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Peripherally-Derived Regulatory T Cells in Mouse Autoimmune Diabetes

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Peripherally-Derived Regulatory T Cells in Mouse Autoimmune Diabetes

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To my parents, Catherine and Dan.

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CONTRIBUTIONS TO THE PRESENTED WORK

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Peripherally-Derived Regulatory T Cells in Mouse Autoimmune Diabetes

Daniel Ryan Holohan

ABSTRACT

Regulatory T cells (Tregs) are part of the suppressive arm of the immune system, playing an important role in maintaining immune homeostasis and preventing excessive inflammatory responses. As autoimmune diseases become increasingly prevalent, it is important to clarify the role of Tregs in etiology and their potential for use in cell therapy. Characterization of Tregs has revealed specialized subsets that have unique functions in controlling tissue-specific inflammation. Of particular interest is the distinction between Tregs that differentiate within the thymus (tTregs) versus the periphery (pTregs). While tTregs recognize self-antigen, pTregs are generated to induce tolerance to non-self-antigens such as those from commensal microbiota and self-antigens not presented in the thymus. pTregs could play a unique non-redundant role in type 1 diabetes (T1D), a chronic autoimmune disease, based on recent work showing connections between microbiota and T1D incidence as well as the generation of neoantigens within the pancreas. Therefore, we created a new model to determine the importance of pTregs in T1D by deleting a key genetic enhancer of *Foxp3* for pTreg generation (conserved noncoding sequence CNS1) within the autoimmune prone non-obese diabetic (NOD) mouse. CNS1 deletion does not alter Treg frequencies or characteristics in young female NOD mice in most organs except the large intestine lamina propria, where increased Helios expression and reduced RORγt expression suggest a shift towards tTregs. Despite increased insulitis, these mice do not exhibit increased T1D

incidence. Surprisingly, we also observed that Tregs in the islet do not recognize peripherally-derived neoantigens made from insulin fusion products, and the reactivity of Tregs and Tconvs is not changed in prediabetic NOD CNS1$^{-/-}$ females. Lastly, preliminary data suggest NOD CNS1$^{-/-}$ females do not show defects in maternal-fetal tolerance but could exhibit significantly reduced Treg frequencies in the spleen and increased frequencies in the pancreatic and mesenteric lymph nodes. We concluded that CNS1-dependent pTregs do not play a significant role in the NOD mouse model, with tTregs being the key regulators of T1D. Additionally, Tregs within pancreatic islets largely recognize thymically-presented antigens, and pTregs are not frequently generated against pancreatic neoantigens.

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CHAPTER 1: INTRODUCTION

1.1 CENTRAL TOLERANCE

The adaptive immune system has the tremendous ability to generate tailored responses to the constantly evolving pool of potential pathogenic antigens in the environment. Within T cells, multiple copies exist of the gene segments that comprise the α and β T cell receptor (TCR) chains, and through somatic recombination of these segments as well as the random pairing of α and β chains themselves, massive diversity is generated, leading up to ~10^{15} potential TCRs in the human T cell repertoire. However, the realized specificity in individuals is substantially lower and is closer to ~2.5 x 10^7 unique TCRs (1). This reduction in diversity is due to negative selection of T cells as they undergo development within the thymus. Immature thymocytes undergo clonal deletion if their TCRs bind too strongly to the antigen-presenting major histocompatibility complex (MHC), preventing the development of autoreactive T cells that are nonspecifically activated through interactions with our MHC alone (2,3). Deletion of autoreactive cells is further aided by medullary thymic epithelial cells (mTECs) that express the autoimmune regulator *(AIRE*) transcription factor, which mediates the promiscuous expression of tissue-specific antigens such as insulin within the thymus. AIRE-expressing mTECs allow for further refinement of the TCR repertoire by preventing the development of T cells that specifically recognize self-antigens, and deletion of this gene causes systemic autoimmunity (4–6). Overall, these processes contribute to central tolerance of self by the immune system.

1.2 PERIPHERAL TOLERANCE AND REGULATORY T CELLS

Although negative thymic selection is highly effective, central tolerance alone is not sufficient to prevent autoreactive T cells from entering the periphery. Therefore,

there are several mechanisms in place that promote peripheral tolerance. One of the key cell types that aid in maintaining peripheral tolerance is regulatory T cells (Tregs). Tregs are an immunosuppressive subset of CD4+ T cells that were initially identified due to their strong expression of the high affinity α chain (CD25) of the receptor for interleukin-2 (IL-2), a key survival and proliferation cytokine for T cells (7). Unlike other T cell subsets, Tregs do not produce their own IL-2 despite being highly dependent on IL-2 signaling (8,9). Tregs are also unique compared to other T cell subsets since they recognize self-antigen but do not undergo negative selection within the thymus (10,11).

This alternative differentiation pathway is dependent on strong costimulation through the T cell activating CD28 pathway (12) and the expression of the fate-determining forkhead box P3 (Foxp3) transcription factor, which confers stable cell identity and suppressive function (13–15). Stable Foxp3 expression in Tregs is conferred in part by epigenetic control of specific CpG motifs located in Treg-specific demethylated regions (TSDRs) in the 5' untranslated region of the *Foxp3* locus (16). Early work showed that through its forkhead (FKH) domain, Foxp3 is a transcriptional repressor that inhibits cytokine production by binding adjacently to regulatory regions where the activating transcription factor nuclear factor of activated T cells (NFAT) binds in cytokine genes (17). Later, Foxp3 was also shown to directly interact with NFAT and another activating transcription factor, NF-κB, to inhibit expression of IL-2, IL-4, and IFN-γ (18), allowing Tregs to be activated by self-antigen without producing potentially pathogenic cytokines. Surprisingly, Foxp3 can also interact with NFAT to activate the expression of key genes for Tregs, including CTLA-4 and CD25 (8), showing that Foxp3 has a broad range of functions in maintaining Treg function and identity.

Mutations in Foxp3 or depletion of Tregs causes systemic autoimmunity in both mice and humans, showing the necessity of mechanisms outside of central tolerance in order to maintain normal immune homeostasis. Foxp3-deficient mice die within 3-4 weeks post-birth and have scaly skin similar to eczema as well as severe anemia (19). Due to their scaly appearance, the initial disease in these mice was called “scurfy,” and the causative gene (*Foxp3*) was called *scurfin.* Later, scurfy was linked to extensive lymphoproliferation and systemic infiltration in various organs, including the kidney, heart, pancreas, lung, gastrointestinal tract, spleen, and liver, suggesting that disease is caused by immune dysfunction and not complete immunodeficiency (20). Scurfy was then concretely linked to human immunodysregulation and polyendocrinopathy enteropathy X-linked (IPEX) syndrome based on genetic comparisons of the mouse *Foxp3* and human FOXP3 loci and the corresponding mutations in the FKH domain that cause disease (21). Tregs also play an important role in establishing tolerance during transplantation. In hematopoietic stem cell transplantation (HSCT), Tregs inherently present in the graft mitigate the development of graft-versus-host disease (GVHD), and the addition of freshly isolated or *ex* vivo-expanded Tregs during HSCT further reduces or abrogates GVHD (22,23). For solid organ transplantation, skin graft rejection models show that administration of Tregs can reduce graft rejection, and continued tolerance to skin grafts is mediated by tissue-resident Tregs within the graft (24–26).

Regulatory T cells have various cell-contact dependent and independent suppressive mechanisms for maintaining homeostasis and preventing excessive inflammatory responses. High CD25 expression allows T_{regs} to compete for IL-2 produced by other T cells, inhibiting their growth in the process (27). T_{regs} more

specifically target T cell subsets through the release of cytokines, which different diseases and inflammatory environments requiring different. For instance, Treg-mediated control of colitis through IL-10 (28) and inflammatory bowel disease through IL-35 (29), while in cancer settings, TGF-β seems to play a key role in controlling CD8+ T cells (30). Tregs can also inhibit cells such as dendritic cells (DCs) through direct cell interaction. CTLA-4, an inhibitory receptor, is upregulated on Tregs and outcompetes CD28, a costimulatory receptor that promotes T cell activation, for binding to its target ligands B7-1/B7-2 (also known as CD80/86) (31,32). Binding of CTLA-4 can also lead to the trogocytosis of these ligands (33). The importance of CTLA-4 in Treg function has been shown due to the fact that CTLA-4-deficient mice exhibit similar disease to Foxp3-deficient mice, and CTLA-4 deficiency can be slightly mitigated but not cured if Foxp3 expression is forced in CD4+ T cells in these mice (34). Although Tregs are antigen-specific, it has been shown that activated Tregs are capable of suppressing immune cells in their general vicinity regardless of those cells own antigen specificity (35,36). This bystander suppression is a key feature of Treg's general ability to maintain homeostasis.

Tregs can also acquire specialized functions and characteristics to specifically control inflammation and other processes in tissues. These tissue-resident Tregs have been found in both humans and mice in various contexts (37,38). For example, Tregs can express peroxisome proliferator-activated receptor (PPAR)-γ, a key adipocyte differentiation factor (39). PPAR-γ expression by Tregs facilitates their accumulation in the VAT and ability to control the insulin sensitivity of adipocytes (40). Memory Tregs (mTregs) have also been identified within the skin (41). These cutaneous mTregs can

promote tolerance to skin commensal bacteria as well as induce epithelial stem cell proliferation and differentiation to promote hair follicle regeneration (42,43). Most cutaneous ear Tregs have also been shown to express retinoid-related orphan receptor α (RORα), which facilitates the control of allergic skin inflammation in models of atopic dermatitis induced by the application of calcipotriol and allergens such as ovalbumin and peanut extract (44). The unique markers and locations of these tissue-resident Tregs could provide the basis for more effective targeted therapies.

1.3 TYPE 1 DIABETES

One of the most well-known and studied versions of autoimmunity is type 1 diabetes (T1D). T1D is a chronic and currently incurable disease that is caused by the immune system destroying the insulin-producing beta cells of the pancreas. As many as 1.25 million Americans currently live with T1D, and incidence has increased 21% between 2001 and 2009, showing that it is a growing problem within the United States. 40,000 new patients are diagnosed each year, with onset usually occurring during early adolescence (10-14 years of age). Although there is evidence of genetic susceptibility to T1D, the concordance in twins is ~60%, showing that environmental factors significantly contribute to T1D incidence. One hypothesis is that a precipitating enteroviral infection triggers disease due to molecular similarities of viral and pancreatic antigens or bystander inflammation. The probability of T1D onset can be determined based on the presence of autoantibodies, with approximately 15% of individuals that have detectable autoantibodies against a single islet antigen developing T1D within 10 years. This probability increases to 70% of individuals within 10 years for those with autoantibodies against two or more epitopes, and nearly all of these individuals become diabetic during

their lifetime. Common antigen specificities for autoantibodies in T1D patients include insulin, glutamic acid decarboxylase 65 (GAD65), zinc transporter 8 (ZnT8), and anti-I-A2 (45).

After destruction of about 70-80% of the beta cells, the body is no longer able to appropriately control blood glucose levels, leading to the need for insulin injections or an insulin plump (46). However, dosing of exogenous insulin can be challenging and often leads to patients being hyper- or hypoglycemic, which chronically can cause cardiovascular damage and acutely lead to life-threatening comas (47). In order to reduce the burden of self-care for those with T1D and increase glycemic control, many groups are creating an “artificial pancreas” that automates insulin delivery through the use of continuous glucose monitors. Newer technologies are also attempting to simultaneously administer glucagon. However, current artificial pancreata still require frequent calibration by users and substantial improvements in accuracy to be truly automated, and these technologies do not restore true pancreatic function. As a result, there is much interest in finding new therapies that better maintain glycemic control.

