantibodies detected during T1D development include the insulin autoantibody (IAA), glutamic acid decarboxylase 65 (GAD65), zinc transporter 8 (ZnT8), and islet tyrosine phosphatase 2 (IA-2).

While these islet autoantibodies are believed to be non-pathogenic [1,12,14], some mouse studies have implicated B cells to play an important role in T1D pathogenesis, likely due to their function as antigen presenting cells [14,15]. Nevertheless, these islet autoantibodies have become critical biomarkers to monitor a patient's progression through the stages of T1D. Even during the pre-diabetes stages, two or more autoantibodies can often be detected in patient blood serum, with the first islet autoantibodies typically targeting insulin and/or GAD65 [1]. The risk of diabetes stage progression increases when patients present with beta cell autoantibodies within the first 3 years of life [16,17], and/or with persistently high autoantibody titre [12]. The number of different autoantibodies is another risk factor defining how quickly a patient will develop clinical diabetes. According to The Environmental Determinants of Diabetes in the Young (TEDDY) study, the

likelihood a child will progress to stage 3 T1D within 5 years is almost 50% if they carry 3 different types of beta cell autoantibodies [18].

1.2 Genetic Susceptibility of Type 1 Diabetes

While the cause of T1D still remains unknown, both genetic and environmental factors play a role in determining whether someone will develop the disease. The largest risk factor for T1D development is a person's Human Leukocyte Antigen (HLA) Class II haplotype. According to the Type 1 Diabetes Genetics Consortium genome-wide association study, the Major Histocompatibility Complex (MHC) DNA region encoding the HLA genes contributes over 50% of the genetic risk to developing T1D [19–21].

MHC molecules are proteins expressed on cell surfaces that present peptides to T cells to mediate immune responses. In humans, HLA Class I molecules (HLA-A, B, C) are expressed on almost all cell types and present endogenous peptides, whereas HLA Class II molecules (HLA-DP, DR, DQ, DM, and DO) are primarily expressed on specialized cells known as Antigen Presenting Cells (APCs) and present peptides derived from exogenous proteins [22,23]. The HLA genes are the most polymorphic in the human genome [22,24], resulting in a diverse catalogue of HLA alleles, also known as haplotypes. The HLA class II haplotypes HLA-DR3-DQ2 and HLA-DR4-DQ8 are predicted to be the largest risk factor in developing T1D [22,25–27]. Indeed, 55% and 70% of T1D patients have been found to carry the DR3-DQ2 and DR4-DQ8 haplotypes, respectively [28]. The HLA-DR and HLA-DQ susceptible haplotypes have also been associated with islet autoantibody development. Children with the HLA-DR4-DQ8 haplotype are more likely to develop insulin autoantibodies first [1], whereas those with the HLA-DR3-DQ2 haplotype will typically present with GAD65 autoantibodies [1,29].

The non-HLA genes individually contribute to the remaining genetic risk of T1D development. Using genome-wide association studies, over 50 T1D susceptibility loci/genes [20,30] have been associated with T1D development. Many of these genes are related to the immune system, particularly in areas that involve immune tolerance. Immunological tolerance occurs when the immune system works to remain unresponsive, or "tolerant", to specific tissues or substances. When the processes that work to maintain immune tolerance are perturbed, this will typically result in the development of autoimmune diseases. One of these genes is *IL2RA* [20,31], which encodes for the cell surface protein CD25 and is part of the high affinity receptor for IL-2. IL-2 is a key survival signal for regulatory T cells (Tregs), which play a crucial role in mediating immune tolerance and homeostasis. *CTLA-4* and *IL-10* have also been identified as some of the many T1D susceptibility genes [8], and both encode for proteins that play major parts in suppressing inappropriate immune responses.

1.3 Autoimmunity is a Failure of Central Tolerance

Autoimmune T cells arise from a defect in a mechanism called central tolerance, which occurs in the thymus. T cell progenitors from the bone marrow migrate to the thymus, where a subset of those cells will eventually develop into mature $CD4^+$ or $CD8^+$ T cells. However, these immature progenitors must first pass a series of checkpoints before being allowed out into the periphery. As immature T cell progenitors arrive in the thymus, their TCR genes undergo somatic recombination to result in unique TCRs with a wide array of target specificities and binding affinities [32]. At this point in their development, the progenitors express both CD4 and CD8 as they have yet to commit to a single positive lineage. In the cortex of the thymus, specialized cells called cortical thymic epithelial cells (cTECs) express self-peptide-MHC

molecules for the progenitors' TCRs to bind to. If a TCR does not bind, or binds too strongly, the progenitor will be deleted from the thymus by apoptosis [32,33].

Progenitors that pass the first checkpoint express TCRs that bind to MHC molecules with low affinity, and depending on whether the bound MHC molecules were Class I or Class II, the progenitors will commit to either the single $CD8^+$ or $CD4^+$ lineage, respectively [32,33]. The single positive progenitors will then migrate to the medulla, where they will encounter their second checkpoint. Medullary thymic epithelial cells (mTECs) are highly specialized APCs and are critical in eliminating any potential autoimmune progenitors from the final T cell pool. mTECs are unique to the other thymic APCs as they express a nuclear protein called autoimmune regulator (AIRE) [34,35]. This allows for mTECs to express proteins found in other non-thymic cell types of the body and present these peptides onto their MHC molecules. The single positive T cell progenitors are now able to encounter tissue specific antigens (TSAs) without leaving the thymus [32,33]. Again, progenitors with TCRs that bind to presented TSAs with high affinity will be deleted, as they would have resulted in autoreactive T cells; however, a special subset of these $CD4^+$ cells will be spared, provided they also express the nuclear transcription factor FoxP3 [36]. These $CD4^+FoxP3^+$ cells will give rise to the Treg population, which require high affinity TCRs towards TSAs to be able to migrate and protect tissues from immune related attacks.

1.4 Islet Beta Cell Pathogenesis by Autoimmune T cells

Despite central tolerance in the thymus working to delete autoreactive T cell populations, a small population of autoimmune T cells will be successful in bypassing the checkpoints to escape into the periphery. It has been found that polymorphisms in the *INS* gene, which encodes for the insulin protein, influence an individual's genetic risk towards developing T1D. As

previously explained, AIRE allows for mTECs to express various proteins normally found on other cell types outside of the thymus. Therefore, even though insulin production is restricted to beta cells in the pancreas, CD4 and CD8 progenitor T cells can still be screened for TCR reactivity against insulin peptides via AIRE expression. Studies using genome sequencing have found many patients with T1D carry polymorphisms in their *INS* gene [7,37]. These *INS* mutations have been linked to poor expression of insulin by mTECs in the thymus [38,39], resulting in subpar T cell screening and insulin-reactive T cells being allowed to escape. Certain *INS* polymorphisms have also been linked to an inefficiency in generating islet specific Tregs [40], therefore resulting in less Tregs homing to the pancreas to suppress the autoimmunity.

The pathogenesis of T1D begins when beta cell-specific T cells infiltrate pancreatic islets to destroy the beta cells in a process known as insulitis. Under normal conditions, beta cells produce a variety of proteins, such as insulin and GAD [41], which are regularly picked up by the resident pancreatic APCs. The proteins are then processed into peptides and presented on their MHC Class II molecules. In healthy individuals, this presentation of self peptides is unproblematic, as a functional central tolerance and other protective mechanisms in the periphery (such as Tregs) will prevent beta cell-autoreactive T cells from mounting an immune response. However, in patients with T1D, a failure in at least one of these mechanisms will allow for inappropriate T cell activation and subsequent immune cell infiltration. The activated beta-cell-targeting $CD4^+$ T cells will activate and recruit cytotoxic $CD8^+$ T cells and pro-inflammatory macrophages [32,37,38,40], where both macrophages [42] and $CD8^+$ T cells [43,44] work to directly kill the beta cells. Furthermore, interferon production by infiltrating T cells drive the islet cells to overexpress HLA Class I molecules [45], unfortunately increasing their susceptibility to cytotoxic attack.

1.5 Regulatory T Cells (Tregs) Protect Against Autoimmunity

Regulatory T cells (Tregs) are critical for health by preventing the development of autoimmune diseases [46]. The importance of Tregs in maintaining immune homeostasis can be demonstrated by infants with Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) Syndrome. These patients develop a multitude of autoimmune diseases involving multiple organs early in life, such as severe autoimmune enteropathy and T1D [47], and can be fatal within the first year of life without medical intervention. Patients with IPEX all have mutations within the *FOXP3* gene locus, the master transcription factor responsible for proper Treg development [47,48].

While the majority of autoreactive T cells are deleted by central tolerance, a small proportion of the autoreactive T cells are able to escape thymic negative selection [49,50]. As central tolerance primarily focuses on deleting T cells expressing TCRs with strong avidity towards self-peptide-MHC complexes, this allows for T cells with relatively low avidity towards self-peptides to evade detection and escape into the periphery [49,51]. While these cells have the potential to instigate autoimmune responses, they will often be swiftly stopped by the secondary tolerance mechanism, peripheral tolerance.

Tregs are known to be one of the key mediators of peripheral tolerance, as they suppress inappropriate effector T cell responses through a variety of mechanisms. These mechanisms can be either be cell contact-dependent or -independent [52], and allow Tregs to inhibit T cell activation and proliferation, as well as induce apoptosis [53,54]. Tregs can suppress inflammation by producing suppressive cytokines, such as IL-10 and TGF-β [52,55]. IL-10 mediates immune suppression by dampening antigen presentation and co-stimulation on APCs by downregulating their MHC Class II expression [56] and co-stimulatory molecule CD40 expression [57]. IL-10

signalling in effector T cells has also been found to limit their activation and proliferation potential, as well as suppress the production of pro-inflammatory T helper 1 cytokines, IFN-γ and TNF-α [56,58,59]. The potent anti-inflammatory activities of IL-10 have been demonstrated in numerous mouse models [60–63]. For instance, blocking Treg production of IL-10 prevented Tregs from protecting mice against developing severe colitis [61], and the presence of IL-10$^+$ Tregs in the central nervous system of mice have been linked to better protection against experimental autoimmune encephalomyelitis (EAE) [60]. TGF-β also inhibits the proliferation and function of activated effector T cells [55,64,65], and mice born with a TGF-β1 deficiency develop T cell mediated autoimmunity within the first weeks of life [52,63]. Furthermore, TGF-β plays an important role in Treg generation by promoting the expression of FoxP3 [64], and thus has been used to successfully convert effector T cells into CD4$^+$FoxP3$^+$ Treg cells [66,67] with suppressive function *in vitro* [68].

In addition to utilizing inhibitory cytokines, Tregs can suppress effector T cell growth through metabolic disruption by depriving neighbouring T cells of cytokines. IL-2 is an important cytokine for both effector T cells and Tregs, and is required to mediate long-term survival potential [69,70]. Effector T cells must be activated to upregulate the high affinity IL-2 receptor CD25, but Tregs naturally express CD25 even at resting state. It is hypothesized when Tregs congregate at a site of inflammation, they can quickly deplete the local area of IL-2 in order to induce IL-2 deprivation-mediated apoptosis in effector T cells [71]. Tregs can also induce apoptosis by secreting granzyme A, granzyme B, and perforin [55,72]. Upon interaction with their effector T cell targets, activated human Tregs have been shown to direct the release of granules containing granzymes A and B, and perforin into the extracellular space between the Treg and T

cell [73–75]. The perforin molecules will create pores in the target cell membrane and once inside the cell, the granzymes can induce apoptosis in a caspase-dependent or independent manner.

Tregs can also prevent T cell activation by targeting and suppressing APCs, such as dendritic cells (DCs), by expression of classical T cell inhibitory molecules. Tregs constitutively express CTLA-4, a T cell checkpoint inhibitor [76], and CTLA-4 expression has been shown to be critical in preventing spontaneous autoimmune development in mice [77,78]. CTLA-4 has greater affinity for the co-stimulatory molecules CD80 and CD86 on APCs [79], thereby being able to outcompete the T cell co-stimulation protein CD28 for receptor-ligand binding. Thus, Tregs are hypothesized to outcompete effector T cells for APC binding to slow T cell activation [80]. CTLA-4 binding can also induce downregulation of CD80 and CD86 on DCs [81] and has been shown to condition DCs to express a more suppressive phenotype. Following CTLA-4 binding to CD80/CD86, DCs will begin to produce indoleamine 2,3-dioxygenase (IDO) [82,83], an immunosuppressive enzyme. IDO activity by DCs results in the production of highly pro-apoptotic metabolites that also suppress effector T cell growth.

1.6 Treg Dysfunction in Type 1 Diabetes

Using their various mechanisms to mediate immune suppression, Tregs have the potential to control islet-specific T cell activation and stop T1D progression. In fact, autoreactive T cells specific towards islet antigens can be found in the circulation of healthy, non-diabetic individuals [84]; however, Tregs from T1D patients have been shown to be dysfunctional and are less efficient in suppressing effector T cell responses. Tregs derived from patients with T1D have been shown to be less suppressive compared to their healthy Treg counterparts, as T1D Tregs were unable to suppress autologous effector T cell proliferation *in vitro* [85,86]. T1D Tregs also display an

abnormal cytokine profile, as seen in studies of children with T1D. A higher proportion of Tregs from these subjects were found to produce more pro-inflammatory cytokines, such as IL-12, IL-18, and IFN-γ [81,87], whereas Tregs taken from non-diabetic controls produced primarily anti-inflammatory cytokines, such as IL-10 and TGF-β [85,88].

In addition to poor functional responses, T1D Tregs also exhibit a defective phenotype. For instance, Tregs from T1D patients have less stable FoxP3 expression [89] and also express lower levels of GITR [90], a marker of functional Tregs, and high GITR expression is associated with long term Treg survival [91]. Accordingly, Tregs from patients in both the pre-diabetic and clinical diabetic stages have been found to be more prone to undergo apoptosis [92]. Furthermore, while the number of Tregs found in non-diabetic versus diabetic patients are reported to be similar [93], the frequency of activated FoxP3$^+$ Tregs were found to be reduced in T1D patients compared to healthy controls [94]. The inability of T1D Tregs to convert from a resting to activated state has been attributed to a defect in TNFR2 signaling [94].

1.7 Adoptive Treg cell therapy to treat Type 1 Diabetes

Currently, the only widely available treatment for patients living with T1D is insulin therapy to help regulate their blood sugar levels. Pancreas or islet cell transplantations are also viable approaches to manage T1D, but patients must take a regimen of immunosuppressive drugs to prevent graft rejection. Nevertheless, none of these treatments address the fundamental autoimmunity underlying T1D. Even with successful transplantation, the patient will still have an imbalanced immune system containing beta cell-specific effector T cells and defective Tregs that are unable to protect the new graft.

New strategies devised to address beta cell autoimmunity have revolved around restoring the immune balance by either targeting the autoreactive effector T cells or by strengthening immune regulation by enhancing Tregs. One such therapy to sway the immune system towards a more anti-inflammatory phenotype is adoptive Treg cell therapy, where autologous Tregs are transferred to increase Treg frequency in the circulation. Tregs are taken from the blood of patients and FoxP3^{high} Tregs can be isolated by sorting the CD4^{+}CD25^{high}CD127^{low} surface phenotype [88]. The purified Tregs can then be expanded until viable numbers are reached for cell infusion back into the patients.

In 2012, the first clinical trial involving adoptive transfer of *ex vivo* expanded, polyclonal Tregs was completed in children with T1D [95]. Shortly following administration, Treg frequency significantly increased, and the majority of subjects experienced a decrease in insulin dependency up to one year post infusion [96]. A second clinical trial in 2015 assessed the safety of adoptive Treg transfer therapy in adult T1D patients and observed similar results. Transferred Tregs could still be found in blood after one-year post infusion [97], and the Tregs also retained their FOXP3^{+}CD4^{+}CD25^{high}CD127^{low} phenotype. Furthermore, many subjects still expressed detectable levels of C-peptide, a biomarker of insulin production, even after two years post cell transfer [97].

1.8 Antigen-Specific Tregs May be Superior in Treating Autoimmunity

The clinical trials using polyclonal, autologous Tregs have demonstrated that adoptive Treg transfer is both feasible and safe as a potential T1D treatment therapy. In fact, in the non-obese diabetic (NOD) mouse model, infusions of 10 x 10^{6} polyclonal NOD Tregs were successful in reversing T1D onset [98,99]. However, there are some concerns regarding the use of

polyclonal Tregs in adoptive cell transfer therapies in humans. If 10 x 10^6 polyclonal Tregs are required to reverse diabetes in mice, then it is likely billions of polyclonal Tregs would be required for infusion when this therapy is translated into human patients [98]. Given that polyclonal Tregs contain cells with varying specificities, the large numbers of cells needed for infusion could carry the risk of generalized immunosuppression. Furthermore, their lack of specificity would also mean only a small minority of infused polyclonal Tregs would likely be able to migrate to the pancreatic islets to mediate immune suppression.

Studies using autoimmune and transplantation animal models have shown treating with antigen-specific Tregs can be 100 times more effective than polyclonal Tregs in achieving the same therapeutic effect [100]. The enhanced efficacy of antigen-specific Tregs is likely attributed to their enhanced longevity upon successful migration to target tissues. In a skin transplant humanized mouse model, Dawson *et al.* showed that Tregs bearing a chimeric antigen receptor (CAR) towards the MHC Class I molecule HLA-A*02 could migrate and persist in the HLA-A*02^+ skin graft for multiple weeks [101]. In comparison, polyclonal Tregs were undetectable after 3 days post injection into the mice. Therefore, perhaps the superior approach regarding Treg therapy to treating T1D may be to infuse Tregs specific towards beta cell-derived proteins, as fewer cells would be required for successful treatment. Indeed, while 10 x 10^6 polyclonal NOD Tregs could reverse T1D onset in NOD mice [98,99], infusion of as few as 4 x 10^4 Tregs bearing an islet-antigen specific T cell receptor (TCR) was sufficient in both preventing insulitis [99,102–104] and reversing disease [102,105].

As a result, there have been many trials attempting to bolster the antigen-specific Treg population pre-existing in T1D patients by utilizing a variety of novel antigen-specific interventions [106]. The most common autoantigen to be utilized as the therapeutic target is insulin,

and the use of insulin vaccinations to induce antigen specific tolerance have shown some promising results. In the Pre-POINT study, giving high doses of oral insulin to children at high risk for T1D had been shown to expand their insulin-specific Treg population [107]. Immunization using Insulin B chain nanoparticular emulsions in NOD mice induced Treg generation and could protect the mice from developing diabetes [108]. Meanwhile in human trials, Insulin B chain vaccinations also resulted in the expansion of $CD4^+FoxP3^+CD127^{low}$ T cells that produced TGF-β and IL-10 upon antigen stimulation [109], although improvement of beta cell function has yet to be seen.

Alternative approaches to generate antigen-specific Tregs that have yet to reach the clinic include the use of viral gene transfers and nanoparticles. In NOD mice, lentiviral vectors conferring expression of the insulin B_{9-23} epitope to hepatocytes were successful in inducing insulin-specific Tregs by exploiting the liver's natural tolerogenic properties [110]. When combined with low dose anti-CD3 therapy, the combination treatment resulted in T1D reversal. Nanoparticle based therapies have been tested in a variety of autoimmune models, including multiple sclerosis and T1D [106]. The nanoparticles are engineered to display peptide:MHC complexes at high densities and aims to convert autoreactive T cells into antigen specific Tregs. In NOD mice, delivery of NRP-V7-K^d nanoparticles expanded their autoregulatory T cell population, and the treatment could prevent and reverse disease by suppressing the antigen presentation of islet autoantigens in the pancreatic draining lymph node [111]. These results have also been replicated using human $GAD_{555–567(557I)}$-HLA-DR4 nanoparticles to generate $CD4^+$ Tregs from engrafted PBMCs in a humanized mouse model [112].

1.9 Engineering Artificial Antigen Specificity onto T cells

An alternative approach to generate antigen-specific Tregs would be to isolate and expand the cells *ex vivo*. Antigen-specific T cells can be isolated from a pool of polyclonal cells, then cloned and expanded to clinical numbers for infusion. However, Tregs are a fairly rare cell population, as they comprise of only 5-10% of all circulating $CD4^+$ T cells in peripheral blood [113]. Therefore, attempting to isolate naturally-occurring, antigen-specific Tregs will result in very low cell yields and will likely be very difficult to expand to clinically relevant numbers. The expansion will also be a long and costly process, and lengthy *in vitro* Treg expansions may increase the risk of the cells destabilizing towards a more pro-inflammatory, conventional T cell-like phenotype [114]. To circumvent this limitation, many groups have taken to artificially engineering antigen specificity onto polyclonal T cells by either modifying their TCR specificity, or by introducing expression of Chimeric Antigen Receptors (CARs).

Genetically modified TCRs arise from introducing the expression of different TCR α and β chains to alter antigen specificity. One common approach is to transfer the TCR genes of known antigen specificity into polyclonal T cells by virus transduction. This has been done in some cancer immunotherapies where TCRs from tumour infiltrating lymphocytes (TILs) of patients experiencing cancer remission are transferred onto T cells of patients with the same cancer [115,116]. In the context of T1D, generation of islet-specific Tregs has also been conducted by transferring the TCRs from IA-2 specific and insulin-specific T cell clones by lentiviral gene transfer [117]. The engineered Tregs were found to be suppressive *in vitro*, but their potency was noted to be significantly lower compared to viral specific TCRs and required additional methods to isolate the functional islet specific Tregs.

Another caveat to genetically modified TCRs would be their tendency to mispair the endogenous and engineered TCR components. While no clinical trials to date involving genetically modified TCRs have observed any toxicities attributed to TCR mispairing, the potential interchain mispairing may result in defective TCRs incapable of antigen binding, or TCRs with reduced or altered specificity [118]. This can lead to dangerous off-target effects, as effector T cells with mispaired TCRs may recognize self-antigens and could potentially cause graft-versus-host disease (GvHD) [119,120]. Indeed, in a study by Bendle *et al*, mice infused with genetically-modified-TCR transduced T cells experienced lethal GvHD driven by IFN-γ, and was attributed to the formation of self reactive TCRs from mispairing of endogenous and engineered TCR components [120]. Therefore, perhaps a safer and more efficient method of generating antigen-specific Tregs may be to utilize CAR technology instead.

CARs are recombinant molecules comprised of an antigen binding domain, a transmembrane domain, and an intracellular signalling domain which all work to modify antigen specificity and T cell function when expressed on the cell surface [121,122]. One major advantage of CARs compared to engineered TCRs is their modularity, as each domain can be replaced with new components to adjust antigen specificity and binding efficiency, or even optimize the biological activity of any CAR.

The antigen binding domain is responsible for tailoring antigen specificity and is typically derived from the single chain variable fragment (scFv) of a high affinity antibody [123]. The scFv is linked to the transmembrane and intracellular domains by a region known as the hinge. The hinge can enhance the general stability of CAR expression on the cell surface [122], as well as provide flexibility to the CAR to increase the scFv binding efficiency to complex targets [124,125]. The CAR transmembrane domain is responsible for anchoring the CAR molecules to the

T cell membrane [122] , and many groups have shown the transmembrane domain also helps facilitate CAR dimerization and immune synapse formation to amplify T cell signaling and activation [126,127].

Finally, the intracellular domain of a CAR consists of one or more T cell activation signalling domains. CARs can be categorized into first, second, or third generation CARs depending on the composition of the intracellular domains used. In a first-generation CAR, the sole intracellular domain responsible for T cell activation is the CD3ζ signaling domain. In a second-generation CAR, the signalling domain of a T cell co-stimulatory molecule is joined to the CD3ζ signaling domain. Common co-stimulatory domains used are from the CD28 and TNFR family [122], and depending on the co-stimulatory domain used, T cell activation by CAR stimulation could enhance T cell growth, influence T cell differentiation, as well as tailor biological function [128–130]. The third generation CARs utilize $\geq$ 2 co-stimulatory domains linked to the CD3ζ signaling domain to combine the functional properties of different co-stimulatory signals into a single CAR T cell. While third generation CARs are reported to have enhanced effector functions, the number of CAR molecules expressed on T cell surfaces is often lower compared to cells expressing first or second generation CARs [131].

1.10 Chimeric Antigen Receptor Expression on Tregs

Tregs require CD28 co-stimulation to mediate their expansion and maintain suppressive function [132] . When Tregs were expanded with artificial APCs that provided alternative co-stimulation, they expressed less FoxP3 and were unable to suppress effector cells *in vitro* as well as their CD28 expanded counterparts [132]. Therefore, the CARs described in this thesis will all be

second generation CARs with a CD28 co-stimulatory domain linked to the CD3ζ signaling domain.

The Levings lab has successfully generated second generation CAR Tregs that work to prevent GvHD and allograft rejection of skin transplants in various mouse models [133,134]. CAR Tregs were generated from humans [101,133] and mice [134] by transducing the cells with virus encoding an HLA-A*02 targeting CAR, as the HLA-A*02 molecule is commonly mismatched during organ transplantation. When comparing the suppressive activity of HLA-A*02 CAR Tregs to polyclonal Tregs, the antigen-specific Tregs were more efficient in suppressing the growth of peripheral blood mononuclear cells (PBMCs) and T cells *in vitro* [101,133,134] when Tregs were cultured with HLA-A*02 expressing cells.

The Levings lab has also shown the HLA-A*02 CAR Tregs are effective *in vivo*. In a xenogeneic GvHD mouse model, mice were injected with human HLA-A*02^{+} PBMCs and treated with either HLA-A*02 CAR Tregs or Tregs bearing an irrelevant CAR (Her2 CAR). Mice that were treated with the HLA-A*02 CAR Tregs exhibited delayed GvHD symptoms and improved survival rates compared to mice treated with Her2 CAR Tregs [101,133]. For the allograft transplant models, mice were transplanted with both HLA-A*02^{+} and HLA-A*02^{-} skin grafts and injected with HLA-A*02 CAR Tregs or Her2 CAR Tregs. Sicard *et al.* observed that only the HLA-A*02 CAR Tregs were capable of delaying skin graft rejection, and could reduce the frequency of graft-specific antibodies and B cells [134]. When monitoring CAR Treg migration via bioluminescent imaging, Dawson *et al.* observed the HLA-A*02 CAR Tregs quickly migrated to the HLA-A*02^{+} skin graft only, while the polyclonal Tregs could not [101]. The Levings lab has demonstrated CAR technology can be utilized to tailor antigen specificity and enhance Treg function. Therefore, I will be using my prior experience working with HLA-A*02 CAR Tregs to

generate CAR Tregs specific towards beta cell peptides complexed with MHC Class II molecules for use as a T1D therapeutic in murine Tregs.

1.11 Diabetic Mouse Models: The Non-Obese Diabetic Mouse

The non-obese diabetic (NOD) mouse model is the main animal model for the study of T1D, primarily due to the similarities of T1D development between NOD mice and humans. Like humans, NOD mice develop insulitis very early in life and the disease is also largely T cell mediated. From as early as 3 weeks of age, innate immune cells such as dendritic cells and macrophages [135,136] infiltrate the pancreas. Studies in NOD mice have shown this initial infiltration is important for the initiation of the diabetogenic T cell response, as depletion of monocytes and dendritic cells significantly delay diabetes onset [137,138]. The infiltration of $CD4^+$ and $CD8^+$ T cells occurs between 4-6 weeks of age, and both subsets are required for diabetes to progress [139]. NOD mice also suffer from a defect in central tolerance and have autoreactive T cells towards islet related antigens. Like humans, these autoreactive T cell clones have been found to target similar diabetes related autoantigens, such as insulin and GAD65 [139].

The genetic risk of developing T1D in NOD mice has also been attributed to their MHC Class II molecule. Just as patients with T1D predominantly express the HLA-DR3-DQ2 and HLA-DR4-DQ8 MHC Class II haplotypes, NOD mice express the MHC Class II molecule I-A^{g7}, an ortholog of the human HLA-DQ [139]. Studies on the I-A^{g7} molecule revealed a polymorphism unique to the NOD mouse strain that is required for T1D development [140]. When the polymorphism was adjusted to be aligned with other mouse strains [141,142], or when NOD mice were engineered to express the MHC Class II molecule I-E instead [142,143], NOD mice were protected from developing diabetes. While it is still unclear in precisely how the I-A^{g7}

polymorphism contributes to disease progression, it has been hypothesized this polymorphism is responsible for the defect in central tolerance. The I-A^{g7} molecule is reported to express poorly on cell surfaces, as well as being a weak peptide binder [144,145]. Thus, diabetogenic NOD T cells might escape into the periphery due to incomplete presentation of the self-peptide repertoire.

While polymorphisms in the MHC gene is the dominant genetic risk factor in NOD mice, multiple other gene loci have been found to contribute to T1D development. Over 50 disease loci have been identified, which are grouped under the term insulin-dependent diabetes, or "*Idd* loci". The *Idd3* gene locus contains the *IL2* gene, and data from NOD mice suggests the polymorphisms found in *Idd3* results in reduced IL-2 production by effector T cells. IL-2 is critical for Tregs, thus a loss of IL-2 will contribute to the diabetes progression in NOD mice by impeding Treg survival [88,146]. Another important *Idd* gene locus specifically impacting Tregs is *Idd5.1*, which encodes for the *CTLA-4* gene. As mentioned previously, CTLA-4 is used by Tregs to suppress the growth of effector T cells and induce a more tolerogenic phenotype in APCs. NOD mice are reported to express lower levels of CTLA-4 compared to other mouse strains [147]. Furthermore, they can also express a splice variant of *CTLA-4*, which encodes for defective CTLA-4 molecules which lack the CD80/CD86 binding domain [148].

1.12 Diabetic Mouse Models: BDC2.5 Transgenic T Cell Receptor

To discover the islet autoantigens that trigger diabetes development in the NOD mouse model, T cells were isolated and cloned by their ability to react with NOD islet cell suspensions [149]. One of the most widely known diabetogenic clones identified by this method is the BDC2.5 TCR, named after its location of discovery at the Barbara Davis Center [149,150]. The BDC2.5 T cell clone has become an invaluable tool to investigate disease progression *in vivo*, due to its

consistent ability to quickly induce diabetes in mice following adoptive transfer. While it would take between 4-6 months for approximately 50% of female NOD mice to spontaneously develop disease [149], one dose of BDC2.5 T cells is sufficient to induce diabetes 2 weeks post injection [99,102].

The BDC2.5 TCR was sequenced and used to generate a transgenic mouse strain derived from NOD mice [151]. Thus, NOD.BDC2.5 transgenic mice carry $CD4^+$ T cells that uniformly express the BDC2.5 TCR. While adoptive transfer of BDC2.5 $CD4^+$ T cells into other mice is highly diabetogenic, the BDC2.5 mice themselves are relatively resistant to developing T1D compared to NOD mice [152]. As Tregs are also $CD4^+$, it is likely their modified antigen specificity now allows for enhanced homing to the islets to better protect against the effector T cells.

When the identity of the islet antigen responsible for activating BDC2.5 T cells was still unknown, peptide libraries were generated and screened to identify peptide mimotopes capable of stimulating BDC2.5 T cells *in vitro* [153]. To date the identified peptide mimotopes with the highest reactivity are still frequently used to stimulate and expand BDC2.5 T cells *in vitro* [102–104]. Mass spectrometric analysis of purified beta cell fractions later revealed the probable antigen of BDC2.5 T cells to be Chromogranin A [154], a secretory protein encoded by neurons and endocrine cells, including islet beta cells. This possibility was supported when BDC2.5 T cells were found to be unable to produce IFN-γ after culture with APCs and islets from *Chga*$^{-/-}$ mice [154].

The WE-14 peptide is a natural cleavage product of Chromogranin A [155]; sequencing of this peptide fragment showed that the WE-14 peptide shared an amino acid motif with many of the highly reactive BDC2.5 peptide mimotopes [153,154]. While WE-14 was predicted to bind poorly to I-A^{g7}, adding WE-14 peptide into cultures containing BDC2.5 T cells and I-A^{g7}-expressing APCs resulted in IFN-γ responses [154]. However, the BDC2.5 T cell response to the

WE-14 peptide was less potent compared to the response towards total islet cell extracts [156]. This led to the conclusion that there must be an additional post-translational modification to WE-14 to create the natural diabetogenic epitope that stimulates BDC2.5 T cells *in vivo*.

One novel form of post-translational modification is peptide fusion, where different peptides degraded by the proteasome spontaneously crosslink together to become a single hybrid product [157]. In 2016, Delong *et al.* generated a library of hybrid peptides consisting of insulin peptide fragments fused to random peptides derived from secretory granules. One hybrid was a fusion between insulin and WE-14, and when added to BDC2.5 T cell and APC cocultures, it strongly stimulated BDC2.5 T cells, even at low nanomolar concentrations [158]. Thus, it was hypothesized the natural islet antigen targeted by the BDC2.5 TCR was a hybrid peptide (HP) between WE-14 and insulin. Indeed, mass spectrometry analysis has confirmed the presence of this so-called 2.5HP in mouse beta cells [156], and 2.5HP reactive T cells have also been found in the spleens, pancreas, and islets of NOD mice with increasing frequency as mice developed diabetes [156,159].

1.13 Summary and Synopsis of Research Questions

Type 1 diabetes is an autoimmune disease where autoreactive T cells target and kill the insulin-producing beta cells of the pancreas. If left untreated, the chronic insulin deficiency will result in hyperglycaemia and lead to severe complications, such as organ failure and blindness [1, 6]. Patients that are susceptible to developing T1D have been found to express a multitude of genetic risk factors such as polymorphisms in genes involved in insulin processing [7,37], immune tolerance [8,20,31], and most importantly, the expression of specific T1D-susceptible MHC Class II haplotypes [20,23].