1.4 THE NON-OBESE DIABETIC MOUSE MODEL

The first and now primary mouse model used to model spontaneous insulin-dependent diabetes mellitus (IDDM) is the non-obese diabetic (NOD) mouse model. This strain was derived from JCL-ICR mice, which are prone to getting cataract eyes with microphthalmia. Initial NOD progeny showed high incidence of T1D, with 80% of females and 20% of males becoming diabetic by 30 weeks (48). Continued characterization of this strain showed that these mice are also prone to other diseases such as autoimmune thyroiditis (49), autoimmune peripheral polyneuropathy (50), and

non-organ specific autoimmunity such as hemolytic anemia (51). The genetic risk factors for these diseases was determined through outbreeding to C57BL/6 (B6) mice, which revealed that more than 20 insulin-dependent diabetes (IDD) loci exist in NOD mice (52). One of the earliest and strongest causative agents identified was I-Ag7 (53), an MHC II molecule known for having a β chain that binds to a broad range of peptides with lower average affinity, potentially impacting thymic deletion of autoreactive T cells (54). Human MHC II serotypes HLA-DQw8 and HLA-DR4 are also associated with increased rates of T1D susceptibility (55–57). Other IDD loci, such as IDD3, have revealed polymorphisms similar to what has been observed in human genome-wide association studies (GWAS) for T1D (58), including *IL2RA* (59), *CTLA4* (60), and the tyrosine phosphatase gene *PTPN22* (61). Later characterization revealed specific loci that make NOD mice prone to other autoimmune diseases such as peripheral neuropathy (62). CD28-deficient NOD mice as well as NOD mice deficient for the CD28 ligands CD80 and CD86 exhibit exacerbated type 1 diabetes due to a severe impairment in Treg generation (63), and specific deficiency for CD86 results in peripheral polyneuropathy (50).

Beyond similarities in the genetic risk factors for T1D, NOD mice also have similar presentation and progression in the development of disease as humans. Development of diabetes in NOD mice mimics human T1DM based on two "checkpoints." The first checkpoint at 2-4 weeks is characterized by priming of immune cells within the pancreatic lymph nodes and followed by infiltration into the pancreatic islets (insulitis) without β cell damage at 4-6 weeks, a shift that is also correlated with changes in the genetic profiles of splenic immune cells to an active immune cell group.

Then, starting around 10 weeks, NOD mice start to exhibit T1D (64–66). This switch from infiltration to islet destruction has also been shown through NOD mice with MHC I selectively deleted on pancreatic β cells, which show that ultimate destruction does not occur despite infiltration, suggesting a potential role or CD8+ T cells (67). T1D can also be acutely triggered by depleting Tregs in NOD mice. Administration of the chemotherapeutic cyclophosphamide, which depletes all proliferating cells but particularly impacts Tregs in the pancreatic infiltrate, causes rapid T1D (68,69), and direct depletion through the use of the transgenic NOD mice that express diphtheria toxin receptor in Foxp3+ Tregs (Foxp3-DTR mice) also rapidly induces T1D (70).

1.5 REGULATORY T CELL THERAPY FOR TYPE 1 DIABETES

Early work in the NOD mouse model has shown that Tregs are a viable option for use in cell therapies for T1D. Administration of expanded polyclonal Tregs are capable of controlling T1D, but more efficient control is also possible through the use of antigen-specific Tregs. The CD4+ T cell TCR clone BDC2.5, which recognizes a secretory protein associated with pancreatic islets called chromogranin A (ChgA), has been used to study the role of T cells in the different steps of T1DM progression through NOD TCR transgenic mice (71–73). Comparisons between NOD and NOD BDC2.5+ Tregs in controlling T1D induction in NOD scid gamma (NSG) mice receiving pathogenic NOD BDC2.5+ T cells revealed that antigen specificity conferred much more potent suppression of disease, with adoptive transfer of as few as $5x10^3$ NOD BDC2.5+ Tregs being able to prevent T1D induction in some mice and $5x10^4$ NOD BDC2.5+ Tregs protecting all mice from disease (36,74,75). In the NOD $CD28^{-/-}$ mouse model that develops both T1D and autoimmune pancreatitis (AIP) in the exocrine pancreas,

adoptive transfer of NOD BDC2.5+ Tregs prevents or delays T1D progression without inducing global immunosuppression. Furthermore, adoptive transfer did not impact AIP in the nearby but anatomically distinct exocrine pancreas, providing evidence that targeted autoimmune control is possible (76).

Human Treg therapy is still in the early stages of development but shows signs of promise. Phase I trial results show that adoptive transfer of expanded autologous polyclonal Tregs is safe, leading to no adverse events. Furthermore, the transferred Tregs were stable and long-lasting, and in some patients, C-peptide levels persisted out to 2+ years, suggesting a potential therapeutic effect as well (77). Later, adoptive transfer of Tregs into children with new-onset T1D showed that 8 out of 12 patients met the criteria for remission after one year with 2 no longer requiring exogenous insulin compared to 2 out of 10 patients treated with control (78,79).

Other groups are also identifying and expressing TCRs in Tregs to redirect their antigen specificity to ones relevant in T1D. Preclinical *in vitro* results show that islet antigen-specific transgenic Tregs are capable of suppression in response to cognate antigen, but their activation was weaker compared to other TCRs identified, suggesting optimization might be required to achieve higher affinity receptors (80). High affinity TCRs for islet autoantigens were also shown to lead to greater suppressive capacity for Tregs compared to lower affinity TCRs, further suggesting that optimal signaling is key for the potential of antigen-specific Treg therapy (81). Lastly, in light of the success of engineering antibodies with T cell signaling domains to create chimeric antigen receptors (CARs) for cancer immunotherapy, people are exploring how to optimize CAR

signaling domains and targets for Treg therapy, with early work showing potential success with an anti-HLA CAR Treg for transplantation (82).

1.6 PERIPHERALLY-DERIVED VS THYMICALLY-DERIVED TREGS

Although most Tregs are thought to be thymically-derived (tTregs), Foxp3 expression can be induced in naïve CD4+ T cells in the periphery to generate stable Tregs as well (pTregs). In the absence of tTregs, pTregs can be generated *in vivo* through oral or intraperitoneal administration of antigen and are sufficient to induce tolerance in a hyper-IgE allergy and asthma model (83). Prolonged exposure to antigen administered subcutaneously via an osmotic pump is also sufficient to generate pTregs (84). Foxp3 induction *in vitro* (iTregs) has been demonstrated by supplementing the media of activated naïve CD4+ T cells with the transforming growth factor β (TGF-β) (85), and the effects of TGF-β are enhanced with coadministration of retinoic acid (RA) (86). However, TGF-β alone is not sufficient for stable induction of Foxp3 expression in cells, since iTregs do not have same TSDR patterns as normal Tregs and lose Foxp3 expression when restimulated in the absence of TGF-β (87). Proper TSDR patterns and increased stability of iTregs can be promoted when vitamin C is also supplemented with TGF-β, which also improves the suppressive capacity of iTregs in an allogeneic skin transplantation model (88). In contrast, when pTregs are generated *in vivo*, proper TSDR patterns are observed and stable Foxp3 expression is maintained, showing that there are additional factors necessary for proper pTreg differentiation (16,87).

Together, TGF-β and RA have been shown to also aid in the extrathymic generation of Tregs *in vivo*, particularly in gut-associated lymphoid tissue (GALT) such as the lamina propria of the intestine and mesenteric lymph nodes (mLN) (89,90). The

GALT is thought to be particularly enriched for pTregs that recognize and tolerize both commensal bacteria and dietary antigens, with around 75% of the Tregs in the colon and small intestines being pTregs (91). These pTregs have TCRs distinct from Treg TCRs in other tissues, and when these TCRs are transgenically expressed within the thymus, they do not lead to Treg generation (92). The large majority of pTregs within the colon also express the transcription factor retinoic acid-related orphan receptor-γt (RORγt). Although RORγt expression is typically associated with Th17 cells, these pTregs are stable suppressors that induce RORγt expression in response to certain microbiota, providing specialized functions in controlling gut inflammation (93–95).

RORγt+ pTregs also have a less diverse but unique TCR repertoire that is more similar to Th17 cells than Tregs or other Tconv subsets (96). Overlap in the TCR repertoire of Tregs and Th17 cells might be due to the fact that some commensal strains can also be pathogenic under certain circumstances, meaning tolerance would need to be broken to resolve disease. This hypothesis has been supported by work studying the pathobiont *Helicobacter* species, which can drive Treg development and responses during homeostasis but then promote largely Th17 responses during colitis. Interestingly, overlap of Treg and effector T cell (Teff) TCRs reactive to *Helicobater* species increased during colitis, supporting the notion that the Treg TCRs might be from peripherally-differentiated CD4+ T cells during homeostasis (97).

Although there is substantial work showing the overlap of pTreg and Tconv TCRs within the GALT, the uniqueness of the pTreg repertoire in other tissues is less clear. When comparing adoptively transferred Tregs isolated from a mouse with pTregs generated from adoptive transfer of Tconvs, there was little to no overlap in their TCR

repertoires despite having similar transcriptional signatures (98). In contrast, when looking at *in vivo* Tregs and Tconvs without the use of adoptive transfer, the TCR repertoire of Tregs is largely distinct from Tconv cells and overlaps with the TCRs of Treg thymocytes. However, these approaches rely the use of transgenic models where the TCRβ chain is fixed and only limited TCRα chains are analyzed, with one study specifically generating a TCRα minilocus that restricts TCRα diversity (99–101). Furthermore, these studies primarily look in the spleen and lymph nodes, which may not be enriched in pTregs as much as gut-resident Tregs.

1.7 APPROACHES FOR STUDYING PERIPHERALLY-DERIVED TREGS

Many early demonstrations of pTreg generation *in vivo* have utilized transgenic TCRs or adoptive transfers into T cell-deficient mice to identify them. Outside of these contexts and the work done with the GALT, it has been more challenging to track pTregs during homeostasis, leading to the search for pTreg-specific markers. Two of the key markers used so far to distinguish tTregs and pTregs have been the transcription factor Helios and the membrane-bound receptor Neuropilin-1 (Nrp1, also known as CD304). Helios expression was first characterized as a marker for tTregs due to the fact that all CD4+CD8-Foxp3+ cells within the thymus express it, but in the periphery, this number is reduced to 70%. Additionally, *in vitro* assays revealed that Helios was not upregulated during iTreg generation (102). Nrp1 was identified through the use of transgenic TCR systems *in vivo,* where RAG-deficient mice that express the 1B3 TCR reactive to myelin basic protein (MBP) have Tregs in the periphery despite no Tregs being detected within the thymus. Nrp1 expression in these pTregs is much lower than in Tregs isolated from RAG-sufficient mice, where Nrp1 is found on ~60% of Tregs

in the lymph nodes (103). In a co-published paper, another group independently validated Nrp1 as a marker for tTregs based on adoptive transfer and *in vivo* differentiation of OVA-specific naïve CD4+ T cells (104). However, the use of negative markers for pTreg identification has been challenging in other contexts due to the fact that Helios and Nrp1 are also upregulated during activation (105–107) and within tissue-resident Tregs (108).

Experiments characterizing the enhancer regions within the *Foxp3* locus of C57BL/6J (B6) mice have provided a more concrete means for distinguishing pTregs and tTregs. Within the locus, there are 3 conserved non-coding sequence (CNS) elements, termed CNS1, CNS2, and CNS3, upstream of the *Foxp3* promoter that control Foxp3 expression. Targeted deletion of these regions revealed that CNS3 is a "pioneer element" important for the initial induction of Foxp3 in both the thymus and periphery, while CNS2 is necessary for the maintenance of Foxp3 expression in dividing cells. Deletion of one element, CNS1, selectively affected induction of Foxp3 in naïve CD4+ T cells *in vitro* without perturbing thymic expression of Foxp3 (109). This region contains Smad-binding motifs downstream of the TGF-β signaling pathway, providing a direct connection to the use of TGF-β in pTreg generation.

B6 CNS1$^{-/-}$ also blocked increases in Treg percentages in peripheral tissues with age, resulting in Th2-mediated pathology in the lungs and intestinal tract (110). Continued characterization of the B6 CNS1$^{-/-}$ mouse revealed defects in gut pTreg generation to metabolites produced by commensal bacteria, specifically the short-chain fatty acids (SCFAs) butyrate and propionate (111). B6 CNS1$^{-/-}$ mice also exhibit Th2 responses that impair colonization of specific border-dwelling microbial constituents,

altering the gut metabolic profile and causing reductions in body weight (112). Lastly, pregnant B6 CNS1$^{-/-}$ females bred to BALB/c males show defects in maternal-fetal tolerance, with increases in embryo resorption and reduced Treg percentages in measured in maternal draining lymph nodes and embryonic tissue (113).

1.8 PERIPHERALLY-DERIVED TREGS AND AUTOIMMUNITY

Although work has been done to show the role of pTregs in homeostasis, less is known about the function of pTregs versus tTregs in autoimmunity. One system that has shown non-redundant roles for pTregs and tTregs is the BALB/c Foxp3^{K276X} mouse line, which exhibits systemic autoimmunity that is lethal within 3 weeks. Adoptive transfer of purified Tregs into BALB/c Foxp3^{K276X} mice is sufficient for survival, but these mice still show signs of systemic autoimmunity, including lymphocyte infiltration in the colon, lungs, and liver; elevated serum concentrations of TNF-α, IL-17A, and IFNγ; and lack of weight gain compared to normal BALB/c mice. However, co-transfer of purified Tregs with naïve CD4+ T cells or iTregs mitigated the symptoms of lingering autoimmunity, despite the fact that iTreg transfer alone is not sufficient for survival. Therefore, although tTregs most likely are the primary regulators of immune homeostasis, pTregs still play a unique role in preventing aspects of autoimmunity (98).

For specific autoimmune disease contexts, tTregs seem to be the dominant regulators of disease. In the experimental allergic encephalomyelitis (EAE) mouse model of multiple sclerosis, B6 mice are inoculated with myelin oligodendrocyte glycoprotein (MOG) peptide. Normally, MOG is not present in the periphery due to the blood-brain barrier, so introduction of MOG sparks disease development. MOG-treated B6 CNS1$^{-/-}$ mice do not have more severe disease, and Treg frequencies within the

brain are not altered, suggesting that pTregs are not typically present in this setting (110). Similarly, it was shown that when pTregs and tTregs are sorted based on Nrp1 expression, transfer of only TCR transgenic tTregs and not pTregs protected mice from EAE (103). Within the NOD mouse background, one group mutated the Smad3 binding region of CNS1, which would hinder pTreg generation. These NOD Foxp3^{CNS1mut} mice exhibited a significant reduction in GALT Treg frequencies in 6-month-old mice, but this reduction did not increase these mice's susceptibility to colitis. Also, although it was not explicitly mentioned, there was no mention of increased T1D incidence (114).

The relative importance of pTregs and tTregs in T1D is less clear and depends on what markers are used to distinguish them. For instance, one study showed that BDC2.5+Nrp1^{lo} pTregs are just as effective as BDC2.5+Nrp^{hi} tTregs in controlling T1D in NOD CD28$^{-/-}$ mice (103). However, in another study using a fluorescent reporter of tTregs, co-transfer of TCR transgenic BDC2.5tg+ pTregs with BDC2.5tg+ CD4+ T was sufficient to control T1D in NOD RAG1$^{-/-}$ mice while BDC2.5tg^{+} tTregs were not (115). Lastly, when comparing the endogenous TCRα chains that pair with the BDC2.5 TCR, there was little to no evidence found of peripheral conversion of Tconvs into Tregs based on largely separate TCR repertoires (116). However, these models rely on the use of TCR transgenic lines and adoptive transfer models, meaning that the role of unmanipulated, polyclonal pTregs versus tTregs is even less well-characterized at the moment, suggesting the need for a new model.

Despite the lack of direct evidence, characteristics of T1D do suggest a potential role for pTregs. Posttranslational modifications to human pancreatic proteins in the periphery such as disulfide bond formation in human insulin A-chain (117),

transglutamination of chromogranin A (Chga) (118), and transglutamination and citrullination of GAD65 (119) increase T cell recognition of these antigens. In mice, there is additional evidence of increased reactivity to known pancreatic autoantigens through the creation of hybrid insulin peptides (HIPs) formed by the fusion of proinsulin C-peptide with ChgA or islet amyloid polypeptide 2 (IAPP2) (120). These neoantigens could drive pTreg differentiation in order to maintain peripheral tolerance. pTregs could also potentially explain the links observed between the gut microbiome and T1D. However, when looking at the antigen specificity of cells within pancreatic islets, HIPs were largely recognized by Tconvs instead of Tregs, and Tregs were largely reactive to natural insulin peptides. In fact, the presence of HIP-reactive Tconvs increased with time, indicating that they could be a biomarker of disease progression and potential causative agents (121,122).

pTregs could also explain the link between T1D and the gut microbiome that has been observed in recent studies. Microbiome composition can be used to predict development of T1D in 8 week old NOD mice despite lack of changes in mononuclear cells in peripheral blood (123), and the microbiota are distinct for non-T1D individuals compared to recent onset patients and patients with varying levels of autoantibodies (124). The impact of the microbiome on T1D has been directly linked to the T cell compartment as well since autoreactive T cells cross-react with microbial peptide mimics in both mice and humans. Islet-specific glucose-6-phosphate catalytic subunit-related protein (IGRP) has a microbial mimotope made by *Fusobacteria* that can activate IGRP-reactive CD8+ T cells and promote T1D development in NOD mice (125), and human $ZnT8_{186\text{-}194}$ auto-reactive CD8+ T cells cross-react with a *Bacteroides*

stercoris mimotope (126). There is no direct evidence of microbial mimotopes for pancreatic autoantigens by CD4+ T cells currently, making it an exciting area to explore with new approaches.

1.9 RESEARCH PURPOSE

Due to the strong role of microbiota and other environmental factors impacting the onset and incidence of T1D, there is much interest in determining the relative roles of pTregs versus tTregs in controlling disease. However, distinguishing these subsets continues to be challenging when the currently established markers are controversial and potentially unreliable. Therefore, the first aim of this study was to create a model that would allow us to objectively determine the role of pTregs in T1D in a marker-independent fashion by directly deleting the *Foxp3* CNS1 region within the NOD mouse background. After we functionally validated the *Foxp3* CNS1 deletion, we then sought to characterize pre-diabetic NOD CNS1$^{-/-}$ mice in various lymphoid and non-lymphoid tissues to see if CNS1 deletion altered Treg frequencies or characteristics at homeostasis. Lastly, we wanted to analyze the impact of CNS1 deletion on T1D disease development and maternal-fetal tolerance.

CHAPTER 2: THYMICALLY-DERIVED FOXP3+ REGULATORY T CELLS ARE THE PRIMARY REGULATORS OF TYPE 1 DIABETES IN THE NON-OBESE DIABETIC MOUSE MODEL

2.1 ABSTRACT

Regulatory T cells (Tregs) are an immunosuppressive population that are identified based on the stable expression of the fate-determining transcription factor forkhead box P3 (Foxp3). Tregs can be divided into distinct subsets based on whether they developed in the thymus (tTregs) or in the periphery (pTregs). Whether there are unique functional roles that distinguish pTregs and tTregs remains largely unclear. To elucidate these functions, efforts have been made to specifically identify and modify individual Treg subsets. Deletion of the conserved non-coding sequence (CNS)1 in the *Foxp3* locus leads to selective impairment of pTreg generation without disrupting tTreg generation in the C57BL/6J background. Using CRISPR-Cas9 genome editing technology, we removed the *Foxp3* CNS1 region in the non-obese diabetic (NOD) mouse model of spontaneous type 1 diabetes mellitus (T1D) to determine if pTregs contribute to autoimmune regulation. Deletion of CNS1 impaired *in vitro* induction of Foxp3 in naïve NOD CD4+ T cells but not the development of T1D or glucose tolerance despite increased pancreatic insulitis in pre-diabetic female NOD CNS1$^{-/-}$ mice. CNS1 deletion did not alter Tregs in most lymphoid and non-lymphoid tissues analyzed except for the large intestine lamina propria, where a small but significant decrease in RORγt+ Tregs and corresponding increase in Helios+ Tregs was observed in NOD CNS1$^{-/-}$ mice. However, preliminary data suggest that in aged females, Treg frequencies decrease in the spleen and increase in the mLN and pLN. Furthermore, the proportions of autoreactive Tregs and conventional T cells (Tconvs) within pancreatic islets were unchanged. Lastly, preliminary data from timed intercross breeding of female NOD and NOD CNS1$^{-/-}$ mice did not reveal a defect in maternal-fetal tolerance. These results

suggest that pTregs dependent on the *Foxp3* CNS1 region are not the dominant regulatory population controlling T1D in the NOD mouse model.

2.2 INTRODUCTION

Regulatory T cells (Tregs) are an immunosuppressive subset of CD4+ T cells important for the maintenance of self-tolerance (7,127). Tregs were initially marked by their high expression of the α chain (CD25) of the interleukin-2 (IL-2) receptor and then later identified as constitutively expressing the fate-determining forkhead box P3 (Foxp3) transcription factor, which confers stable cell identity and suppressive function (13–15). Mutations in Foxp3 or Treg deletion in adult mice leads to systemic autoimmune pathology in human and mice, including type 1 diabetes (T1D) (21,34,63,69,70).

A number of studies have shown that Tregs can develop both in the thymus (tTreg) and post-thymically in the periphery (pTreg), especially in the gut. During negative selection in the thymus, high affinity, self-reactive cells can escape clonal deletion by differentiating into Tregs, which is facilitated through CD28 costimulation and Foxp3 expression (10,128). In contrast, naïve conventional T cells (Tconvs) exposed to self-antigens and the microbiome in the periphery can differentiate into Tregs, especially in mucosal tissues and in context of transforming growth factor-beta (TGF-β) and other pro-Treg environmental factors (85,86,89,90). Direct evidence of pTreg generation can be seen when antigens were administered in the periphery by osmotic pumps (84), oral administration (83), or targeted antigen delivery to dendritic cells (129).

Previous studies have shown that Tregs are defective in both mice and humans that develop T1D. Thymic development and the Treg repertoire have been shown to be altered in NOD mice (130,131), and reducing T-cell receptor (TCR) diversity in NOD mice alleviates T1D, connected to a lack of insulin beta chain (InsB:9-23) autoreactive T cells (132). In fact, recent studies have identified islet-antigen-specific Tregs that can control disease progression (121). However, it remains unclear where these Tregs are generated and which subset of Tregs are defective. Finally, fusion peptides made by post-translational modifications of insulin derivatives and other proteins also create neoantigens that more strongly bind to potentially autoreactive cells in NOD mice (118,120,122) and humans (117,119,120,133). Although the role of these neoantigens and their recognition by Tconvs and Tregs remain unclear, their existence suggests that peripherally-derived antigens not typically found in the thymus might drive T1D and potentially pTreg generation. Interestingly, adoptive transfer experiments have shown non-redundant roles for tTregs and pTregs in maintaining tolerance (98).