T1D pathogenesis is initiated when autoreactive T cells encounter islet APCs presenting endogenous beta-cell derived peptides on their MHC molecules [41]. When the T cells become activated, this will result in a cytotoxic attack directed towards the islet beta cells, mediated primarily by autoreactive $CD8^+$ T cells and pro-inflammatory macrophages [42,43,44]. In healthy individuals, autoreactive T cells that have managed to escape into the periphery are silenced by Tregs, key immune mediators that inhibit inappropriate immune responses [52]. However, it has been found patients with T1D carry dysfunctional Tregs, with many defects found involved in cell activation and suppression [84-90].

Currently the most accessible treatment available for T1D patients is insulin therapy, but there have been increasing efforts to research and develop therapeutic strategies that will address the causal underlying autoimmunity. One such strategy is adoptive Treg transfer therapy where Tregs are expanded *ex vivo* then transferred back into a patient, thus increasing the number of Tregs to allow for better suppression of ongoing autoimmunity. While phase I clinical trials with diabetic patients have established the feasibility and safety of adoptive polyclonal Treg transfer as a potential therapy for humans [95,96], studies in mouse models have suggested using antigen specific Tregs would be more effective. Pre-diabetic and diabetic mice injected with Tregs bearing a islet antigen specific TCR have been shown to prevent diabetes development [99,102–104] and even reverse disease [102,105] much more swiftly than mice injected with polyclonal Tregs.

CARs have become a popular tool to engineer antigen specificity due to their modular nature and high antigen specificity. The Levings lab has expertise in generating antigen specific Tregs by utilizing CARs [101,133,134,160] to better tailor Treg specificity and function. For instance, infusion of human Tregs expressing a CAR targeting a common transplant antigen can greatly delay rejection of transplanted allografts by preventing xenogeneic GvHD [101,121]. Thus, the goal

of my work was to generate antigen-specific Tregs expressing CARs targeting islet peptides complexed to MHC Class II molecules for the treatment of murine T1D. I collaborated with the Fife lab from the University of Minnesota, which generated two monoclonal antibodies specific for p63:I-A^{g7} or Insulin $B_{10\text{-}23}$:I-A^{g7}. At the Levings lab, the antibodies were converted into CARs and I optimized methods to test their function *in vitro*.

Patients with T1D have an autoimmune disorder where their autoreactive T cells target and kill their pancreatic beta cells. Tregs have long been considered to be key mediators in suppressing inappropriate immune responses, and diabetic mouse models have shown Tregs engineered to target the pancreas are superior in preventing diabetes development. Thus, I hypothesized that CAR Tregs specific for peptide:I-A^{g7} complexes would be functionally superior to polyclonal Tregs when stimulated with relevant antigen. To test this hypothesis, I first had to ask whether the engineered CAR Tregs were antigen specific. If they are antigen specific, does this specificity translate to biological cell function when stimulated with the relevant antigen? Finally, does their antigen specificity also enhance their suppressive capacity compared to polyclonal Tregs?

Chapter 2: Materials and Methods

2.1 CARs and Retrovirus

To generate the antigen binding domain of the CARs, the variable regions of the heavy and light chains were cloned from hybridomas produced by the Fife lab [161]. Mice were immunized with p63:I-A^{g7} (RTRPLWVRME) or $InsB_{10–23}$:I-A^{g7} (HLVERLYLVCGEEG) monomers with repeated boosters over a period of 45 days. The spleens of immunized mice were harvested and stained with either p63:I-A^{g7} or $InsB_{10–23}$:I-A^{g7} tetramers conjugated to streptavidin-phycoerythrin (PE). The tetramer specific B cells were then isolated by magnetic enrichment using anti-PE beads and fused with myeloma fusion partners to generate hybridomas (Fig. 1A). Antibodies were screened by harvesting hybridoma supernatants for ELISA to confirm peptide:I-A^{g7} specificity.

Hybridoma clones validated for antigen specificity were sequenced and the DNA sequences were then converted into an scFv format and fused to a Myc epitope tag to allow for cell surface detection. The extracellular domains were fused to the hinge (derived from mouse CD8), transmembrane (derived from mouse CD28), and intracellular domains. The intracellular domains were comprised of a fusion of mouse CD28 and CD3ζ signalling domains. The CARs were named 1B2 (targets $InsB_{10–23}$:I-A^{g7}) and FS1 (targets p63:I-A^{g7}) and cloned into a murine stem cell virus (MSCV)–based retroviral vector. Retroviral particles were produced by using the Platinum-E (Plat-E) Retroviral Packaging Cell Line transfected with the pCL-Eco Retrovirus Packaging Vector, according to the manufacturer's recommendations (Cell Biolabs).

2.2 $CD4^+$ Treg and Conventional T cell Sorting, Transduction, and Expansion

Spleens and (popliteal, axillary, mandibular, and mesenteric) lymph nodes were harvested from 8-12-week-old NOD.FOXP3 GFP mice. Mice were kept under pathogen-free conditions at the animal facility at British Columbia Children's Hospital Research Institute. The organs were crushed and strained to isolate single cells, and $CD4^+$ cells were magnetically enriched using mouse $CD4^+$ T cell isolation kits (STEMCELL Technologies). Live regulatory T cells (Tregs) and conventional $CD4^+$ T cells (Tconvs) were sorted by fixable viability dye eF780 (Thermo Fisher Scientific) and CD4 antibody staining (clone RM4-5, BD Biosciences), as well as high and low FoxP3-GFP expression (for Tregs and Tconvs respectively) using the MoFlo Astrios cell sorter (Beckman Coulter). Both sorted Tregs and Tconvs were cultured in ImmunoCult™-XF T Cell Expansion Medium (STEMCELL Technologies) supplemented with 50 μmol/L of β-mercaptoethanol and 100 units/mL of Penicillin/Streptomycin (Thermo Fisher Scientific). Sorted Treg cultures also contained 1000 U/mL of IL-2 (Proleukin) and 50 nmol/L of rapamycin (Sigma-Aldrich), whereas Tconv cultures only contained 100 U/mL of IL-2. Tregs and Tconvs were stimulated with mouse T-Activator CD3/CD28 Dynabeads™ (ThermoFisher Scientific) at a bead to cell ratio of 3:1 and 2:1, respectively.

Two days post sorting, Tconvs were transduced with the 1B2-, FS1-, or Her2-encoding retrovirus. Lipofectamine™ 2000 (2 μg/mL, ThermoFisher Scientific) and hexadimethrine bromide (Polybrene, 1.6 μg/mL, Sigma) were added to increase the efficacy of retroviral transduction, and cells were centrifuged for 90 minutes at 805 x g at 32°C. Tregs were transduced using the same method, but on day 3 post sorting.

Cell cultures were monitored and split accordingly depending on media colour and cell density. IL-2 and rapamycin were replaced when cultures were split. On day 7, CD3/CD28

Dynabeads^{TM} were magnetically removed and both Tregs and Tconvs were rested for 2 days with a decreased dose of IL-2 (300 U/mL and 30 U/mL respectively) before use for functional *in vitro* assays.

2.3 Flow Cytometry

All antibody staining was conducted at room temperature for 30-40 minutes away from light. The cell phenotyping, proliferation, and suppression assays were read using the Cytoflex (Beckman Coulter), whereas the activation assays were read using the Fortessa (BD Biosciences). All resulting flow data was analyzed using FlowJo Software versions 10.1.6 and 10.7.1.

On Day 5 and 7 post cell sort, transduction efficiency was assessed by cell surface staining using mouse anti-CD4-BV605 (clone RM4-5, BD Biosciences), anti-Myc-AF647 (clone 9E10, UBC Ablab), and live cells were detected using the fixable viability dye eF780 (eBioscience). To evaluate CAR antigen specificity, CAR Tregs and Tconvs were stained with a panel of peptide:I-A^{g7} tetramers (provided by the Fife lab). The tetramers used were Insulin B_{10-23}:I-A^{g7}, p63:I-A^{g7}, 2.5HP:I-$A^{g7}$and Hen Egg Lysozyme (HEL_{11-25}):I-A^{g7}, and all tetramers were conjugated with BV421 Streptavidin (Biolegend).

To assess Treg purity, both Tregs and Tconvs (for use as negative controls) were stained with anti-CD4-BV605 and fixable viability dye eF780. Cells were then fixed and permeabilized using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) for 40 minutes at room temperature in the dark. Intracellular staining using anti-FoxP3-PE (clone FJK-16s, eBioscience) and anti-Helios-eF450 (clone 22F6, eBioscience) was performed in Permeabilization Buffer (eBioscience).

Both proliferation and activation assay samples were stained with anti-Myc-AF647 and fixable viability dye eF780. Proliferation assays were stained with anti-CD4-BV605, whereas activation assays were stained with anti-CD4-PE-Cy7 (clone RM4-5, BD Biosciences). Proliferation of CAR Tregs and Tconvs was analyzed by the dilution of the CPD eF450 signal, whereas CAR Treg and CAR Tconv activation was read out by staining with anti-CD69-BV785 (clone H1.2F3, Biolegend), anti-LAP-PE (clone TW7-16B4, eBioscience), and anti-CTLA4-BV605 (clone UC10-4B9, Biolegend). Suppression assays were also stained with anti-CD4-BV605 and fixable viability dye, and BDC2.5 T cell proliferation was also read out by the dilution of CPD eF450 signal (eBioscience), whereas CAR Treg proliferation was read out by the dilution of CPD eF670 signal (eBioscience).

For the *in* vivo experiment, peripheral blood from the saphenous vein (60-80 µL blood) was collected to determine the cell engraftment of the injected Tconvs. Erythrocytes were lysed using the 10X RBC Lysis Buffer (eBioscience) and anti-mouse Fc block (BD Biosciences) was added to the remaining leukocytes. Following Fc blocking, cells were stained with anti-CD8-PE (clone 53.6-7, invitrogen), anti-CD45-eF450 (clone 30-F11, invitrogen), anti-CD4-BV605, and labelled with fixable viability dye eF780. To determine whether the injected CAR Tconvs were still expressing the CAR, cells were also stained with anti-Myc-AF647. After staining, 123count eBeads™ (ThermoFisher Scientific) were added to all samples to allow for absolute counting of cells by flow cytometry. The samples were then read out using the LSRII (BD Biosciences) and results were analyzed using FlowJo Software versions 10.1.6 and 10.7.1.

2.4 *In vitro* Activation, Proliferation, and Suppression Assays

All functional assays were conducted on day 9 post sort, after two days of cell resting (Fig. 3B). CAR Tregs and Tconvs were collected, counted, and washed prior to processing in preparation for their respective assays. To collect cells for use as APCs, spleens were harvested from NOD.FOXP3 GFP negative mice and crushed to isolate the splenocytes. T cells were then depleted from the splenocyte population by magnetic selection using the Mouse CD90.2 Positive Selection Kit (STEMCELL Technologies).

For use in activation assays, depleted splenocytes were labelled with cell proliferation dye CPD eF450 before being pulsed with 15 µM Insulin P8E (HLVERLYLVAGEEG), p63 (RTRPLWVRME), 2.5HP (LQTLALWSRMD) or Hen Egg Lysozyme ($HEL_{11\text{-}25}$ AMKRHGLDNYRGYSL) peptides and cultured with 1B2 CAR, FS1 CAR, or untransduced cells at a 1:1 ratio. Treg cocultures were supplemented with 100 U/mL IL-2 and incubated overnight at 37°C. The Treg activation markers CD69, LAP, and CTLA-4 were then assessed by flow cytometry the following day.

For use in proliferation assays, T-cell-depleted and non-labelled splenocytes were irradiated by X-ray at 2000 rad. Meanwhile, collected CAR Tregs and Tconvs were labelled with Cell Proliferation Dye CPD eF450 (eBioscience). The irradiated splenocytes were pulsed with 10 µM Insulin P8E, p63, 2.5HP, or HEL peptides and cultured with labelled CAR Tregs or Tconvs at a 1:1 ratio to assess for antigen dependent proliferation. Proliferation assay cocultures were incubated at 37°C and antigen dependent cell proliferation was assessed by flow cytometry following 3 days of incubation. CAR Tregs also received supplemental 100 U/mL IL-2 one day into incubation.

To set up suppression assays, the spleen and lymph nodes were harvested from BDC2.5 mice to isolate for T cells bearing the BDC2.5 TCR. The organs were crushed to isolate cells and $CD4^+$ T cells were enriched by using the EasySep™ Mouse $CD4^+$ T Cell Isolation Kit (STEMCELL Technologies). To discriminate CAR Tregs from BDC2.5 T cells, BDC2.5 T cells were labelled with the Cell Proliferation Dye CPD eF450 (eBioscience), and CAR Tregs were labelled using the CPD eF670 (eBioscience). Fifty thousand BDC2.5 T cells were added to each well and CAR Tregs were plated by serial dilution from a ratio of 2 CAR Tregs : 1 BDC2.5 T cell to 1 CAR Treg : 16 BDC2.5 T cells. Splenocytes were harvested and treated as in the proliferation assay and 100,000 irradiated splenocytes were plated and pulsed with 10 nM p63 peptide to stimulate BDC2.5 T cell proliferation. Splenocytes that were plated with 1B2 CAR Tregs were also pulsed with 10 µM Insulin P8E peptide to stimulate the 1B2 CAR Tregs. Suppression assay cocultures were incubated at 37°C and suppression of BDC2.5 T cell proliferation was assessed by flow cytometry following 3 days of incubation.

To measure cytokine production, supernatants from suppression assays were collected and frozen after the 3 days of incubation. Supernatants from select conditions were thawed and cytokine concentration was determined by using the mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences) and analyzed by the FCAP Array Software v. 3.0.1 (Soft Flow).

2.5 *In vivo* Experiments

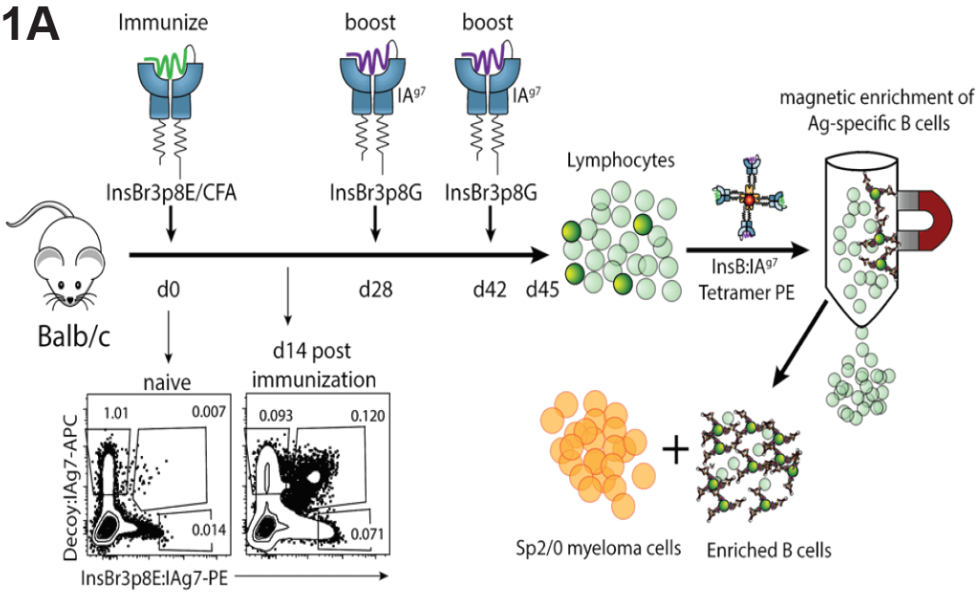
4×10^6 CAR Tconvs were transferred to male NSG mice (The Jackson Laboratory, bred in house) by tail-vein injection. Mice that did not receive any cells served as the negative controls. Following cell injection, the overall health of the mice was monitored by body weight, fur texture, posture, activity level, and grimace scale one-two times per week. Blood glucose was

checked on a weekly basis to monitor for diabetes development via the OneTouch Ultra® 2 Blood Glucose Meter (Lifescan). A mouse was considered diabetic if their blood glucose readings exceeded 15 mmol/L for three consecutive readings/days. After 7 weeks, all remaining mice were euthanized. Sections of spleen, pancreas, liver, lung, duodenum, ileum, and colon were collected for histology and fixed in 4% paraformaldehyde overnight. Afterwards, all tissues samples were transferred to 70% ethanol and stored at 4°C.

2.6 Statistics

Analysis of results were performed using Prism 7 software (Graphpad). Results were considered statistically significant when $p \leq 0.05$. Statistical analyses of activation and proliferation were calculated using one-way ANOVA with Tukey's multiple comparison's post tests. Significance of Treg mediated suppression of BDC2.5 T cell proliferation was determined by two-way ANOVA with Tukey's multiple comparison's post test and suppression of cytokine production was calculated by two-way ANOVA with Bonferroni's multiple comparisons post test.

1A



1B

Wild type Ins B_{10-23} :	H L V E A L Y L V C G E R G
Modified Ins B_{10-23} : Insulin p8E	H L V E R L Y L V C G E E G
p63 peptide:	R T R P L W V R M E

1C

p:MHC II	Clone Name	Isotype	K_D
$InsB_{10-23}$:I-A^{g7}	1B2	IgG1, κ	8.5E-09
p63:I-A^{g7}	FS1	IgG1, κ	1.7E-11

Figure 1. Background information of islet-peptide:MHC II CAR Generation.
(A) Representative immunization schedule of mice to induce and isolate islet-peptide:MHC Class II specific B cells and their subsequent isolation and fusion into hybridoma cells. (B) Amino acid sequences of CAR peptide targets. Amino acids in red are predicted to bind in the MHC binding groove by filling the anchor positions. (C) Comparison of anti-islet-peptide:MHC II antibody binding affinities to their target antigen.

Chapter 3: Results

3.1 Generation and expression of islet-peptide:I-A^{g7} MHC Class II CARs

I aimed to generate CARs specific towards known diabetogenic beta cell peptides in the context of MHC Class II molecules. As detailed in Methods, collaborators from the Fife lab generated monoclonal antibodies against peptide bound to MHC Class II by immunizing mice with recombinant peptide:MHC monomers (Fig. 1A) [161]. The peptides I was interested in developing antibodies towards were the p63 peptide, a mimotope of the Chromogranin A cleavage product WE-14 [153,154], and the InsB$_{10–23}$ (P8E) peptide (Fig. 1B). Insulin P8E is a mutant where the 22nd amino acid of the wild type Insulin B peptide (arginine) is mutated into glutamic acid. This mutation was predicted to enhance peptide binding to the MHC binding groove and would improve InsB$_{10–23}$ presentation by I-A^{g7} [162]. In the Fife lab, hybridomas were screened for their specificity and affinity (Fig. 1C), and the top performing antibodies were sequenced and converted into single chain antibodies. The monoclonal antibodies (mAb) that were chosen were the FS1 mAb (specific towards p63:I-A^{g7}) and the 1B2 mAb (InsB$_{10-23}$:I-A^{g7}). Both scFvs were cloned into a retroviral vector [134] to create second-generation CARs encoding the CD8 hinge, CD28 transmembrane, and CD28 and CD3ζ intracellular domains (Fig. 2A). A Myc-epitope tag was also added to allow detection of CAR expression on the cell surface.

Preliminary testing of the 1B2 and FS1 CARs was conducted by transient transfection of HEK 293T cells, and I was able to detect cell surface CAR expression by flow cytometric staining of the Myc-epitope tag (Fig. 2B). I then utilized p63:I-A^{g7} and InsB$_{10-23}$:I-A^{g7} tetramers to ensure the conversion into single chain antibodies did not compromise the antigen binding capabilities of the FS1 and 1B2 CARs. As seen in Fig. 2C, both the FS1 and 1B2 CARs were

able to bind specifically to the p63:I-A^{g7} and InsB$_{10-23}$:I-A^{g7} tetramers respectively and did not bind to the irrelevant Hen Egg Lysozyme (HEL):I-A^{g7} tetramer.

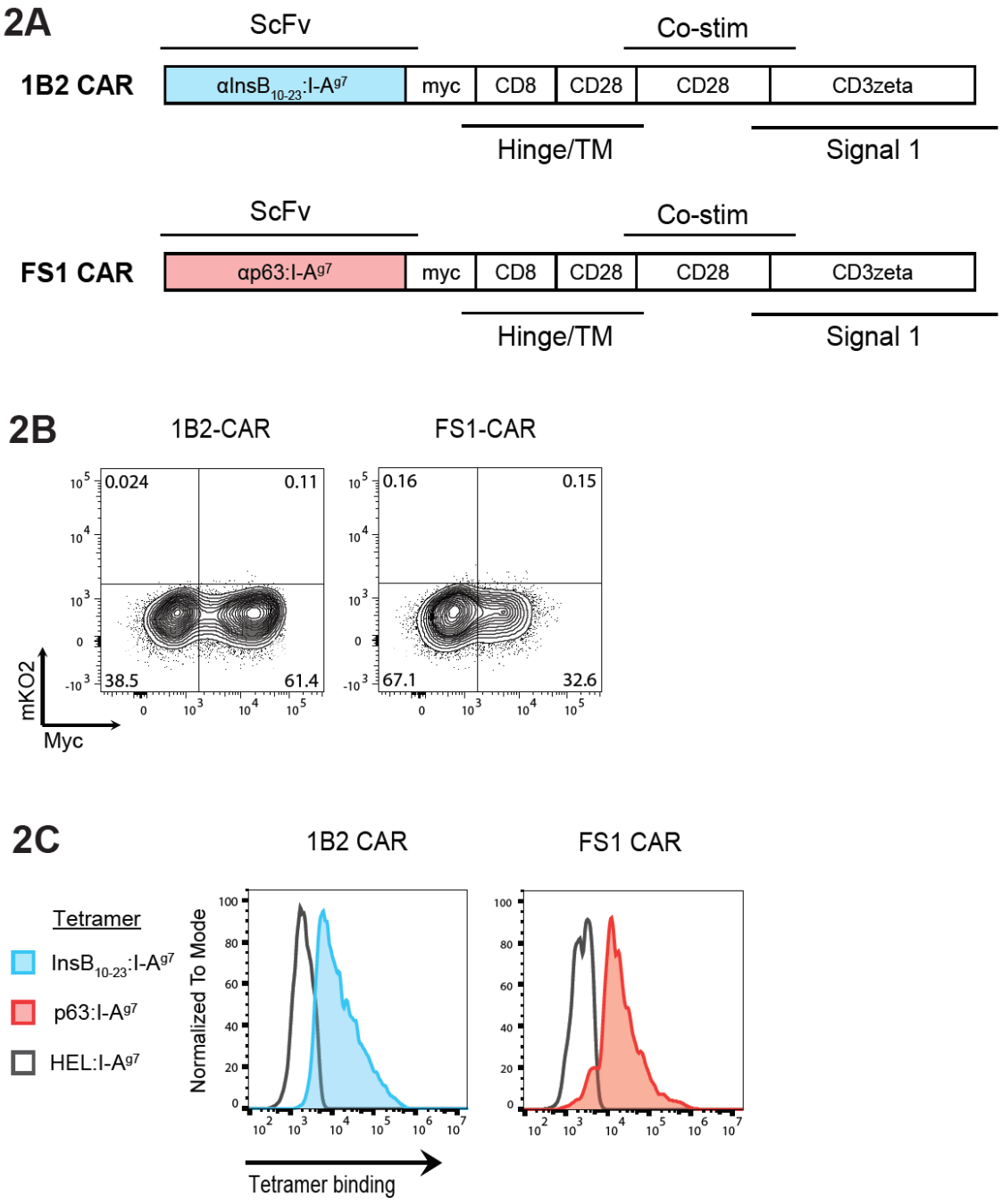


Figure 2. Generation and Validation of islet-peptide:MHC II CARs.
(A) Schematic of domains in the 1B2 and FS1 CARs. ScFv, Single chain variable fragment; Myc, Myc-tag; TM, transmembrane; Co-stim, Co-stimulation. (B) Validation of CAR expression on HEK 293T cells following transient transfection. Surface expression of both CARs were assessed by flow cytometry of the Myc-tag. (C) 293T cells expressing the 1B2 or FS1 CAR were stained with either their relevant tetramer (Insulin B$_{10-23}$:I-A^{g7} and p63:I-A^{g7}, respectively) or an irrelevant tetramer (HEL:I-A^{g7})

3.2 Generation of islet-peptide:I-A^{g7} MHC Class II CAR Tregs

To generate the 1B2 and FS1 CAR Tregs, I sorted CD4^{+}GFP^{+} cells harvested from spleens and lymph nodes of NOD.FOXP3-cre mice (Fig. 3A). The mice have been engineered to express a FOXP3-EGFP/cre BAC transgene conferring expression of a fusion protein containing green fluorescent protein (GFP) fused to a humanized cre recombinase [163]. The transgene expression is under the control of the *FoxP3* promoter, thus the sorted CD4^{+}GFP^{+} cell population will result in an almost pure population of Tregs, as FoxP3 is the master transcription factor for Tregs. CD4^{+}GFP^{-} cells were also sorted in parallel for use as conventional T cell (Tconv) controls. Following sorting, Tregs were stimulated using anti-CD3/28 Dynabeads^{TM}, and cultured in the presence of a high concentration of IL-2 (1,000 U/ml) and rapamycin (to limit expansion of any contaminating Tconv cells). The activated Tregs were then transduced with 1B2 CAR-, or FS1 CAR-encoding retrovirus and expanded for 4 additional days (Fig. 3B). In addition to untransduced Tregs, Her2 CAR Tregs were also generated for use as an irrelevant CAR control, as the Her2-specific scFv targets the human Her2 antigen which is not expressed in mice.

After 7 days, Tregs that were transduced with the 1B2 CAR or FS1 CAR had high levels of CAR cell surface expression, as determined by flow cytometric staining of the Myc-epitope tag (Fig. 3C). To determine the specificity of each CAR, Tregs were stained with relevant and irrelevant peptide:MHC Class II tetramers. The 1B2 CAR Tregs only bound the InsB$_{10–23}$:I-A^{g7} tetramer, while the FS1 CAR Tregs only bound the p63:I-A^{g7} tetramer (Fig. 3D). None of the CARs bound to the irrelevant HEL:I-A^{g7} tetramer, suggesting that both 1B2 and FS1 CAR Tregs are highly specific to their relevant antigen.

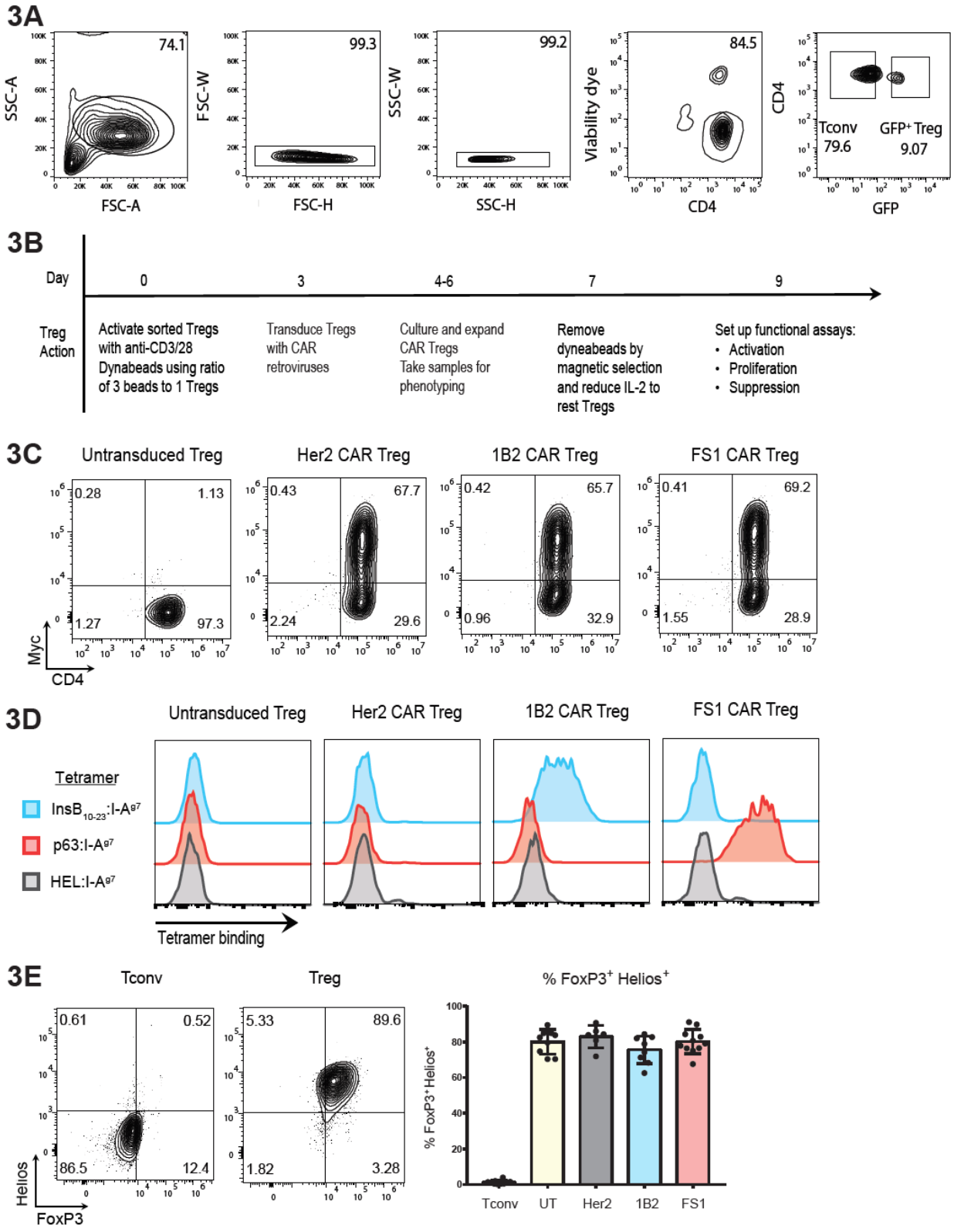


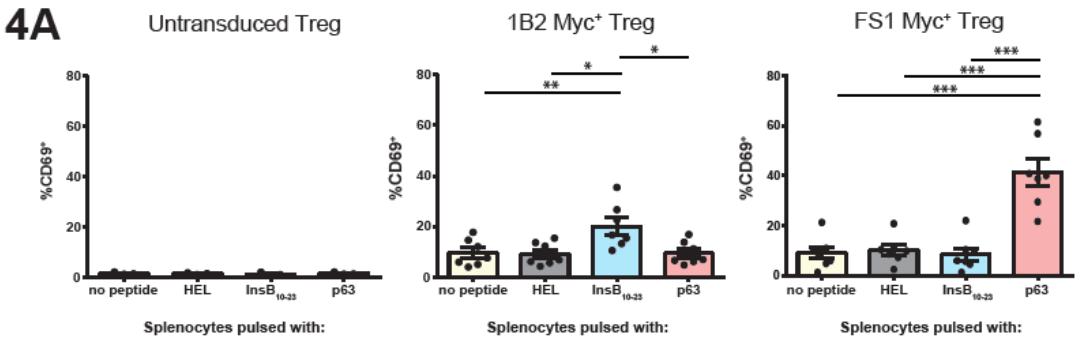
Figure 3. Generation of 1B2 and FS1 CAR Tregs.
(A) Sorting strategy to isolate for FoxP3-GFP^{+} Tregs from CD4 enriched cells. (B) Workflow of CAR Treg manufacturing detailing expansion, transduction, and resting process prior to *in vitro* functional assay set up. (C) Representative flow analyses of CAR surface expression on Tregs (*n*=6-11) by detection of the Myc tag. (D) CAR Tregs were stained with relevant and irrelevant peptide:MHC II tetramers to assess for CAR specificity. (E) Expression of FoxP3 and Helios was assessed on Day 7 post sort to evaluate Treg purity during expansion (mean ± SD, *n*=6-11).

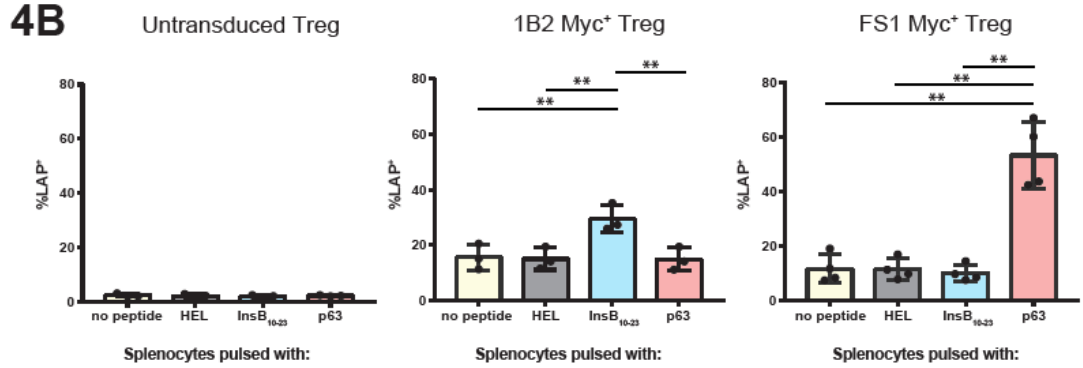
To ensure the CAR Tregs remained stable during expansion, samples were taken for flow cytometric analysis of FoxP3 and Helios expression. Helios is another transcription factor expressed in Tregs and has been found to be positively correlated with Treg lineage stability [164]. The deletion of Helios within Tregs has been shown to promote conversion to an effector T cell phenotype, with increased production of proinflammatory cytokines and enhanced antitumour immunity [165]. During the 7 days of stimulation and expansion, all CAR Tregs retained high (>70%) FoxP3 and Helios expression compared to the Tconv controls (Fig. 3E). Furthermore, CAR Treg expression of FoxP3 and Helios was comparable to untransduced NOD Tregs, suggesting neither virus transduction nor CAR expression altered the phenotype of NOD Tregs *in vitro*.