Identification of phenotypic or molecular markers that distinguish tTregs from pTregs *in vivo* has proven more challenging. Expression of the surface marker Neuropilin-1 (Nrp1, also known as CD304) (103,104) and the transcription factor Helios (102) have been linked to tTregs; however, Helios and Nrp1 can also be markers of activation (105–107), and Nrp1 expression has been observed in some pTregs generated during adoptive transfer experiments and in tissue resident Tregs (108). As a result, efforts have been made to identify distinct regulatory elements that differentially control Foxp3 expression in the different Treg subsets.

Genetic studies by Rudensky and colleagues showed that there are 3 conserved non-coding sequence (CNS) elements, termed CNS1, CNS2, and CNS3, upstream of the *Foxp3* promoter that control Foxp3 expression. One element, CNS1, was shown in C57BL/6J (B6) mice to selectively affect pTreg development without perturbing thymic expression of Foxp3 (109). This region contains Smad-binding motifs downstream of the TGF-β signaling pathway, which has been implicated in the development of induced Tregs (iTregs) differentiated from naïve CD4+ T cells *in vitro* and gut-derived pTregs through mechanisms such as the activation of latent TGF-β through interactions with integrin $\alpha_v\beta_8$ (134). Administration of TGF-β *in vivo* can also enhance the antigen-specific generation of pTregs (129,135). B6 CNS1$^{-/-}$ blocked increases in Treg percentages in peripheral tissues with age, resulting in Th2-mediated pathology in the lungs and intestinal tract (110). Continued characterization of the B6 CNS1$^{-/-}$ mouse revealed defects in pTreg generation and gut microbiome colonization in CNS1$^{-/-}$ mice (111,112) as well as in maternal-fetal tolerance (113).

There have been limited studies on the relative roles of pTregs and tTregs in controlling autoimmunity. Studies of experimental allergic encephalomyelitis (EAE) in a B6 CNS1$^{-/-}$ mouse model suggested that pTreg deficiency did not exacerbate disease (110), and adoptive transfer of TCR transgenic tTregs but not pTregs protected mice from EAE (103). These results are consistent with another group who showed that a Smad3 binding mutation within *Foxp3* CNS1 of NOD mice did not affect susceptibility to colitis, and although not stated explicitly, the authors did not note an increased incidence of T1D (114). In contrast, other studies have shown that in NOD CD28$^{-/-}$ mice, adoptive transfer of islet antigen-specific pTregs was equally effective as tTregs in

controlling T1D (31), and in one study, co-transfer of TCR transgenic BDC2.5tg+ pTregs with BDC2.5tg+ CD4+ T was sufficient to control T1D in NOD RAG1$^{-/-}$ mice (115). In sum, these results suggest that tTregs are most critical in preventing and reversing autoimmunity and that in most cases pTregs are not effective. However, in the case of unmanipulated, polyclonal Tregs, the results are less clear.

Therefore, the goal of this study was to address the role of polyclonal pTregs in controlling autoimmune diabetes. pTreg development in the autoimmune-prone NOD mouse model was directly impaired through the use of the CRISPR/Cas9 gene editing system to selectively delete the CNS1 region of *Foxp3*. The results suggest that polyclonal tTregs are sufficient to control T1D. In parallel, another group generated a NOD CNS1$^{-/-}$ model using the CRISPR/Cas9 system. Their results differed, suggesting that pTregs were indeed critical for control of autoimmune NOD diabetes, with NOD CNS1$^{-/-}$ males and females exhibiting higher rates of T1D incidence (136). Our results suggest that tTregs are the dominant regulators of T1D in the NOD mouse model, but under certain conditions such as targeted antigen-specificities or certain external factors, pTregs may play a role in the progression of T1D.

2.3 RESULTS

2.3.i CNS1 deletion in NOD mice inhibits in vitro *iTreg generation*

The CNS1 region of *Foxp3* was directly deleted in the NOD mouse background using the CRISPR-Cas9 genome editing system (Fig 2.1A). Single guide RNAs (sgRNAs) were generated based on the 705 bp region that spans the regulatory element previously described in the B6 *Foxp3* locus, namely from +2003 to +2707 bp from the transcriptional start site of *Foxp3* (109). PCR and DNA sequencing confirmed

the deletion of a 736 bp sequence (+1976 to +2711), which spans the CNS1 region (Fig 2.1B). To verify that the genetic deletion corresponded with a functional defect, we performed an *in vitro* Foxp3 induction assay using TGF-β. This assay demonstrated an impairment in the generation of iTregs (Fig 2.1C), confirming that genetic deletion of the CNS1 region in the NOD background conferred the previously described functional defect observed in the B6 background.

2.3.ii Treg quantification and phenotyping in NOD CNS1$^{-/-}$ mice

In order to directly address the specific consequences of *Foxp3* CNS1 deletion on pTreg generation, pre-diabetic female NOD mice aged 8-13 weeks were sacrificed and the *in vivo* Treg compartment was analyzed. Overall Treg percentages were comparable between NOD and NOD CNS1$^{-/-}$ mice in both lymphoid and non-lymphoid tissues, including the thymus, spleen, mesenteric lymph nodes (mLN), pancreatic islets, and large intestine lamina propria (LI LP), with a small but significant decrease in Treg percentages in the pancreatic lymph nodes of NOD CNS1$^{-/-}$ females (Fig 2.2A). However, in the original paper characterizing the B6 CNS1$^{-/-}$ mouse, decreases in Treg frequencies were not evident until the mice were aged out to 6-9 months. Therefore, Treg frequencies were also analyzed in 30-week NOD and NOD CNS1$^{-/-}$ females that did not become diabetic. Surprisingly, preliminary results show that Treg frequencies were only reduced in the spleen, and within the pLN and mLN, Treg frequencies were increased (Fig 2.2B). We also characterized the Tregs present based on markers such as Nrp1 and Helios expression. As expected, Nrp1 and Helios expression within the thymus were comparable between NOD and NOD CNS1$^{-/-}$ females (Fig 2.2C-D). We examined whether a decrease in the Nrp1^{-} and Helios^{-} populations in the periphery

would be evident based on previous results in B6 mice (Fig 2.2E). However, there was no significant difference in the percentage of Nrp1^{-}Helios^{-} (Fig 2.2F), Nrp1^{-} (Fig 2.2G), or Helios^{-} (Fig 2.2H) Tregs in the mLN and pLN.

Previous studies have suggested that the LI LP is largely comprised of Helios^{-} pTregs, with a majority being positive for the transcription factor retinoic acid-related orphan receptor-γt (RORγt) (93,94). These RORγt+ Tregs are induced by the presence of complex microbiota and provide anti-inflammatory functions (95) and have been shown to have a distinct repertoire (96). For example, RORγt^{+} pTregs responsive to the short-chain fatty acid (SCFA) butyrate are generated in the gut (111) where they function to establish border-dwelling bacteria that protect against microbial exposure and maintain normal metabolite profiles (112). Therefore, we hypothesized that there would be a reduction in the Helios^{-} and RORγt^{+} pTreg populations in NOD CNS1$^{-/-}$ females. Cells expressing Helios versus RORγt were mutually exclusive in Tregs isolated from the gut of both NOD (Fig 2.2I) and NOD CNS1$^{-/-}$ (Fig 2.2K) mice. There was a small but significant shift in the percentage of the Helios^{-} as well as RORγt^{+} Treg populations in NOD CNS1$^{-/-}$ mice (Fig 2.2J and Fig 2.2L, respectively). The implications of these differences are unclear and may reflect changes in the ability of the pTregs to develop in response to exposure to microbiota.

2.3.iii Islet antigen-reactive Tconvs and Tregs are unchanged in NOD CNS1$^{-/-}$ mice

Previous studies have shown that defects in Tregs in local tissues result in the expansion of autoreactive Tconv cells. Therefore, we examined the relative number of antigen-specific T cells in the pancreatic islets of NOD and NOD CNS1$^{-/-}$ female mice. To assess antigen-specific T cells, I-A^{g7}-peptide tetramers was used to stain cells

specific for two insulin beta chain (InsB:9-23) variants: p8E, which represents the natural InsB peptide sequence; and p8G, which contains a mutation mimicking a proposed C-terminal InsB:9-20 truncation that shifts the MHC register of presentation for the epitope (137). Insulin peptide is thought to be produced within the thymus by medullary thymic epithelial cells (mTECs) expressing the autoimmune regulator (*Aire*) transcription factor, driving thymic selection of T cells (4). I-A^{g7}-peptide tetramers were also used to stain for reactivity against post-transcriptional HIPs generated from the fusion products of proinsulin C-peptide with chromogranin A (ChgA) or islet amyloid polypeptide 2 (IAPP2) (120).

MHC II-islet peptide-specific tetramer staining revealed that Tconvs were more reactive to the HIP peptides than InsB peptides (Fig 2.3A). This result is consistent with the idea that InsB-reactive Tconvs would be deleted within the thymus while the so-called HIPs could represent peripherally-derived neoantigens that escape negative selection. Moreover, when comparing tetramer staining in Tconvs between NOD and NOD CNS1$^{-/-}$ females, no statistical difference was observed between the percentage of cells reactive to either the InsB:9-23 p8E (Fig 2.3C) or p8G variants (Fig 2.3D). The same was true for the Ins:ChgA 2.5HIP (Fig 2.3E) and the Ins:IAPP2 6.9HIP (Fig 2.3F) tetramers.

Thus, we hypothesized that HIP-reactive Tregs would represent a pTreg population derived from HIP-reactive Tconvs, which would predict that there would be fewer HIP-reactive Tregs in the islets of NOD CNS1$^{-/-}$ mice. However, Tregs isolated from pancreatic islets harvested from wild type NOD mice did not react with either the HIP2.5 or the HIP6.9 tetramers, suggesting that there may not be a significant

percentage of pTregs in the islets overall. In contrast, Tregs reactive with the p8E and p8G tetramers were higher as compared to Tconvs (Fig 2.3B). Furthermore, there was no statistical difference observed between NOD and NOD CNS1$^{-/-}$ females in the percentage of Tregs reactive to the p8E (Fig 2.3C), p8G (Fig 2.3D), 2.5HIP (Fig 2.3E), and 6.9HIP (Fig 2.3F) tetramers. These data support the idea that CNS1 deletion does not alter the relative percentage of autoreactive Tconvs or Tregs within the pancreatic islets. Furthermore, the Treg compartment within the pancreatic islets seem to be comprised of largely tTregs

2.3.iv No difference in glucose tolerance, time of T1D onset, or disease incidence in NOD CNS1$^{-/-}$ mice despite differences in insulitis

The ultimate potential consequence of the effects of disruption of pTreg induction was read out by the impact on T1D development in the NOD background. Female NOD and NOD CNS1$^{-/-}$ pancreata were collected at 6, 10, and 14 weeks of age for histology to measure insulitis (Fig 2.4A). Histological analysis of the isolated islets showed that there was some increase in insulitis in NOD CNS1$^{-/-}$ mice as early as 6 weeks and 10 weeks of age in the CNS1$^{-/-}$ mice. However, by 14 weeks, the time of initial development of clinical diabetes in our colony, there was only a marginal difference in insulitis between NOD and NOD CNS1$^{-/-}$ females, suggesting that the deletion of CNS1 did not ultimately lead to an increase in beta cell destruction. In fact, in younger pre-diabetic NOD CNS1$^{-/-}$ female mice, normal glucose tolerance was maintained (Fic 2.4B-C). These results show that the small differences in insulitis between NOD and NOD CNS1$^{-/-}$ mice at earlier ages were not consequential for either acute or chronic changes in blood glucose control. Importantly, these early differences in insulitis did not influence

the onset or incidence of T1D in either female or male NOD CNS1$^{-/-}$ mice, which was not statistically different between the groups (Fig 2.4D).