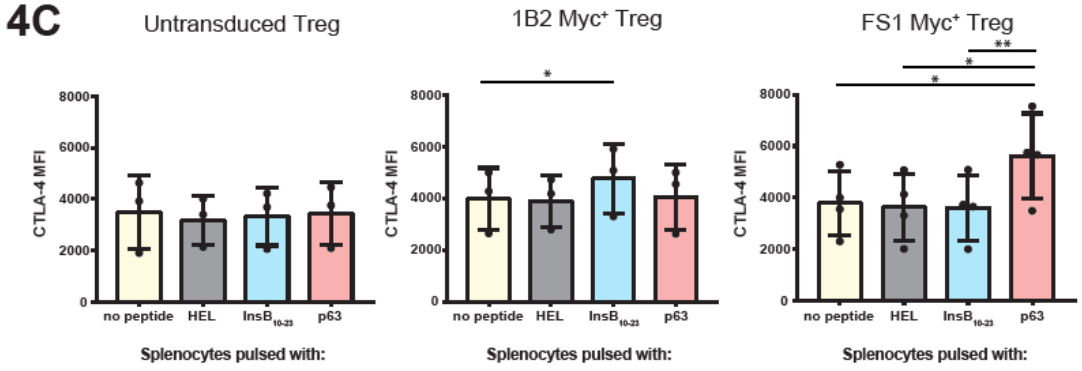
3.3 CAR Tregs are activated in an antigen-specific manner

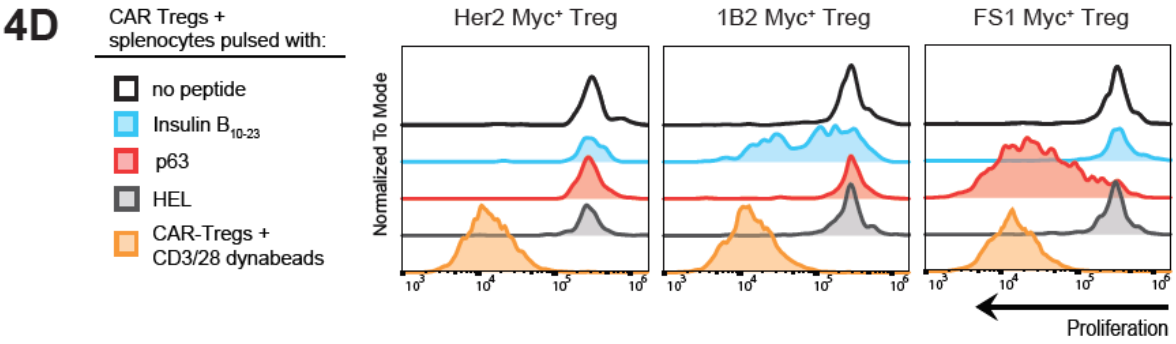
Once I confirmed the CARs were expressed on Tregs, and that the 1B2 and FS1 CARs were specific towards the relevant peptide:MHC Class II tetramer, I then tested whether the CARs mediated antigen-specific effects in Tregs. To assess the function of the 1B2 and FS1 CAR Tregs, cells were cultured overnight with NOD splenocytes pulsed with 15 µM of insulin $B_{10–23}$ peptide, p63 peptide, or HEL_{11-25} peptide. When CARs were cultured with the relevant peptide, both 1B2 CAR and FS1 CAR Tregs increased CD69 (Fig. 4A), LAP (Fig. 4B), and CTLA-4 expression (Fig. 4C). However, when cultured with irrelevant peptides, the expression of these activation markers remained similar to baseline (no peptide added), confirming antigen-specific, CAR-mediated Treg activation.

To test whether CAR stimulation would result in cell proliferation, CAR Tregs were labelled with cell proliferation dye (CPD) and cultured with irradiated NOD splenocytes pulsed


4A
Untransduced Treg
1B2 Myc+ Treg
FS1 Myc+ Treg
%CD69+
no peptide
HEL
InsB10-23
p63
Splenocytes pulsed with:


4B
Untransduced Treg
1B2 Myc+ Treg
FS1 Myc+ Treg
%LAP+
no peptide
HEL
InsB10-23
p63
Splenocytes pulsed with:


4C
Untransduced Treg
1B2 Myc+ Treg
FS1 Myc+ Treg
CTLA-4 MFI
no peptide
HEL
InsB10-23
p63
Splenocytes pulsed with:


4D
CAR Tregs +
splenocytes pulsed with:
no peptide
Insulin B10-23
p63
HEL
CAR-Tregs +
CD3/28 dynabeads
Her2 Myc+ Treg
1B2 Myc+ Treg
FS1 Myc+ Treg
Normalized To Mode
Proliferation

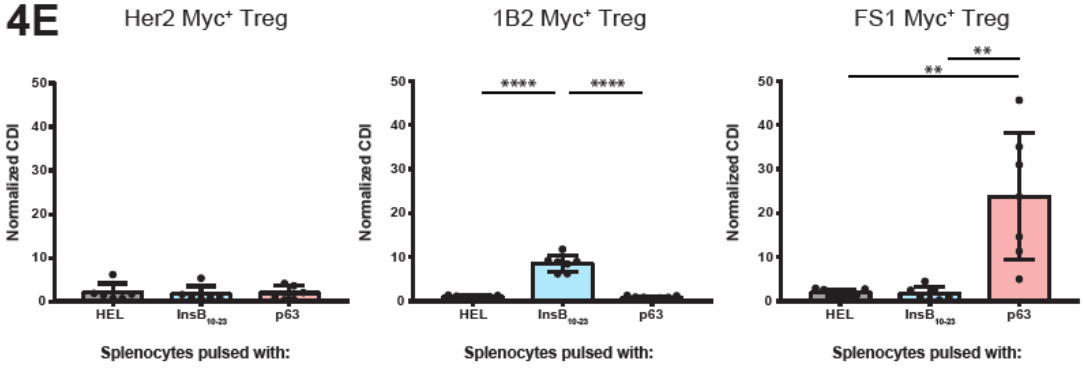

4E
Her2 Myc+ Treg
1B2 Myc+ Treg
FS1 Myc+ Treg
Normalized CDI
HEL
InsB10-23
p63
Splenocytes pulsed with:

Figure 4. 1B2 and FS1 CAR Tregs activate and proliferate in an antigen dependent manner. CAR Tregs were cultured with peptide pulsed splenocytes overnight (mean ± SEM, *n*=3-7) and assayed for expression of (A) CD69, (B) LAP, and (C) CTLA-4. (D) CAR Tregs were cultured with peptide pulsed splenocytes for 3 days and proliferation was assayed by dilution of the CPD fluorescent signal. (E) The cell division index (CDI) was calculated for each peptide condition. CDIs of CAR Tregs cultured with peptide were normalized to the CDI of CAR Tregs cultured without peptide (mean ± SEM, *n*=7). Significance for activation and proliferation were determined by one-way ANOVA with Tukey's multiple comparison's post tests. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

with Insulin $B_{10–23}$ peptide, p63 peptide, or HEL_{11-25} peptide (Fig. 4D and 4E). 1B2 CAR Tregs only proliferated in response to the Insulin $B_{10–23}$ pulsed splenocytes, and FS1 CAR Tregs only proliferated when cultured with p63 pulsed splenocytes. Neither 1B2 nor FS1 CAR Tregs responded to the splenocytes pulsed with the irrelevant diabetogenic peptide, nor to the splenocytes pulsed with the negative control HEL peptide.

3.4 FS1 CAR Tregs suppress BDC2.5 T cells better than polyclonal NOD Tregs

After confirming peptide presentation by APCs can stimulate CAR Tregs in an antigen dependent manner, I then asked whether CAR Tregs would be able to mediate antigen dependent suppression of $CD4^+$ T cells. I aimed to set up an *in vitro* suppression assay that would mimic the conditions of a diabetic mouse, so I used BDC2.5 transgenic T cells as the responder T cell population. Conveniently, the BDC2.5 T cells can also be activated by the p63 peptide, as it is a mimotope of the natural epitope of Chromogranin A [153]. Therefore, I set up a p63 peptide titration to determine the optimal p63 concentration to stimulate BDC2.5 T cell proliferation (Fig. 5A). The BDC2.5 mimic peptides are known superagonists for BDC2.5 T cells [166], thus I had to determine a minimum amount of peptide that could be used to stimulate T cell proliferation, since high levels of stimulation were expected to make the cells resistant to CAR Treg suppression.

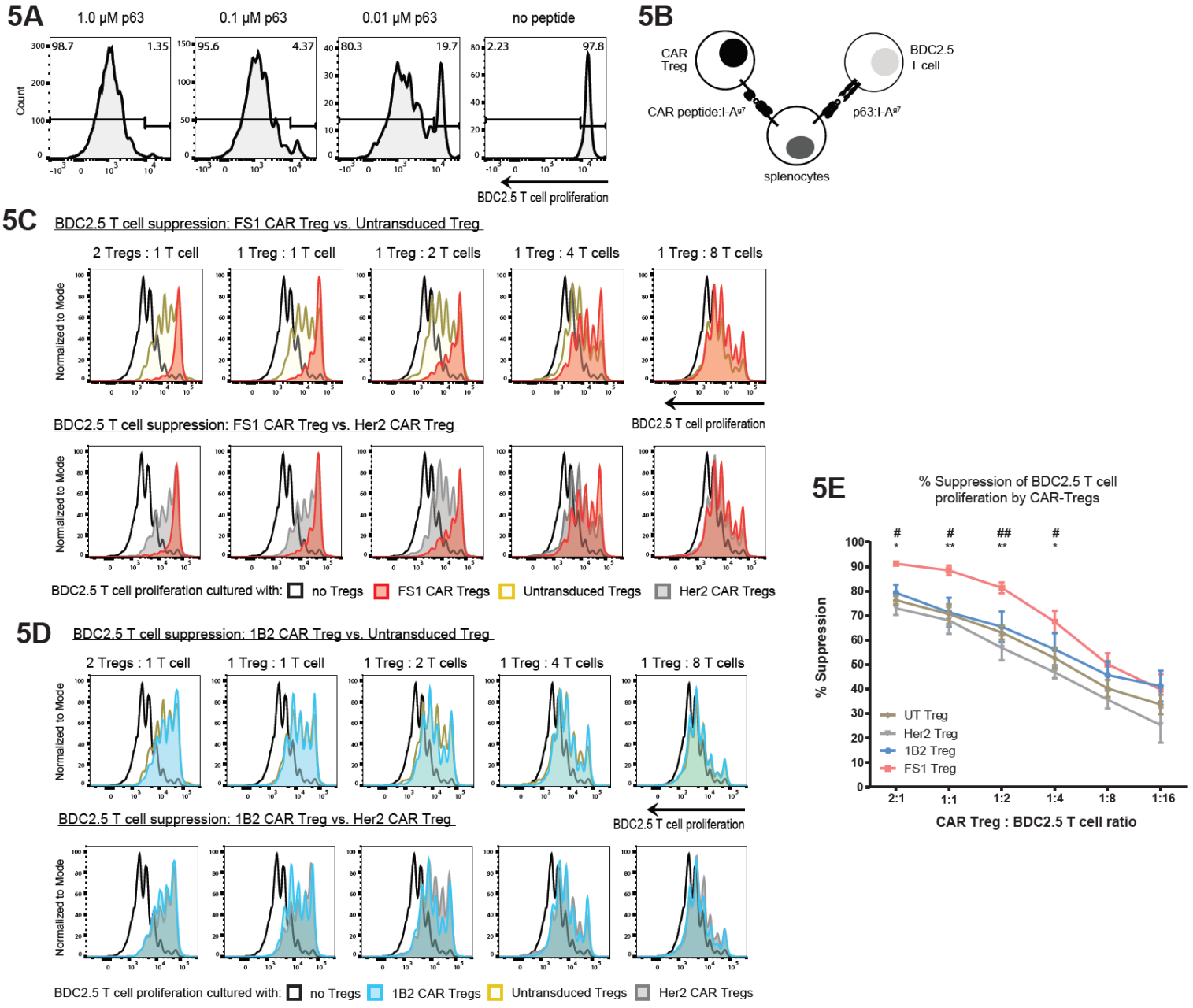


Figure 5. FS1 CAR Tregs mediate enhanced suppression over polyclonal Tregs when stimulated with relevant peptide.

(A) p63 peptide titration to optimize BDC2.5 T cell stimulation. (B) Schematic of suppression assay set up: BDC2.5 T cells were stimulated with p63 and CAR Tregs were stimulated with relevant peptide, both presented by splenocytes. (C) Representative proliferation histograms of BDC2.5 T cells cultured with FS1 CAR Tregs (*n=7*) versus untransduced Tregs (top; *n*=6) or Her2 CAR Tregs (bottom; *n*=3). (D) Representative proliferation histograms of BDC2.5 T cells cultured with 1B2 CAR Tregs (*n*=4) versus untransduced Tregs (top) or Her2 CAR Tregs (bottom). (E) Averaged data (mean ± SEM, *n*=3-7) pooled from independent suppression assays. Significance was determined by two-way ANOVA with Tukey's multiple comparison's post test. *FS1 vs UT; #FS1 vs Her2. *p < 0.05; **p < 0.01

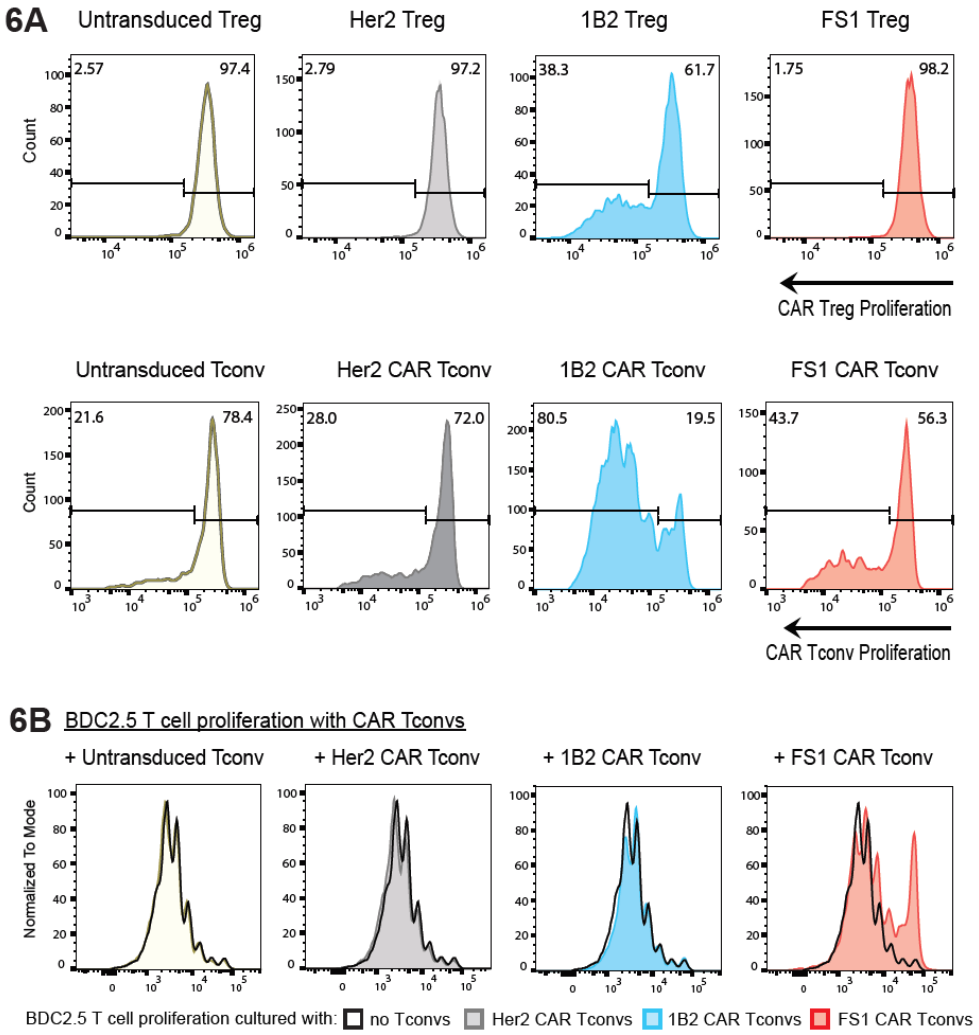


Figure 6. Only 1B2 CAR Tregs and Tconvs proliferate following culture in suppression assays. (A) Representative proliferation histograms of CAR Tregs (top; *n*=3-7) and CAR Tconvs (bottom; *n*=3) at the 2 Treg/Tconv : 1 BDC T cell ratio. Proliferation was assessed by dilution of the cell proliferation dye fluorescent signal. (B) Representative proliferation histograms of BDC2.5 T cells after culture with CAR Tconvs at the 2 Tconv : 1 BDC2.5 T cell ratio (*n*=3).