2.3v Preliminary data suggest no difference in maternal-fetal tolerance or most Treg percentages in NOD CNS1$^{-/-}$ female mice

The last area we explored for the function of NOD CNS1-dependent pTregs was in maternal-fetal tolerance. It has been previously established that Tregs play a key role in protecting allogeneic fetuses from being rejected by the maternal immune system in mice (138) and humans (139,140). Furthermore, in B6 CNS1$^{-/-}$ females interbred with BALB/c males, there are fewer Tregs present in both maternal lymph nodes and the decidua, an embryonic tissue rich in immune cells at the maternal-fetal interface. These reductions were associated with an increase in embryo resorption rates, which was most likely caused by the allogeneic MHC molecules expressed in BALB/c (113). Therefore, we decided to conduct timed interstrain breeding experiments between B6 males and virgin NOD and NOD CNS1$^{-/-}$ females to determine if there are differences in litter size, embryo resorption, and Treg characteristics within the decidua and the following maternal lymphoid nodes: the paraaortic lymph nodes as primary uterine draining sites; the inguinal lymph nodes as potential secondary uterine draining sites; and the brachial lymph nodes, which are non-draining sites for the uterus.

Analysis of pregnant females and their litters at mouse embryonic day 14.5 (E14.5) showed that litter sizes for NOD and NOD CNS1$^{-/-}$ mice were largely comparable (Fig 2.5A). Furthermore, there was not a statistically significant difference in visible resorption rates between NOD and NOD CNS1$^{-/-}$ females (Fig 2.5B). However, since the original phenotype described in pregnant B6 CNS1$^{-/-}$ mice was only evident

when comparing large numbers of mice, this experiment would need to be repeated in order to draw a conclusion confidently (113). When comparing Treg frequencies between pregnant NOD and NOD CNS1$^{-/-}$ mice in maternal spleen, paraaortic lymph nodes, inguinal lymph nodes, and brachial lymph nodes, there were no statistically significant differences observed. This observation also held true for Treg frequencies in the embryonic decidua tissue (Fig 2.5C). Lastly, there were also no notable differences in the percent of Tregs that were Nrp1- (Fig 2.5D) or Helios- (Fig2.5E) in these tissues except within the paraaortic lymph nodes, where there was a significant reduction in the Helios- population in NOD CNS1$^{-/-}$ females. Since the paraaortic lymph nodes are the primary draining sites for the uterus, this reduction could reflect a decrease in CNS1-dependent pTregs specific for fetal alloantigens. This observation is also consistent with our previous findings in the LI LP.

2.4 DISCUSSION

Our data show that deletion of CNS1 in the NOD background does not impact Treg frequencies or expression of known markers associated with pTregs and tTregs in most tissues except in aged mice, which show significant decreases in Treg frequencies within the spleen and increases in the pLN and mLN. Furthermore, deletion does not change the frequency of islet antigen-specific Tregs or Tconvs within the pancreatic islets. This includes InsB:9-23, a self-antigen known to be expressed in the thymus, and HIP tetramers, specific for post-translationally fused peptides generated in the periphery. These results are consistent with recent reports that show that InsB:9-23 tetramer binding is enriched in Tregs while HIP tetramer binding is enriched in Tconvs

(121,122). There are also no differences in glucose tolerance, diabetes onset or incidence when comparing the NOD and NOD CNS1$^{-/-}$ mice. Lastly, preliminary data suggest that CNS1 deletion does not alter maternal-fetal tolerance or Treg frequencies during pregnancy, although there is a reduction of Helios- Tregs within the paraaortic lymph nodes, which are key draining sites for the uterus.

These results are consistent with another group who generated a Smad3 binding mutation within CNS1 of NOD mice, which led to an age-dependent decrease in Treg frequency within the LI LP and other gut associated lymphoid tissues (GALT) in males but did not affect susceptibility to colitis (114). However, another group independently generated a NOD CNS1$^{-/-}$ model using different sgRNAs (136). Their work revealed a similar cellular phenotype where the frequency of Tregs found in most lymphoid and non-lymphoid tissues remained largely the same, but they observed a small but significant decrease in the Treg frequencies within the LI LP instead of the pLN. Interestingly, this alternate NOD CNS1$^{-/-}$ model, there were significant reductions in Helios expression in Tregs in more lymphoid and non-lymphoid tissues than what we observed. Moreover, the alternate NOD CNS1$^{-/-}$ model showed a much starker difference in insulitis at 6 and 10 weeks of age, and although there is not a significant difference in insulitis by 14 weeks of age, overall insulitis was much higher in both their NOD and NOD CNS1$^{-/-}$ females. These results suggest that there may be a role for pTregs early in disease progression but overtime, the tTregs dominate and control disease progression such that depending on the colony and the degree of early beta cell damage, the resulting ability to control disease and maintain normoglycemia may be different. In the case of our mice, tTregs control disease progression preventing clinical

disease, while in the other NOD *Foxp3* CNS1 deletion model a clinical disease phenotype is evident in both male and female mice.

Although many of the results we obtained are consistent with the previous NOD CNS1$^{-/-}$ studies, there are a number of factors that could account for both earlier and more severe insulitis in the CNS1$^{-/-}$ mice described by Kissler and colleagues than those in our colony. One difference between the different NOD CNS1$^{-/-}$ mouse strains is that the CNS1 deletion is not identical. Kissler and colleagues used sgRNAs that span a wider region around the DNA than the ones we utilized. Thus, the resulting edit generated a slightly larger 795 bp deletion (+1949 to +2743 from the *Foxp3* transcriptional start site) (unpublished information from Kissler) than was generated in the animals herein (+1976 to +2711). Whether these additional nucleotides might alter DNA binding of other enhancer proteins remains unclear but might be impactful.

Perhaps the most likely difference between the two mouse models resides in the gut microbiome or other environmental factors. There is extensive literature documenting that innate microbial sensing and gut microbial constituents can shift T1D incidence, explaining differences observed in diabetes incidence among animal facilities (142,143). Specific differences in the gut microbial community of NOD males and females post-puberty also contribute to the differences in T1D incidence seen between the sexes (144,145), and microbiome composition can be used to predict development of T1D (123). The gut microbiome might be linked to T1D due to the fact that the pLN is also a draining site for microbial antigens (146), and autoreactive cells have also shown cross-reactivity between microbial antigens and islet antigens (125,126). In fact, recent characterization of the B6 CNS1$^{-/-}$ mouse directly revealed that defects in gut pTreg

generation alter gut microbiome colonization (111,112). Since there is a change in gut Tregs in both NOD CNS1$^{-/-}$ models generated, the ability to observe a similar disease phenotype in our NOD CNS1$^{-/-}$ model might be masked by differences in gut microbiome composition. Future studies that sequence and compare the microbiome of NOD and NOD CNS1$^{-/-}$ mice generated in the two facilities would be required to address this issue directly.

In summary, our data suggest that tTregs play the dominant role in the control of islet-specific autoimmunity and subsequent development of diabetes. This conclusion is supported by the observation that within the pancreatic islets, there are not many Tregs that recognize tissue-specific, peripherally-generated autoantigens as detected by HIP I-A^{g7} tetramers, consistent with the notion that the Treg compartment within the islets is largely comprised of tTregs. Although our work shows that tTregs are sufficient for controlling T1D in NOD mice, the role of CNS1-dependent pTregs might be influenced by environmental factors based on the differences seen between our model and an independently-generated NOD CNS1 deletion. This observation is consistent with what is known about T1D and could provide further insight into the interplay between the immune system and environment that determines both mouse and human disease susceptibility and outcomes.

2.5 FIGURES

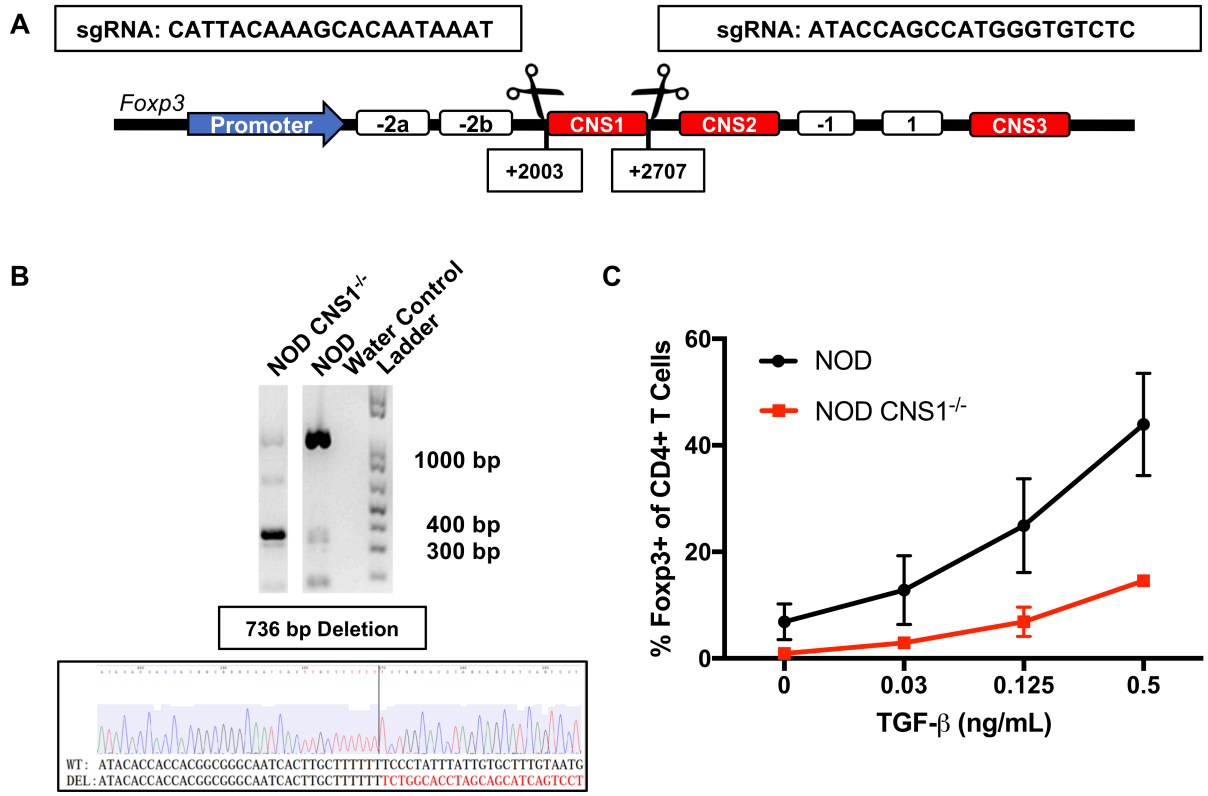

Figure 2.1. Design, generation, and verification of CNS1$^{-/-}$ in the NOD mouse background.

(A) Schematic representation of the Foxp3 locus highlighting the originally defined CNS1 region (+2003 - +2707 from transcription start site) and corresponding sgRNA sequences for CRISPR/Cas9 targeted cutting. (B) Genotyping PCR showing deletion of the CNS1 region in the primary founder and genetic sequencing after CRISPR/Cas9 deletion verifying a 736 base pair deletion in the Foxp3 locus. (C) CD4^{+}CD8^{-}CD25^{-} CD62L^{hi} naïve T cells were sorted from NOD and NOD CNS1$^{-/-}$ mice and stimulated in 96-well plates with plate-coated anti-CD3 (5 µg/mL) and anti-CD28 (0.5 µg/mL) antibodies for 72 hours with hIL-2 (100 IU/mL) in the absence or presence of TGF-β. Cells were than stained for CD4, CD25, and Foxp3 and analyzed with flow cytometry (n=2 mice per genotype, two technical replicates per mouse used).