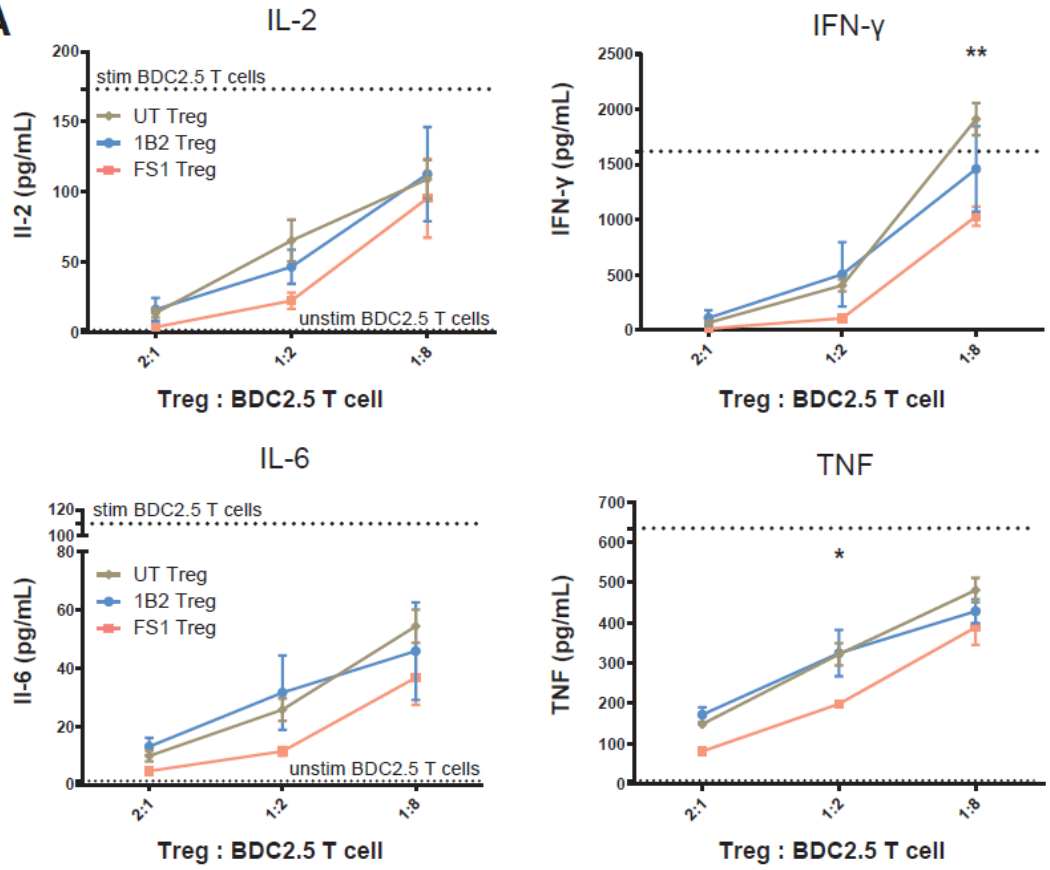
The BDC2.5 T cells were labelled with cell proliferation dye and cultured with irradiated NOD splenocytes pulsed with 10 nM of p63 peptide and a decreasing ratio of CAR Tregs (Fig. 5B). Surprisingly, despite decreasing the p63 peptide concentration by 1000X (compared to the proliferation assays), the FS1 CAR Tregs were still able to suppress BDC2.5 T cell proliferation (Fig. 5C). Furthermore, the FS1 CAR Treg mediated suppression was superior to both Treg

controls, as BDC2.5 T cells proliferated significantly less when cultured with FS1 CAR Tregs compared to untransduced and Her2 CAR Tregs (Fig. 5E).

To evaluate the suppressive capacity of the 1B2 CAR Tregs, splenocytes were pulsed with insulin peptide to stimulate the 1B2 CAR Tregs. However, while 1B2 CAR Tregs were able to suppress BDC2.5 T cell proliferation, I did not observe any differences in their suppressive ability compared to either Treg controls (Fig. 5D and E). The untransduced and Her2 CAR Tregs were able to suppress BDC2.5 T cell growth to the same extent as 1B2 CAR Tregs, despite the extra insulin peptide stimulation provided to the 1B2 CAR Tregs. When analyzing the Treg proliferation in suppression assays (Fig. 6A), I noticed that only the 1B2 CAR Tregs had proliferated. Therefore, while the 1B2 CAR Tregs did not exhibit enhanced suppression of BDC2.5 T cell proliferation, the 1B2 CAR Tregs were activated in response to the insulin peptide. Conversely, neither Her2 nor untransduced Tregs showed any proliferative response towards p63 or insulin, validating the proliferation assay results. The FS1 CAR Tregs also did not proliferate in the suppression assays, likely due to the low p63 concentration in the culture. Yet, FS1 CAR Tregs suppressed BDC2.5 T cell proliferation significantly moreso than the other Treg groups, despite 1B2 CAR Tregs exhibiting a more traditionally activated phenotype. These data suggest that Tregs do not need to be actively proliferating to mediate suppression.

Parallel cultures with CAR Tconvs were also set up as negative controls to confirm whether the observed CAR Treg mediated suppression of BDC2.5 T cells could be attributed to Treg function, or if the lack of BDC2.5 T cell proliferation was merely due to resource competition for space and nutrients. As shown in Figure 6B, BDC2.5 T cells proliferated well in the presence of CAR Tconvs, confirming the suppression observed in the Treg based assays was mediated by the CAR Tregs.

7A


IL-2
stim BDC2.5 T cells
UT Treg
1B2 Treg
FS1 Treg
unstim BDC2.5 T cells
Il-2 (pg/mL)
Treg : BDC2.5 T cell
IFN-γ
**
IFN-γ (pg/mL)
Treg : BDC2.5 T cell
IL-6
stim BDC2.5 T cells
UT Treg
1B2 Treg
FS1 Treg
unstim BDC2.5 T cells
Il-6 (pg/mL)
Treg : BDC2.5 T cell
TNF
*
TNF (pg/mL)
Treg : BDC2.5 T cell

7B

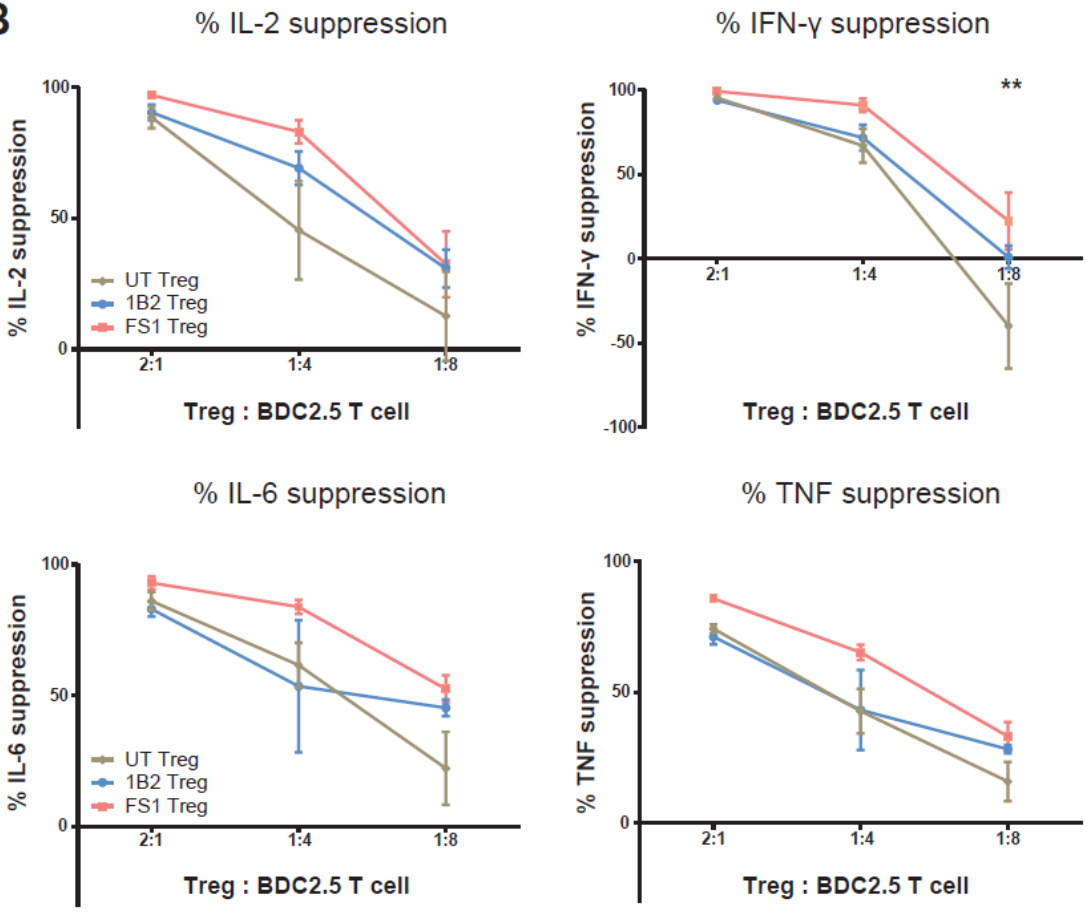

% IL-2 suppression
UT Treg
1B2 Treg
FS1 Treg
% IL-2 suppression
Treg : BDC2.5 T cell
% IFN-γ suppression
**
% IFN-γ suppression
Treg : BDC2.5 T cell
% IL-6 suppression
UT Treg
1B2 Treg
FS1 Treg
% IL-6 suppression
Treg : BDC2.5 T cell
% TNF suppression
% TNF suppression
Treg : BDC2.5 T cell

Figure 7. CAR Tregs mediate suppression of cytokine production.
(A) Supernatants from independent suppression assays were frozen and thawed to measure their cytokine content. The CAR Treg : BDC2.5 T cell ratios selected were 2:1, 1:4, and 1:8 (mean ± SEM, *n*=3). (B) The concentrations of each cytokine were normalized to the cytokine concentrations found in the BDC2.5 T cell only controls to calculate the efficiency of cytokine suppression mediated by each CAR Treg. Significance was determined by two-way ANOVA with Bonferroni's multiple comparisons post test. *FS1 vs UT ; *$p < 0.05$; **$p < 0.01$

3.5 CAR Tregs mediate enhanced suppression of cytokine production compared to polyclonal NOD Tregs

While the classical measurement of Treg function is suppression of lymphocyte proliferation, Tregs inhibit multiple aspects of effector T cell activity [167]. One such effect is suppression of T cell cytokine production, such as IL-2 and IFN-γ. Therefore, I collected supernatants from select suppression assay conditions to measure the effects of Tregs on production of IL-2, IFN-γ, IL-6 and TNF (Fig. 7A). As expected, supernatants collected from conditions with high Treg to BDC2.5 T cell ratios had the lowest levels of cytokines detected. At the 2 Tregs to 1 BDC2.5 T cell ratio, the Treg ratio was so high that there was no difference in suppression of the four cytokines between the different Treg groups. However, antigen-specific cytokine suppression became pronounced at the lower Treg ratios. FS1 CAR Tregs suppressed the IFN-γ and TNF production from stimulated BDC2.5 T cells significantly better compared to the polyclonal untransduced Tregs (Fig. 7A and B). FS1 CAR Treg mediated suppression of IL-6 production also appeared to be more efficient compared to the untransduced Tregs, although the trend was not significant. While the 1B2 CAR Tregs were unable to suppress BDC2.5 T cell proliferation better than untransduced Tregs, the 1B2 CAR Tregs did appear to dampen BDC2.5 T cell cytokine production more efficiently. Similar to the FS1 CAR Tregs, there was a trend towards more efficient 1B2 CAR Treg-mediated suppression of IFN-γ, IL-6 and TNF compared to untransduced Tregs at the low Treg to BDC2.5 T cell ratios.

3.6 FS1 CAR Tregs recognize the natural diabetogenic hybrid peptide presented by I-A^{g7}

In 2016, Delong *et al.* reported the discovery of the natural peptide target of the BDC2.5 T cell receptor [158]. They determined that WE-14, a cleavage product of Chromogranin A, could fuse with a portion of insulin to form a hybrid peptide, called 2.5HP. Given that the FS1 CAR was raised against the p63 peptide, a mimotope of 2.5HP, I wondered whether FS1 would also be able to bind to 2.5HP in the context of the Class II molecule I-A^{g7}. I added a 2.5HP:I-A^{g7} tetramer to my tetramer staining panel and found the FS1 CAR could indeed bind to the natural hybrid peptide (Fig. 8A). While the binding of the FS1 CAR to the 2.5HP:I-A^{g7} tetramer was highly specific, the binding efficiency to the p63:I-A^{g7} tetramer appeared to be superior (Fig. 8B). Measurements of the affinity for the FS1 mAb and 2.5HP:I-A^{g7} are now underway in Dr. Fife's laboratory.

When I discovered the FS1 CAR could target 2.5HP in the context of I-A^{g7}, I realized that the FS1 CAR Tregs now had high potential *in vivo*, as it could recognize a naturally occurring diabetogenic peptide. To determine whether the FS1 CAR would be able to stimulate Treg activation with 2.5HP, I set up functional assays with splenocytes pulsed with the 2.5HP peptide. I hoped to see a similar level of Treg activation and proliferation when the FS1 CAR Tregs were stimulated with the hybrid peptide as when they were cultured with p63 pulsed splenocytes.

Unfortunately, I was unable to observe any activation (Fig. 8C) or proliferation (Fig. 8D) when the FS1 CAR Tregs were cultured with 2.5HP pulsed splenocytes *in vitro*. While the FS1 CAR Tregs were able to bind to the 2.5HP:I-A^{g7} tetramer, the FS1 CAR Tregs were not stimulated by the 2.5HP pulsed splenocytes, as they did not upregulate CD69, LAP, or CTLA-4 expression (Fig. 8C) above baseline. Furthermore, FS1 CAR Tconvs did not proliferate in

response to splenocytes pulsed with 2.5HP (Fig. 8D), whereas they responded positively when cultured with p63 pulsed splenocytes.

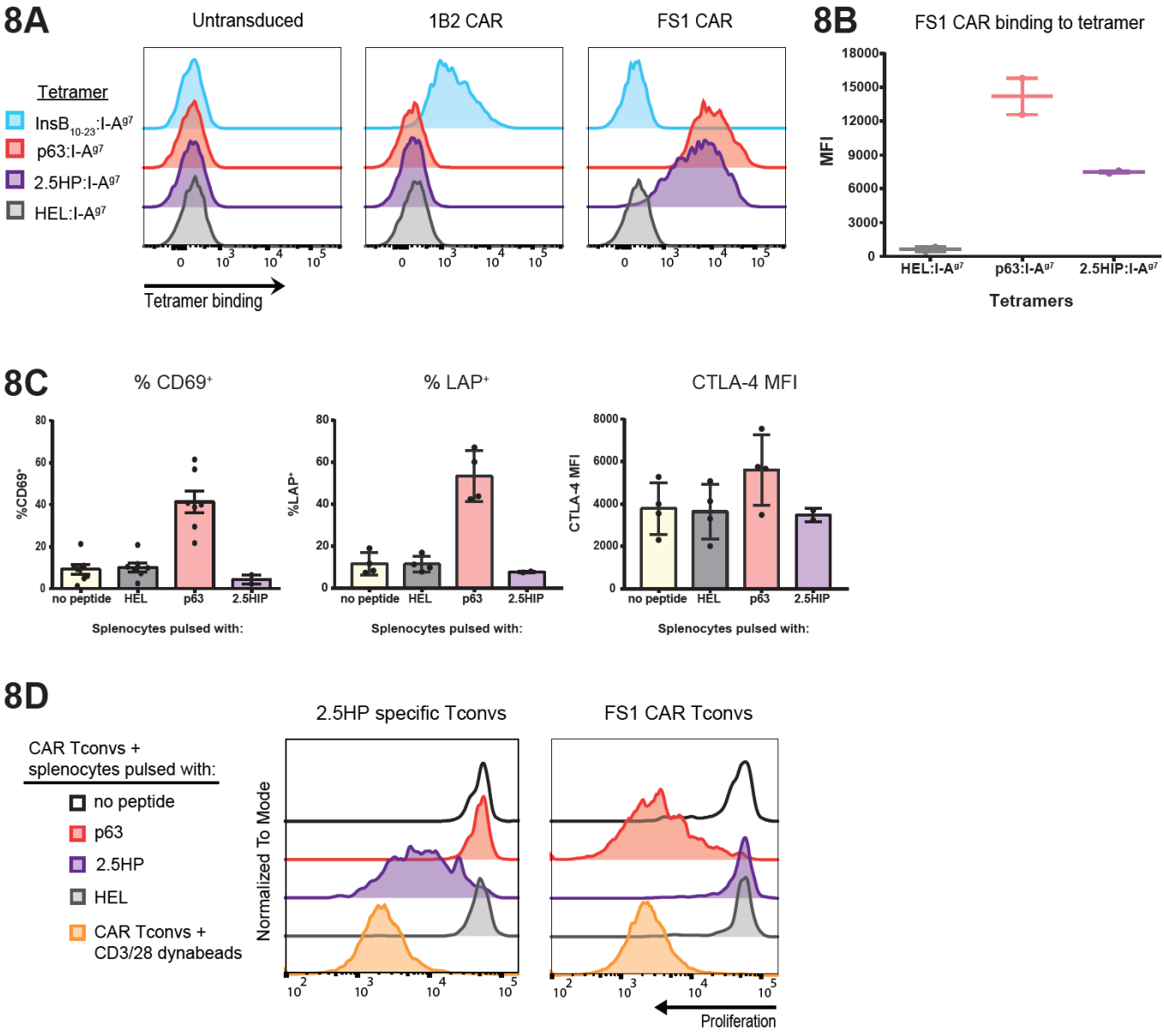


Figure 8. FS1 CAR can bind to 2.5HP:I-A^{g7} but does not induce cell activation.
(A) The 2.5HP:I-A^{g7} tetramer was added to the staining panel to assess if FS1 CAR Tregs could recognize the natural ChgA epitope. (B) Mean Fluorescence Intensity (MFI) comparison of FS1 CAR bound to p63:I-A^{g7} tetramer versus 2.5HP:I-A^{g7} tetramer (mean ± SEM, *n*=2). (C) Expression of activation markers on FS1 CAR Tregs when cultured with splenocytes pulsed with 2.5HP peptide overnight (mean ± SEM , *n*=2 for assays involving 2.5HP). (D) Proliferation histograms of 2.5HP specific Tconvs and FS1 CAR Tconvs after culture with splenocytes pulsed with 2.5HP for 3 days.

3.7 1B2 CAR Tconvs, not FS1 CAR Tconvs, induce GvHD-like symptoms in NSG mice

To determine how well each CAR could function *in vivo*, I set up an experiment with immunodeficient NOD/SCID IL-2Rγ^{null} (NSG) mice to determine whether CAR Tconvs could cause diabetes. NSG mice are engineered to be deficient in mature lymphocytes, and lack the expression of functional NK cells, B cells, and T cells [168]. This strain is useful as their lack of T cells will make it easier to detect the circulating CAR Tconvs following injection. Their lack of lymphocytes also provides another advantage in providing our injected cells with space, nutrients, and other resources that may not be offered in an immunocompetent mouse, due to the other lymphocytes competing for the same immune niche. I also decided to go forward with a preliminary diabetes induction model using CAR Tconvs as I believed diabetes development to be a faster and easier experimental endpoint to evaluate, compared to diabetes prevention. I had noticed our parallel CAR Tconv controls were more sensitive to antigen *in vitro* (Fig. 9A and 9B) than their Treg counterparts, suggesting injection of CAR Tconvs into mice could potentially have a more efficient biological effect.

NSG mice were injected with 4 x 10^6 1B2 CAR, FS1 CAR, Her2 CAR, or untransduced Tconvs, and monitored for 7 weeks as described in Methods. Surprisingly, the mice receiving the 1B2 CAR Tconvs had a rapid biological effect. Almost immediately after injection, mice that had received 1B2 CAR Tconvs quickly began losing weight (Fig. 9C) and showed acute symptoms of illness, such as decreased activity, facial pain, and distended abdomens. While none of the mice achieved the desired experimental endpoint (blood glucose > 15 mmol/L), I did notice that mice injected with 1B2 CAR Tconvs exhibited the largest week-to-week variance in blood glucose (Fig. 9D). Due to the severity of illness, all animals that had received the 1B2

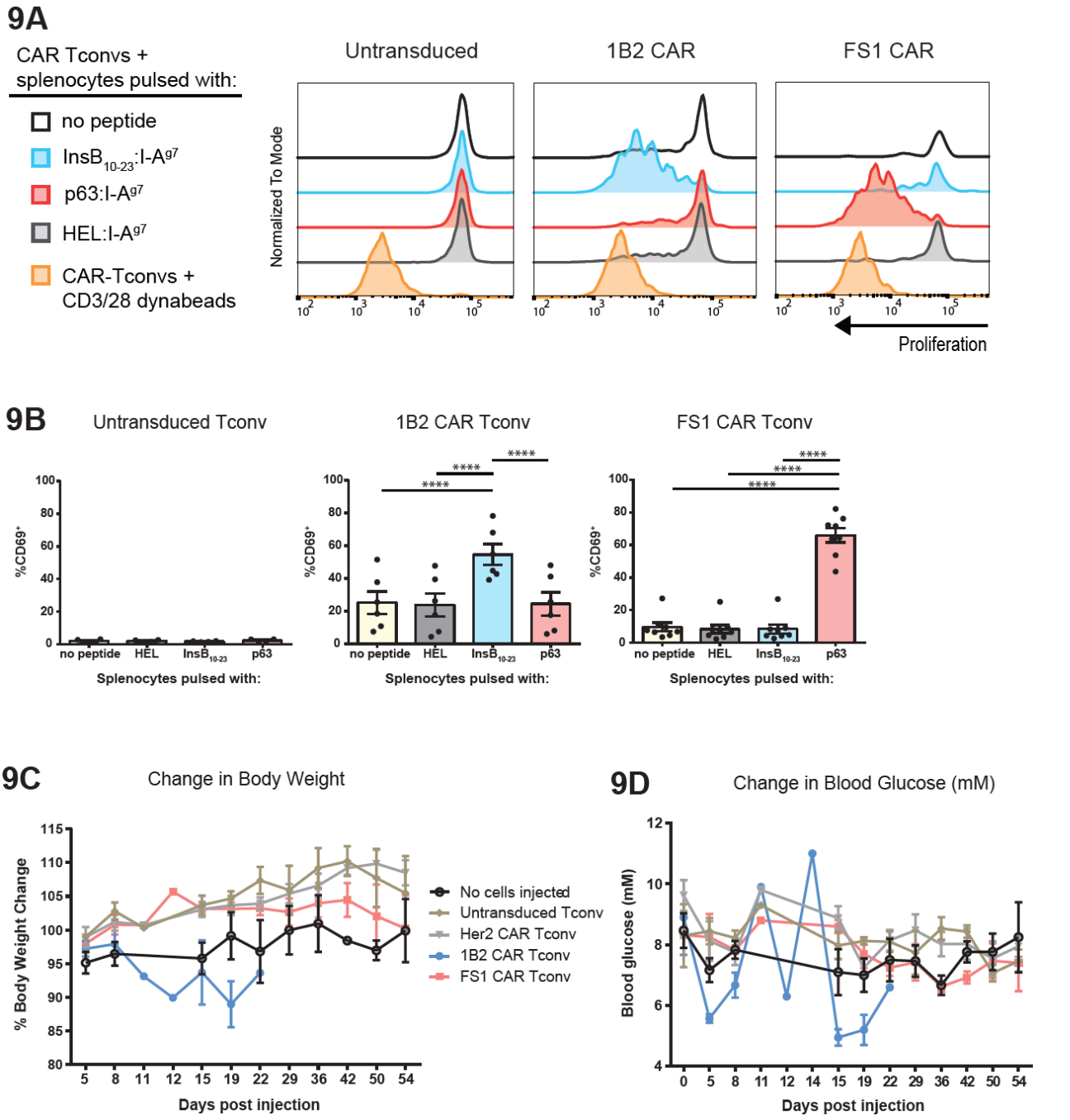


Figure 9. CAR Tconvs are more sensitive to peptide stimulation *in vitro* and may be more suitable for testing *in vivo*.

(A) Proliferation histograms of CAR Tconvs after culture with splenocytes pulsed with 10 µM peptide for 3 days. (B) CD69 expression on CAR Tconvs after culture with splenocytes pulsed with 15 µM peptide overnight (mean ± SEM, *n*=5-8). Significance was determined by one-way ANOVA with Tukey's multiple comparison's post tests; ****p < 0.0001. (C) 4 x 10^6 CAR Tconvs were injected into NSG mice. The body weight of each individual mouse was monitored and plotted as change in percent body weight relative to the start of experiment. (D) The blood glucose of each mouse was monitored to assess for diabetes development. (*n*=4 mice/group from 1 experiment).

CAR Tconv injections were euthanized early in the experiment, so I was unable to ascertain whether the 1B2 CAR Tconvs would have eventually caused diabetes.

Engraftment of the CAR Tconvs was monitored once a week by taking blood from the saphenous vein (Fig. 10A). As NSG mice lack T cells, I presumed any $CD4^+$ leukocytes were the injected CAR Tconvs. I initially analyzed cell engraftment by determining the % $CD4^+$ of mouse $CD45^+$ cells and observed increasing cell engraftment of untransduced and Her2 CAR Tconvs with each subsequent week (Fig. 10B). However, upon enumeration of cells via count beads, I determined mice injected with 1B2 CAR Tconvs actually had the highest number of circulating $CD4^+$ T cells, whereas FS1 CAR, Her2 CAR, and untransduced Tconvs had similar numbers of $CD4^+$ T cells each week (Fig. 10C). Myc expression on $CD4^+$ Tconvs was also monitored each week to assess the stability of each CAR *in vivo* (Fig. 10D). Initially, both 1B2 and FS1 CAR Tconvs retained very high levels of CAR expression following injection, but FS1 CAR expression declined after 3 weeks post injection.

The endpoint of the experiment occurred on day 54 post injection. All remaining mice were euthanized, and parts of the spleen, pancreas, lung, liver, and gut were harvested and preserved for histology. A small segment of the spleen and half of the pancreas were crushed to isolate the Tconvs for flow cytometric analysis. Even after almost 8 weeks post injection, CAR Tconvs could still be found in both the spleen and the pancreas (Fig. 11A). There was no difference in the proportion of $CD4^+$ cells found in either organ between the FS1 CAR Tconvs versus the Her2 CAR or untransduced Tconvs. However, only a small population of $CD4^+$ cells was found in either organ in mice injected with 1B2 CAR Tconvs. This is likely because the mice were euthanized much earlier (day 15-22) compared to the experimental endpoint of day 54. While the population of injected 1B2 CAR Tconvs found in each organ was small, the

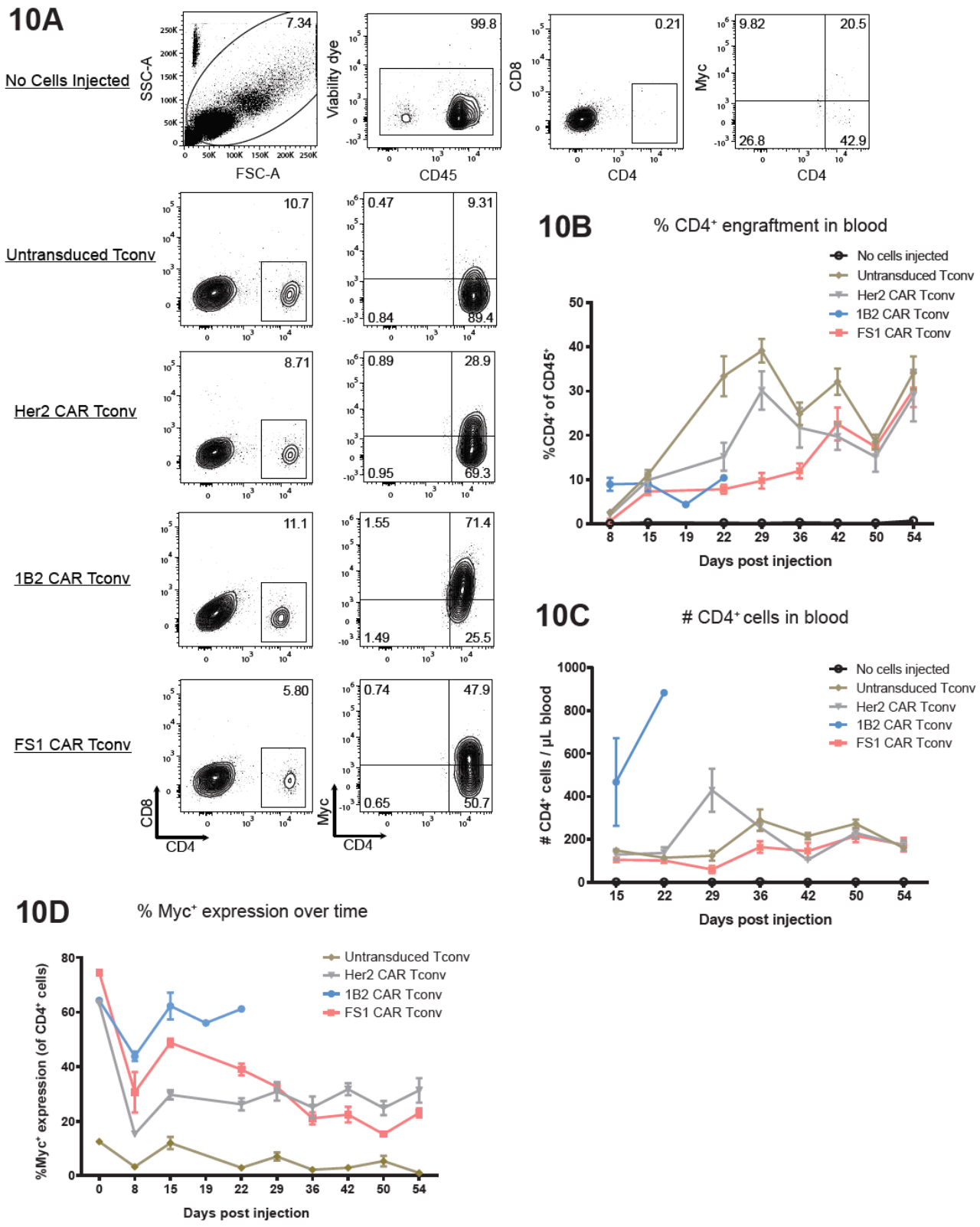


Figure 10. CAR Tconv phenotype following injection into NSG mice.
(A) Representative flow analysis of CAR expression on CAR Tconvs recovered from blood of injected NSG mice (*n*=4 mice/group). $CD4^+$ T cell engraftment in each mouse was monitored for up to 8 weeks post CAR Tconv injection. Engraftment was reported as a proportion (B) and as absolution cell numbers (C). (D) Changes in CAR expression were also monitored by detection of the Myc-tag with flow cytometry.

majority still retained high CAR expression (Fig. 11B). The 1B2 CAR expression in the spleen and pancreas was higher compared to the cells found in the blood (Fig. 10D and 11B), suggesting the environment within in the pancreas and spleen may be better at stimulating 1B2 CAR Tconv activation to maintain higher CAR expression. The FS1 and Her2 CAR Tconvs found in the organs had much lower CAR expression, although their CAR expression could not be directly compared to 1B2 CAR Tconvs due to the time difference between euthanization. Interestingly, while the Her2 CAR expression was fairly similar across blood, spleen, and pancreas on day 54 (between 20-30% Myc$^+$), I observed that the FS1 CAR Tconvs found in the pancreas had almost double the FS1 CAR expression (40%) compared to spleen and blood (20%).