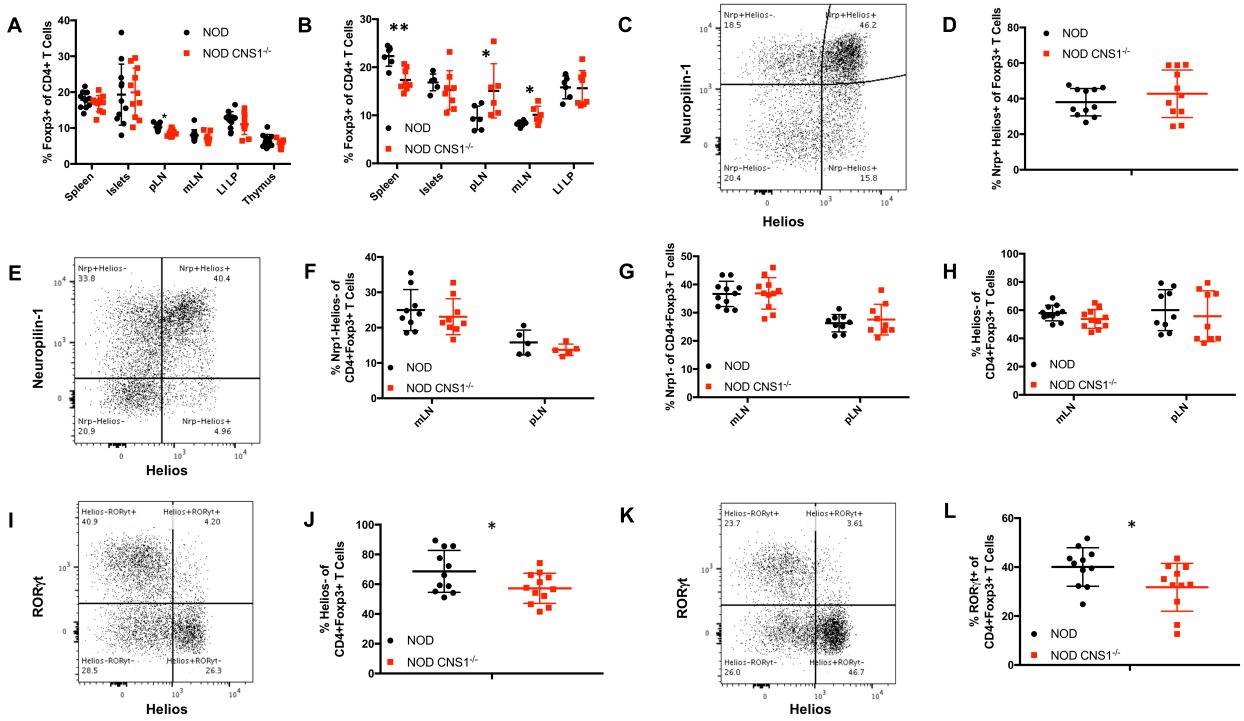

Figure 2.2. Broad characterization of lymphoid and non-lymphoid tissues in NOD and NOD CNS1$^{-/-}$ mice.

(A) Quantification of Treg percentages in various tissues of NOD and NOD CNS1$^{-/-}$ female mice, 8-13 weeks (n=11) (pLN = pancreatic lymph node, mLN = mesenteric lymph node, LI LP = large intestine lamina propria) (B) Quantification of Treg percentages in various tissues of 30 week old female NOD mice (n=6) (C) Representative plot of CD4+CD8-Foxp3+ thymocytes showing Neuropilin-1 (Nrp1) and Helios expression. (D) Quantification of Nrp1+Helios+ CD4+CD8-Foxp3+ Treg thymocytes. (E) Representative plot of CD4+Foxp3+ T cells expressing Nrp1 and Helios in the mesenteric lymph node (mLN). Quantification of Nrp1-Helios- (F), Nrp1- (G), and Helios- (H) Tregs in mLN and pancreatic lymph node (pLN), reflecting potential pTreg populations. Representative plot of CD4+Foxp3+ T cells expressing RORγt and Helios in the large intestine lamina propria (LI LP) of NOD (I) and NOD CNS1$^{-/-}$ (J) mice. Quantification of Helios- (K) and RORγt+ (L) Tregs in the LI LP, reflecting potential pTreg populations. (C) – (L) show NOD and NOD CNS1$^{-/-}$ female mice, 8-13 weeks, (n=11) (* = $p < .05$, ** = $p < .005$).

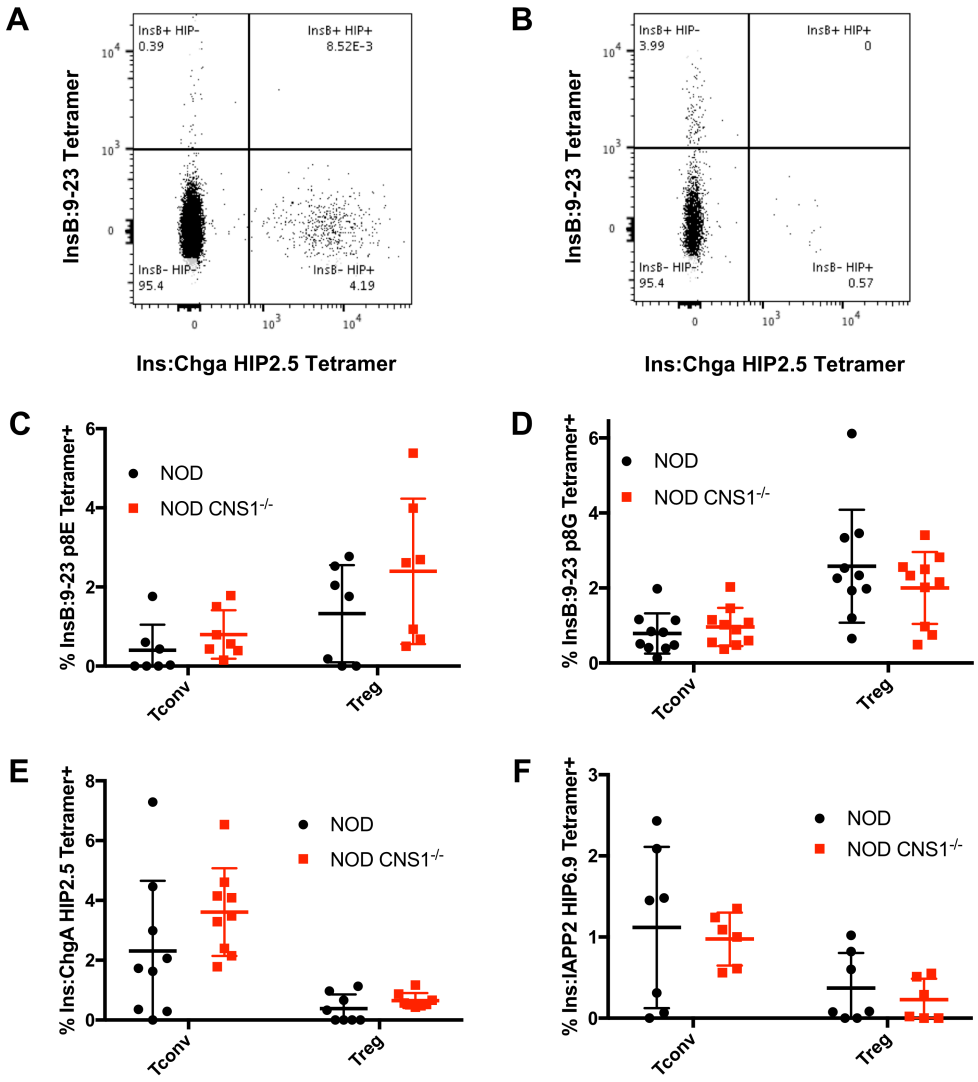

Figure 2.3. Autoreactive Treg and Tconv percentages between NOD and NOD CNS1$^{-/-}$ mice.

Representative tetramer staining plots of CD4+Foxp3- Tconvs (A) and CD4+Foxp3+ Tregs (B) for insulin peptide InsB 9:23 and hybrid insulin peptide (HIP) fusion of insulin and chromogranin A show differential tetramer binding between Tregs and Tconvs. Tetramer staining within pancreatic islets for InsB:9-23 p8E (n=7) (C) and InsB:9-20 p8G (n=10) (D) variants and Ins:ChgA HIP2.5 (n=9) (E) and Ins:IAPP2 HIP6.9 (n=7) (F) is quantified and compared between NOD and NOD CNS1$^{-/-}$ pre-diabetic females aged 8-13 weeks (* = $p < .05$).

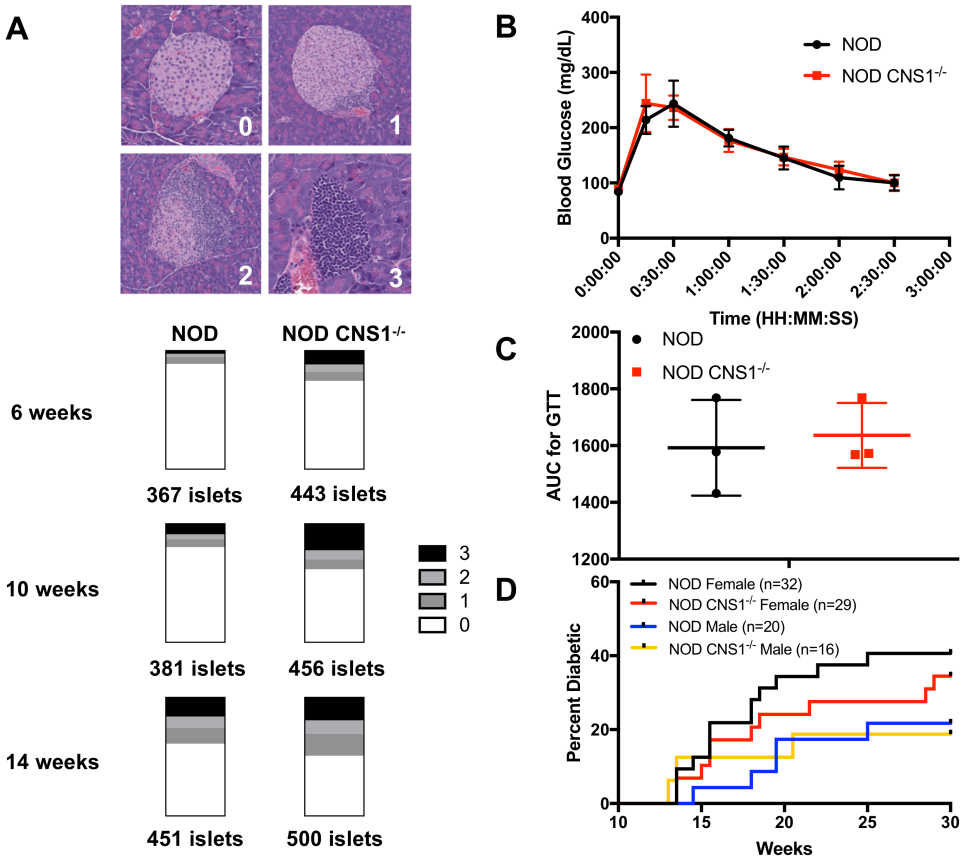

Figure 2.4. Comparison of T1D development and glucose tolerance between NOD and NOD CNS1$^{-/-}$ mice.

(A) Pooled insulitis scoring of female NOD and NOD CNS1$^{-/-}$ mice at 6, 10, and 14 weeks of age (n=3 mice per age and genotype). Representative H&E histology images of pancreatic islets illustrating insulitis scoring rubric are shown at the top (0 = no insulitis, 1 = peri-insulitis or <25% infiltration, 2 is 25%-75% infiltration, and 3 is >75% infiltration or full islet destruction). $p < 0.0001$ for 6 weeks and 10 weeks, and $p = 0.0140$ at 14 weeks based on a chi-square comparison of islets scored as 0 to islets scored as 1, 2, or 3. (B) Glucose tolerance testing of 8-9 week old NOD and NOD CNS1$^{-/-}$ females after 16 hour overnight fast (n=3 per group) (C) Glucose area under the curve (AUC) of glucose tolerance testing shown in (B). (D) Diabetes incidence based on weekly blood glucose measurements comparing NOD and NOD CNS1$^{-/-}$ males and females.

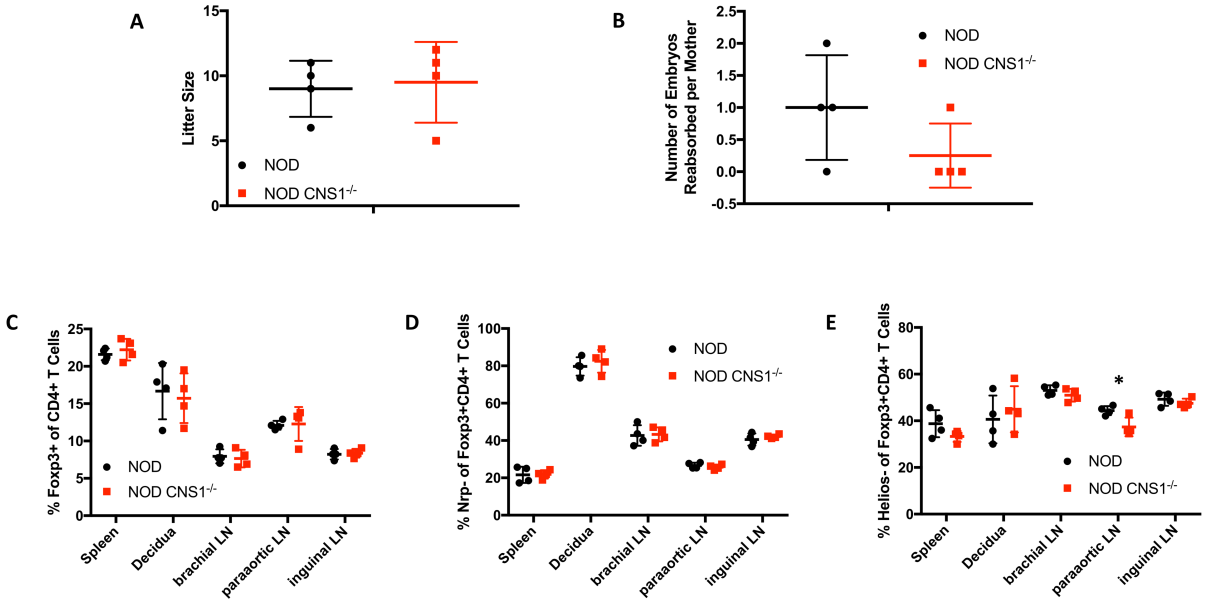

Figure 2.5. Preliminary data for maternal-fetal tolerance in NOD and NOD CNS1$^{-/-}$ females.

(A) Number of viable pups found per pregnant 12-13 week NOD or NOD CNS1$^{-/-}$ female bred to a 12-13 week B6 male at E14.5 (n=4 per group). (B) Number of reabsorbed embryos found per pregnant female. Quantification of overall CD4+Foxp3+ Treg percentages (C) as well as Nrp- (D) and Helios- (E) Treg percentages in nonlymphoid and lymphoid tissues (* = $p < .05$). All tissues listed were collected from the mother except for the decidua, which represent pooled data collected from mouse embryos.

CHAPTER 3: MATERIALS AND METHODS

3.1 MICE

NOD CNS1$^{-/-}$ mice were made by the JAX Model Generation services using the CRISPR/Cas9 system (gRNAs 5'-CATTACAAAGCACAATAAAT-3' and 5'-ATACCAGCCATGGGTGTCTC-3'). Potential founders were genotyped and sequenced using the following primers: 5'- CAGCAGTGCTCTTACCCATG-3' and 5'-CAGTGAGAGCAGTTTAGAGG-3'. The full CNS1 deletion can be seen in Figure 3.1. Founder mouse progeny were crossed to NOD JAX mice for at least three generations before experiments to account for potential off-target CRISPR/Cas9 effects. Hyperglycemia and spontaneous induction of T1D in NOD mice were monitored by weekly blood glucose measurements made with the OneTouch Ultra 2 glucometer and UNISTRIP1 generic blood glucose test strips. Mice with two consecutive blood glucose readings of at least 250 mg/dL were considered diabetic. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

3.2 ISOLATION OF LYMPHOCYTES AND CELL STAINING

Lymph nodes were physically dissociated and filtered to generate single-cell suspensions. Spleens were physically dissociated and incubated at room temperature in ACK red blood cell lysis buffer before filtration. Large intestine lamina propria (LI LP) were digested using the Lamina Propria Dissociation Kit (MACS Miltenyi Biotec) per manufacturer's instructions. Islet isolation was done courtesy of the UCSF Mouse Islet Isolation Facility (147) and then dissociated in Cell Dissociation Buffer Enzyme-Free PBS-based (ThermoFisher Scientific) at 37°C in a tissue culture incubator. Antibodies used for flow cytometry staining are listed in S1 Table. Cell viability was measured

before flow cytometry using the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter) and tracked during flow cytometry using the LIVE/DEAD Fixable Blue Dead Cell Stain Kit for UV Excitation (ThermoFisher Scientific). After viability staining, tetramer staining was performed at room temperature for 1 hour, and then without washing, concentrated surface stain was added for an additional 15 minutes at room temperature. Antibodies used for surface staining can be found in Table 3.1. The following mouse I-A^{g7} tetramers were used courtesy of the NIH Tetramer Core Facility: InsB p8E variant (HLVERLYLVCGEEG) conjugated to APC; InsB p8G variant (HLVERLYLVCGGEG) conjugated to APC; Ins:ChgA 2.5HIP (LQTLALWSRMD) conjugated to APC; Ins:IAPP2 6.9HIP (LQTLALNAARD) conjugated to PE; and DPB1*04:01/DPA1*01:03 control human CLIP 87-101 conjugated to APC, BV421, and PE (PVSKMRMATPLLMQA). Fixation and intracellular staining were done using the eBioscience Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific) per manufacturer's instructions. Stained single-cell suspensions were analyzed using a Fortessa flow cytometer running FACSDiva (BD Biosciences). FSC 3.0 files were analyzed and presented with FLOWJO Software (flowjo.com).

3.3 *IN VITRO* FOXP3 INDUCTION ASSAY

Naïve T cells (CD4+CD25-CD62Lhi) were isolated and stained as described above and then FACS-Aria sorted into fetal bovine serum (FBS). Cells were resuspended in complete (penicillin/streptomycin, sodium pyruvate, HEPES, NEAA, and β-mercaptoethanol) DMEM plus 10% FBS with human IL-2 (100 IU/mL) and TGF-β (0, 0.03125, 0.125, and 0.5 ng/mL) and cultured in 96-well plates coated with anti-CD3 (5 µg/mL) and anti-CD28 (0.5 µg/mL) for 72 hours at 37°C in a tissue culture incubator.

Cells were than stained for CD4, CD25, and Foxp3 (S1 Table) and analyzed using a Fortessa flow cytometer running FACSDiva (BD Biosciences). FSC 3.0 files were analyzed and presented with FLOWJO Software (flowjo.com).

3.4 HISTOLOGY AND PANCREATIC INSULITIS SCORING

Pancreata were fixed in 10% neutral buffered formalin solution overnight and dehydrated in 70% ethanol. Histology was performed by HistoWiz Inc. (histowiz.com) using a Standard Operating Procedure and fully automated workflow. Samples were processed, embedded in paraffin, and sectioned at 6 μm with 175 μm steps between slices. Three sections per mouse pancreas were collected. Haemotoxylin and Eosin (H&E) staining was performed, and then sections were dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems). Islets were scored blinded to genotype and age with at least 100 islets scored per mouse. Insulitis was scored as none (0), peri-insulitis or less than 25% infiltration (1), 25-75% infiltration (2), or greater than 75% infiltration (3). Islet scoring was done in the open source software QuPath (qupath.github.io) (148).

3.5 GLUCOSE TOLERANCE TEST

Glucose tolerance was tested in 8-9-week-old NOD and NOD CNS1$^{-/-}$ mice after a 16 hour overnight fast. Mice were challenged with injections of 1M glucose in PBS (2 g glucose/kg of total body weight) intraperitoneally and blood glucose was measured with the OneTouch Ultra 2 glucometer and UNISTRIP1 generic blood glucose test strips at baseline and then 15, 30, 60, 90, 120, and 150 minutes post-challenge.

3.6 MATERNAL-FETAL TOLERANCE BREEDING

12-13 week virgin female NOD and NOD CNS1$^{-/-}$ mice had dirty bedding from 12-13 week male B6 mouse cages placed in their cages 3 nights before breeding. The night before breeding, 12-13 week B6 males were singly housed in fresh cages. The day of intended breeding, either a NOD or NOD CNS1$^{-/-}$ 12-13 week virgin female was added to each singly housed male cage at 5 PM to establish timed intercross breeding. The next morning, females were checked for vaginal plugs to determine potentially pregnant females. Out of 9 breeding cages established for both NOD and NOD CNS1$^{-/-}$ females, 4 for each genotype had vaginal plugs the next morning, and all females with plugs became pregnant. At embryonic day 14.5, the pregnant females were sacrificed and tissues listed were collected for flow analysis. Due to small cell amounts, the decidua of all the pups for a single mother were pooled for analysis.

3.7 STATISTICS AND METHODS OF ANALYSIS

Data analyses were performed using GraphPad Prism 7 software (graphpad.com/scientific-software/prism), and values at $p < .05$ were deemed significant. For comparison of the insulitis scores, the chi-square test was used to calculate if there was a significant difference in the proportion of islets without any peri-insulitis or insulitis (score = 0) to islets with peri-insulitis or full insulitis (score = 1, 2, or 3). All other statistical comparisons were done using a two-tailed, unpaired t test.

3.8 FIGURES

5’-AATCACTTGCTTTTTTTCCCTATTTATTGTGCTTTGTAATGCATGTGTCTTTTAGGTCTTTAGATTACTCTTTTCTTGTGGGGCTTCTGT
GTATGGTTTTGTGTTTTAAGTCTTTTGCACTTGAAAATGAGATAACTGTTCACCCCATGTTGGCTTCCAGTCTCCTTTATGGCTTCATTTT
TTCCATTTACTGCAGAGGTCAAAAGTGTGGGTATGGGAGCCAGACTGTCTGGAACAACCTAGCCTCAACTCAAGTCATCTGTGTGAAT
TTTACCCAGGCTCTTAACCTCTCTGTACCTCCATTTCCTCGTATGTACTGTGATGATTATAACAGTACCTACCTCAGAGGATCTTTCTGA
GGATTATTTTTATTAATGATGGTAGGTGCTCAGCACAAGGCCAAACAACAATGATAGACATTAAAACGTATCTCTCTAGTGGGTCTGG
AAATTATTCTAGAGCGTCTGATGACAGCGACATTTCAAGTGGGCAGGGAGGTATTGGTGGGAAAGTGGGCTATCTACCCAGTCACTT
TATTTTCCCCTAATTGTCTCAGAATCATTTGTTAATCTGTCCTGCACTGTTCCTCATGTTGAAATGTTGTGTTCATCACAAATTCCATTCC
CTCTGTGCATGGGTCTCTGCCACGGTTTTCTACTCTAATCTGCTCCTTAGTGTTTATTCTTGTACAAAGCCCACACTATTTTTCTGATGTT
GCTTTGCAAAACAATTCAATACCAGCCATGGGTGTCTCTGGCACCTA-3’

NOD CNS1 Deletion
CNS1 Region

Figure 3.1. Sequence of CNS1 deletion in NOD mice.