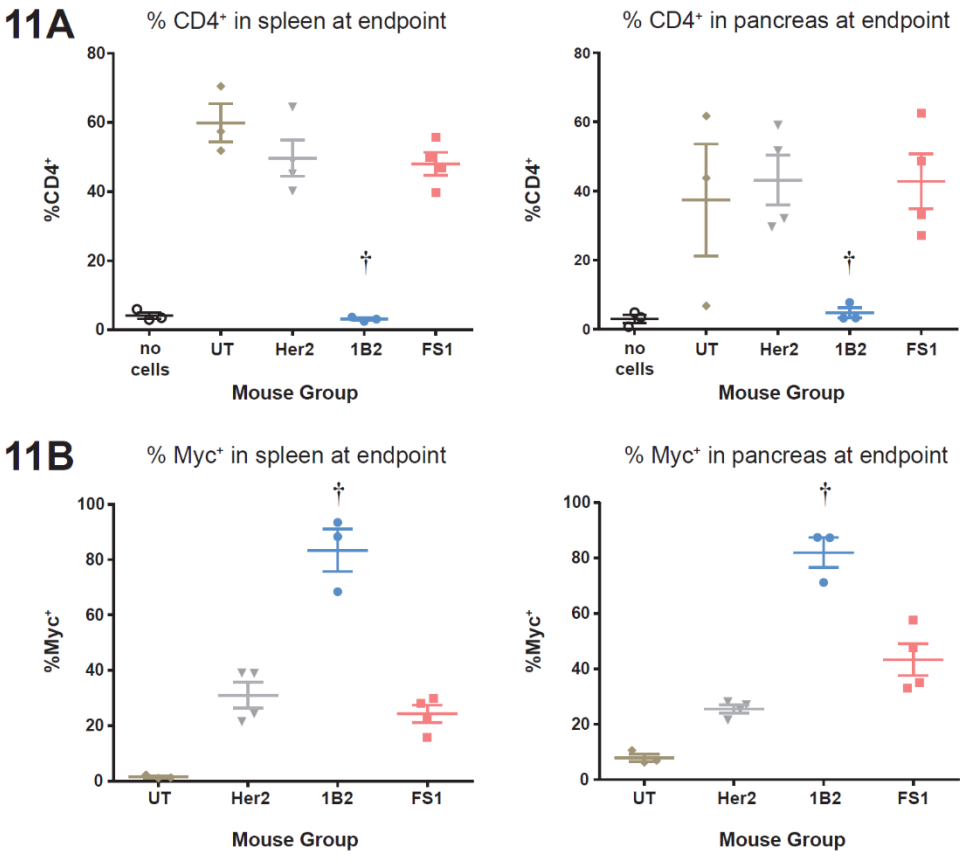


Figure 11. Isolation of CD4$^+$ T cells found in spleens and pancreas of injected mice at endpoint. Mice were euthanized on day 54 (†1B2 mice were euthanized early between day 15-22 due to poor health) and samples from each spleen and pancreas were taken to analyze their contents by flow cytometry (*n*=4 mice/group). (A) The proportion of CD4$^+$ T cells found in the spleen (left) vs. the pancreas (right). (B) CAR expression on CD4$^+$ T cells found in the spleen (left) and pancreas (right) was determined by the detection of the Myc tag.

Chapter 4: Discussion

There is currently a lack of therapies available to patients living with type 1 diabetes that aim to treat the underlying autoimmunity of the disease [1,6,169]. Thus, a growing field of research for T1D therapies involves immunomodulation to promote the shift towards a tolerogenic immune state [5]. Tregs are critical in maintaining immune homeostasis, and early clinical data has shown adoptive polyclonal Treg transfer therapies to be safe in humans, but with limited efficacy [95–97]. However, preclinical research has demonstrated that antigen-specific Tregs are superior to polyclonal Tregs in preventing and reversing disease in pre-diabetic and diabetic mice [102–105]. These antigen-specific Tregs bear a TCR specific towards islet antigens, but recent work in cancer immunotherapy has illustrated the power of CARs to redirect antigen specificity and modulate T cell function [118,170,171]. In this thesis, I aimed to generate antigen-specific CAR Tregs from NOD mice specific towards islet antigens complexed to MHC Class II molecules.

Monoclonal antibodies targeting $InsB_{10\text{-}23}$:I-A^{g7} and p63:I-A^{g7} were converted into CARs named 1B2 and FS1, respectively. Both 1B2 and FS1 CARs expressed well on NOD Tregs following retroviral transduction and mediated activation and proliferation in an antigen dependent manner. However, when testing whether CAR expression would result in enhanced suppression of effector T cell proliferation, there was no difference observed between the 1B2 CAR Tregs versus the polyclonal NOD Tregs. Both Her2 CAR and untransduced Tregs exhibited a very high level of suppression of BDC2.5 T cell proliferation, even though the Tregs were supposed to remain unstimulated. My proliferation and activation results had shown both Her2 CAR and untransduced Tregs were unable to respond to APCs pulsed with p63 peptide. Therefore, it is unlikely the high suppression from polyclonal Tregs is due to p63 stimulus.

Why were the polyclonal Tregs activated? It is possible they were receiving stimulation from endogenous peptides presented by the cocultured splenocytes. The spleen does not have afferent lymph vessels [172], and is therefore connected directly to the bloodstream in order to recruit leukocytes. As beta cells secrete insulin into the blood to mediate cellular adsorption of glucose, it is possible that some antigen presenting cells from the spleen will pick up the protein and present insulin peptides on their MHC Class II [173]. Furthermore, even though the splenocytes were sourced from NOD mice that did not display any diabetes symptoms, the immune cell infiltration of islets begins within the first weeks of life [136–138]. The early destruction of beta cells could also release additional insulin or other islet related antigens into the bloodstream, increasing the likelihood of splenocytes encountering islet antigens for presentation.

It has been shown that insulin-specific T cells make up a significant component of the T cell repertoire in NOD mice [174]; therefore, it is likely a portion of the NOD Tregs may bear insulin-specific TCRs. If the APCs in my suppression assays are presenting endogenous insulin, they may be stimulating the insulin-specific Treg population just enough to suppress BDC2.5 T cell proliferation. However, I have shown neither Her2 CAR nor untransduced Tregs upregulate early activation makers or proliferate in response to APCs pulsed with high levels of insulin peptide. These data suggest that if this insulin-specific population does exist within polyclonal Tregs, they must occur at a very low frequency and remain undetected in *in vitro* proliferation assays. Furthermore, while endogenous insulin-specific Tregs may partially explain why the polyclonal Tregs could suppress effector T cell proliferation, it still does not resolve why the 1B2 CAR Tregs were unable to exhibit enhanced antigen dependent suppression. The 1B2 CAR Tregs were provided additional insulin peptide, yet the 1B2 CAR Tregs displayed a similar suppressive capacity as both Her2 and untransduced Tregs.

As mentioned previously, Tregs mediate cellular suppression by various mechanisms through cellular contact, soluble factors, or metabolic disruption [52]. While it is still unclear which mechanisms are more biologically relevant when comparing *in vitro* versus *in vivo* studies [167], there is evidence suggesting cell-contact dependent mechanisms may be more important for *in vitro* suppression assays. It has been shown Tregs are unable to suppress T cell proliferation when the cells were separated by a membrane [175], and Treg supernatant alone could not suppress T cell proliferation *in vitro* [176,177]. One of the many contact dependent mechanisms of suppression is CTLA-4 engagement of CD80/86 on APCs. In my activation assays, I observed that 1B2 CAR Tregs and untransduced Tregs expressed similar levels of CTLA-4 (Fig. 4C). Even when 1B2 CAR Tregs were cultured with APCs pulsed with insulin, CTLA-4 upregulation was not particularly striking in comparison to their upregulation of CD69 (Fig. 4A) and LAP (Fig. 4B). Therefore, perhaps the similar suppressive capacity between the untransduced, Her2 CAR, and 1B2 CAR Tregs is partially due to similar levels of CTLA-4 expression.

I have noticed in all three *in vitro* functional assays that FS1 CAR Tregs appear to consistently perform better than 1B2 CAR Tregs. The FS1 CAR Tregs exhibited larger increases in CD69, LAP, and CTLA-4 expression (Fig. 4A-C), they proliferated moreso in response to relevantly pulsed splenocytes (Fig. 4D and E), and they suppressed both BDC2.5 T cell proliferation (Fig. 5C and E) and cytokine production (Fig. 7A and B) better than 1B2 CAR Tregs. The enhanced suppression demonstrated by FS1 CAR Tregs is even more striking when considering 1000X less peptide is added to stimulate the FS1 CAR Tregs compared to 1B2 CAR Tregs. The Fife lab had determined that the FS1 mAb affinity towards p63:I-A^{g7} is much higher than the 1B2 mAb affinity towards $InsB_{10-23}$:I-A^{g7} (Fig. 1C). In cancer immunotherapy, increasing the CAR affinity towards its target antigen can increase their capacity to recognize

low-density antigens [178]. CAR T cells expressing a lower affinity CAR targeting EGFR were only capable of killing cells over-expressing EGFR, whereas the cytotoxicity of T cells expressing a higher affinity CAR did not change regardless of antigen density [179]. Since peptide-MHC complexes are considered to be relatively low-density antigens [180], the superiority of the FS1 CAR may be due to its higher affinity causing increased sensitivity towards p63:I-A^{g7} stimulation.

One interesting finding I made was that although the FS1 mAb was raised towards the p63 peptide in the context of I-A^{g7}, when re-formatted into a CAR, it could also bind to the 2.5HP:I-A^{g7} tetramer. Prior testing by the Fife lab had found that the FS1 CAR was unable to recognize the p31 peptide, another chromogranin A mimotope that only differs from p63 by 2 amino acids. Therefore, this suggests that FS1 CAR binding is highly dependent on the identity, size, and charge of the exposed resides that make up the FS1 epitope. The first amino acid in p31 is a tyrosine [181], p63 has an arginine, and 2.5HP has a leucine, and they are all predicted to bind to P1 of the MHC binding groove, which is a position often involved in TCR recognition [182]. Therefore, these amino acids are likely to be exposed residues and will heavily influence the strength of FS1 binding. Tyrosine and leucine are hydrophobic, but arginine is positively charged under physiological conditions. This could greatly enhance the binding energy of FS1, resulting in stronger electrostatic interactions between FS1 and p63:I-A^{g7}. Another consideration is tyrosine is very bulky compared to leucine and arginine, due to its aromatic side chain. In combination with its weak interactions, tyrosine may further block FS1 binding to p31 by steric hinderance. On the other hand, the 2.5HP epitope still allows for FS1 binding as its smaller size would not prevent FS1 from interacting with the epitope, even though its electrostatic interactions may be weaker compared to the p63 epitope.

Although the FS1-CAR could bind to the 2.5HP:I-A^{g7} tetramer, this did not translate to functional activity *in vitro* with 2.5HP pulsed APCs. While the affinity measurements of the FS1 CAR towards 2.5HP:IA^{g7} have yet to be determined, I predict its affinity will be much lower than the affinity towards p63:I-A^{g7} due to the predicted poor electrostatic interactions of 2.5HP as well as my comparison of FS1 tetramer binding MFIs (Fig. 8B). Perhaps the difference in affinity is large enough that the FS1 CAR is unable to recognize naturally presented 2.5HP complexed to I-A^{g7}, especially when considering that peptide-MHC complexes are low-density antigens. It is also likely the strong binding of the FS1 CAR to the 2.5HP:I-A^{g7} tetramer may have been artificially enhanced by the "avidity effect" [183], where multimerization of peptide:MHC significantly extends the duration of the receptor-multimer interaction. These data have significant implications for our understanding of CAR Treg biology and suggest that the affinity of the scFv is a key consideration, especially for low-density target antigens.

I set up an *in vivo* study to determine whether the peptide:I-A^{g7} targeting CAR Tconvs would cause diabetes. While MHC Class II expression is classically described as restricted to antigen presenting cells only, it has been shown MHC Class II can be induced in other cell types as well [184–186]. In the NOD mouse model, pancreatic beta cells were found to increase their expression of I-A^{g7} during inflammation by IFN-γ exposure [187]. Therefore, I did not expect any of the CAR Tconvs to induce any form of illness. Since I injected non-diabetic NSG mice, the I-A^{g7} expression on their islets should have been minimal, and both CARs were specifically designed to only target islet APCs. In fact, Zhang *et al.* has shown a single infusion of $CD8^+$ effector T cells expressing an $InsB_{9\text{-}23}$:I-A^{g7} targeting CAR in pre-diabetic NOD mice was sufficient to delay hyperglycemia by 5 weeks compared to irrelevant CAR controls [188]. They hypothesized the $InsB_{9\text{-}23}$:I-A^{g7} CAR redirected the cytotoxic CAR T cells to kill the APCs

presenting the $InsB_{9-23}$ peptide, thereby preventing the activation of diabetogenic T cells and delaying diabetes onset.

I was surprised to observe how pathogenic effects of the 1B2 CAR Tconvs after cell injection. The mice experienced substantial weight loss, fluctuations in blood glucose, and a large expansion of injected $CD4^+$ cells. Upon organ harvest, I noticed the liver, spleen, and pancreas were noticeably inflamed, resembling a GVHD/cytokine storm like disease. I wondered if this could be indicative that the 1B2 CAR was not as specific as I initially thought. However, the tetramer staining and functional *in vitro* assays have consistently shown the 1B2 CAR only binding to $InsB_{10-23}$:I-A^{g7} tetramers, and 1B2 CAR bearing cells could only activate and proliferate in response to APCs pulsed with $InsB_{10-23}$ peptide. Given that insulin is present in the bloodstream, I then wondered whether APCs residing outside of the pancreas might present insulin peptides. Indeed, it has been recently found in NOD mice that circulating B cells and other blood leukocytes are able to present insulin peptides [173]. The APCs were isolated from blood after a glucose challenge and could stimulate antigen-specific T cell responses after immediate coculture with diabetogenic T cells [173]. Although NSG mice lack B cells, they retain expression of an innate immune cell population that can process antigen for presentation to T cells [168]. It may be possible that the 1B2 CAR Tconvs were activated systemically by the APCs in the blood, causing symptoms reminiscent of cytokine release syndrome seen by CAR T cells in cancer immunotherapy [189]. However, it is also possible the 1B2 CAR Tconvs were activated by an unknown systemic antigen *in vivo*.

On the other hand, transfer of FS1 CAR, Her2 CAR, and untransduced Tconvs appeared to be non-toxic, as the mice maintained a normal body weight, and appeared physically active and healthy. The injected cells could still be found in the circulation almost 2 months post cell

injection (Fig. 10B and C) and were able to expand within the mice without causing disease. I was also able to find a population of injected $CD4^+$ cells in the spleen and pancreas after 54 days post injection, whereas the $CD4^+$ population was still quite low in the 1B2 mice that were euthanized much earlier. An interesting observation was the increased CAR expression by FS1 CAR Tconvs that were found in the pancreas, compared to the spleen and blood. Even though the FS1 CAR Tconvs did not appear to recognize any of the naturally presented diabetogenic peptides (insulin or 2.5HP) *in vitro*, this finding suggests that the FS1 CAR may recognize a natural target in the pancreas to maintain the higher CAR expression.

Unfortunately, as I did not induce diabetes in our NSG mice prior to injection, I was unable to determine whether any of the CAR Tconvs (apart from 1B2) were exhibiting any effect on a diabetes-relevant phenotype. Given Zhang *et al.* has shown some promising results with their $InsB_{9\text{-}23}$:I-A^{g7} CAR T cells, if I were to try the *in vivo* experiment again, I would use pre-diabetic NOD mice. Then, I would be able to properly assess whether infusion of islet-peptide:I-A^{g7} CAR Tconvs could kill the islet-peptide presenting APCs to prevent diabetogenic T cell activation and delay diabetes. While this *in vivo* experiment could be conducted using diabetic NSG mice (diabetes can be quickly induced by injecting NSG mice with BDC2.5 T cells), their immunodeficiency may potentially result in subpar CAR Tconv activation. In addition to T cells, NSG mice are engineered to lack B cells, and mouse studies have implicated B cells as important APCs for the development of murine T1D [14,15,173]. Furthermore, NSG mice also have defective macrophages and dendritic cells [168], and their functional immaturity may result in inefficient antigen presentation and thus, weakened CAR Tconv responses.

In summary, I have successfully generated two CAR constructs (1B2 and FS1) that are specific to islet peptides complexed to the mouse MHC Class II I-A^{g7}. Both CAR constructs

were expressed on the surface of Tregs and Tconvs, and could induce antigen dependent cell activation and proliferation when stimulated with the relevant antigen. While only the FS1 CAR Tregs suppressed BDC2.5 T cell proliferation beyond the levels of background suppression, both 1B2 and FS1 CAR Tregs suppressed inflammatory cytokine production. I also discovered the FS1 CAR, originally raised against an artificial peptide, had the potential to recognize the natural diabetogenic peptide found in NOD mice.

Thus, I believe the 1B2 and FS1 CAR Tregs may have the therapeutic potential to delay T1D onset in pre-diabetic NOD mice. Preliminary *in vivo* work using CAR Tconvs showed that the infusion of FS1 CAR Tconvs was non-toxic, and CAR Tconvs were able to survive and maintain CAR expression for an extended period in an NSG mouse model. While the 1B2 CAR Tconvs did induce illness in mice, the response also suggests that the 1B2 CAR is capable of strong activation by natural antigen recognition. Altogether, our *in vitro* and *in vivo* work with 1B2 and FS1 CARs lay the groundwork for a proof-of-concept where human CAR Tregs targeted towards islet-peptide:MHC Class II complexes are a feasible and efficacious therapy for Type 1 diabetes. Adoptive transfer therapy with antigen-specific Tregs can suppress the autoimmune attack causing the disease, which may lead to disease remission and rescue of any remaining insulin producing islet beta cells.

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