Displayed here is the +1960 to +2722 region from the transcriptional start side of Foxp3. The original CNS1 region identified in B6 mice (+2003 to +2707) is highlighted in yellow, while the sequence of the deleted region generated in the NOD background with CRISPR/Cas9 (+1976 to +2711) is bolded in red.

3.9 TABLES

Table 3.1. Reference list of flow cytometry antibodies used for analysis.

Antibody	Clone	Vendor	Catalog Number
CD3 BUV737	17A2	BD Biosciences	564380
CD4 BV605	RM4-5	BioLegend	100548
CD8 PerCP-Cy5.5	53-6.7	eBioscience	48-0081-82
CD25 PE-Cy7	PC61.5	eBioscience	25-0251-82
CD45 BUV395	30-F11 (RUO)	BD Biosciences	564279
Foxp3 FITC	FJK-16s	eBioscience	11-5773-82
Foxp3 eFluor450	FJK-16s	eBioscience	48-5773-82
Helios PE	22F6	BioLegend	137216
Helios Pacifc Blue	22F6	BioLegend	137220
Neuropilin-1 (CD304) APC	Polyclonal	R&D Systems	FAB566A
Neuropilin-1 (CD304) PE-Cy7	3DS304M	invitrogen	25-3041-80
RORyt PE-CF594	Q31-378 (RUO)	BD Biosciences	562684

CHAPTER 4: DISCUSSION

4.1 FUTURE DIRECTIONS

4.1.i Compare the microbiomes in both NOD CNS1$^{-/-}$ models generated

The most surprising result of our study is that our NOD CNS1$^{-/-}$ model does not exhibit increased T1D incidence while another independently generated NOD CNS1$^{-/-}$ model does. To explore our hypothesize that the gut microbiome is the cause of this difference, we would perform 16sRNA sequencing to compare the microbial constituents of our NOD and NOD CNS1$^{-/-}$ mice to first see if the communities are altered as would be predicted based on work done in the original B6 CNS1$^{-/-}$ strain (112). We would then compare the microbiomes of our mice to the NOD and NOD CNS1$^{-/-}$ mice maintained by the other group to establish the baseline differences in NOD microbial constituents and determine if the magnitude and types of differences observed in the NOD CNS1$^{-/-}$ strains depends on the model used. Lastly, we would either cohouse these two NOD CNS1$^{-/-}$ strains in both facilities or use antibiotics and fecal gavages to inoculate each model with the microbiome present in the other. These experiments would allow us to control environmental effects to determine if they are the cause for the differences in the T1D incidence observed between the two NOD CNS1$^{-/-}$ strains. Furthermore, microbiome analysis could reveal key microbial strains that either produce tolerogenic pTregs or abrogate the effects of pTreg-mediate tolerance.

4.1.ii Create a CNS1$^{+/-}$ reporter model to study in vivo *competition between NOD and NOD CNS1-deficient Tregs*

Although we did not observe large differences in Treg frequencies or marker expression in NOD CNS1$^{-/-}$ mice, it is possible that there is compensation that occurs during homeostasis. Adoptive transfer of B6 CNS1$^{-/-}$ naïve CD4+ T cells that express

transgenic TCRs specific for gut antigens showed that although initial Foxp3 induction was impaired out to 1 week post-transfer, by week 5, substantial Foxp3 expression was seen in the mLN and the colon, which was not due to the selective expansion of a small subset of Foxp3+ T cells (108). Therefore, even if CNS1 deletion might impair initial pTreg generation, it is possible that with time, this defect is overcome through other enhancers or signaling pathways. It is also possible that within the NOD mouse model, tTregs expand to fill the niche. As a result, it would be interesting to see if T cells expressing the *Foxp3* gene with the CNS1 deletion are at a selective disadvantage compared to T cells with a normal copy of Foxp3.

One means to address this question *in vivo* at homeostasis is through the use of the NOD Foxp3-IRES-RFP reporter line (149). The *Foxp3* gene is located on the X chromosome, and in female mammals that carry two copies, each cell will randomly inactivate one copy. X-inactivation has been taken advantage of previously to compare cells expressing wildtype *Foxp3* versus the CNS1-deficient *Foxp3* gene in heterozygous B6 females (B6 CNS1$^{WT/-}$) (110). In this system, the CNS1-deficient *Foxp3* locus also contained a GFP reporter that was used to identify which Tregs expressed the modified *Foxp3* gene. It was hypothesized that based on random X-inactivation, half of tTregs in these mice should express the wildtype gene while the other half would express the CNS1-deficient gene; however, if CNS1 deletion confers a defect in pTreg generation, fewer or no pTregs would express the CNS1-deficient *Foxp3* gene, skewing the ratio towards the wildtype gene in pTreg-rich environments. In B6 CNS1$^{WT/-}$ aged 3-4 weeks, it was revealed that within the thymus where there should only be tTregs, half of the cells expressed either copy of the Foxp3 gene. In contrast, within the GALT where

pTregs are thought to be enriched, more cells expressed the wildtype *Foxp3* gene, suggesting that either more tTregs were present or that there was a loss of pTregs. By using the NOD Foxp3-IRES-RFP reporter to identify which Tregs express the wildtype *Foxp3* gene, we could conduct similar experiments to see if the ratio is skewed in particular tissues in younger mice. Additionally, it would be interesting to use this reporter system to further explore what is happening in aged NOD CNS1$^{-/-}$ mice when Treg frequencies in the spleen, pLN, and mLN are significantly altered. Lastly, this approach could allow us to more easily determine potential pTreg vs. tTreg markers if in the full NOD CNS1$^{-/-}$ mouse compensation masks the defect.

4.1.iii Clarify the role of pTregs in therapies that prevent or reverse T1D

Beyond characterization of CNS1-dependent pTregs in the NOD mouse model during homeostasis, it would be interesting to explore whether CNS1 deletion could impact certain therapeutic approaches for T1D. In particular, the NOD CNS1$^{-/-}$ model could provide insight into what occurs during diabetes reversal and remission in NOD mice that receive FcR-nonbinding (FNB) anti-CD3 therapy (150,151). The exact mechanism by which FNB anti-CD3 treatment induces diabetes reversal is unclear; however, it is believed that it preferentially depletes pathogenic Tconvs while preserving Tregs, which then confer protection (152). pTregs have been implicated in this system since blocking TGF-β signaling abrogates diabetes reversal (153). Furthermore, immunosuppressive TGF-β signaling might specifically be facilitated through pTreg generation (154). However, other work has suggested that Tregs enriched in Helios expression are selectively protected from depletion by FNB anti-CD3 treatment, and there was no evidence of pTreg generation or general Treg expansion (152).

Additionally, administration of low-dose exogenous IL-2 has been shown to prevent and reverse T1D. One study showed that treatment increases the proportion of Tregs present in the spleen, pLN, and pancreatic islets, potentially by increasing Treg survival to prevent T1D onset (155). Another group showed that low-dose IL-2 selectively increases Treg percentages within pre-diabetic NOD mice, and later treatment can reverse T1D in recent onset NOD mice by increasing the expression of Foxp3 and CD25 without changing Treg percentages in these tissues (156). The NOD CNS1$^{-/-}$ model provides a great tool for clarifying whether CNS1-dependent pTregs are necessary for diabetes prevention or reversal with these treatments.

4.1.iv Determine whether pTregs can be generated against pancreatic neoantigens and the explore the efficacy of HIP-reactive TCRs in Tregs

The observation that pTregs might not be induced against HIPS within the pancreatic islets during homeostasis could have significant implications for the use of antigen-specific Tregs in cell therapy for T1D. In the absence of pTreg generation, it is possible that HIPs and other pancreatic neoantigens cannot be tolerized through bystander suppression of tTregs within the pancreatic islets, making them the key drivers of pathogenesis in T1D. Therefore, it would be interesting to explore whether pTregs can be generated against HIPs under the proper conditions *in vitro* or if *in vivo* pTreg generation can be encouraged. If not, TCRs specific for HIPS can be isolated from Tconvs and transgenically expressed in Tregs to determine if they are effective in activating Tregs and facilitating targeted Treg suppression. This approach has already proven successful through the use of the mouse BDC2.5 TCR, which binds strongly to

the ChgA HIP. These TCRs could also be compared to TCRs expressed by Tregs that recognize thymically-expressed antigens to determine their relative efficacy in therapy.

4.2 CONTRIBUTIONS TO THE FIELD OF AUTOIMMUNITY

This study independently generated a new NOD mouse model that can be used to study the role of CNS1-dependent pTregs in T1D and a broad range of other autoimmune diseases to which NOD mice are prone. With this model, we corroborated the recently published results of another group that made its own NOD CNS1$^{-/-}$ line, showing that prediabetic CNS1-deficient NOD females exhibit reduced pTreg marker expression within the LI LP as well as increased insulitis. Surprisingly, these two models had contrasting results for the effect of CNS1 deletion on T1D incidence, with our deletion producing no change while the other model exhibits increased incidence in both male and female NOD CNS1$^{-/-}$ mice. However, we validated that increased insulitis, female NOD CNS1$^{-/-}$ are not impaired in their ability to control acute changes in blood glucose. This difference between the models provides the first evidence that the functional role of CNS1-dependent pTregs in controlling T1D could be impacted by environmental factors such as the microbiome.

Our work also provided additional insights not initially explored in the other NOD CNS1$^{-/-}$ model. First, we more deeply characterized antigen specificity within pancreatic islets to show that tTregs seem to be the dominant subset in this tissue, with CNS1 deletion not significantly altering the antigen specificity of Tregs or Tconvs for both self and tissue-specific neoantigens. Surprisingly, we also showed for the first time that in aged NOD CNS1$^{-/-}$ mice that in contrast to the B6 CNS1$^{-/-}$, the percent of Tregs among CD4+ T cells in the mLN and pLN increases, with only the spleen showing a decrease

in Treg frequency. Lastly, we are the first group to provide preliminary data on the impact of CNS1 deletion in NOD maternal-fetal tolerance, suggesting that pregnant NOD CNS1$^{-/-}$ have comparable litter sizes, embryo resorption rates, Treg frequencies, and pTreg marker expression except for the uterine-draining paraaortic lymph nodes, which showed an increase in Helios+ Tregs in pregnant NOD CNS1$^{-/-}$ mice.

Finally, the NOD CNS1$^{-/-}$ model provides additional support to the conclusions made from studying EAE in the B6 CNS1$^{-/-}$ model and colitis in the NOD CNS1^{mut} model, which suggest that pTregs do not play a significant role in controlling autoimmunity. However, our results do show that gut pTregs are impacted by CNS1 deletion in young mice, reinforcing that the gut might be the primary site for pTreg generation. Therefore, the main function of pTregs might be in inducing tolerance to commensal bacteria and not to self-antigens. Alternatively, pTregs might primarily be induced to prevent the development of allergies to food and other innocuous allergens. Based on these findings, it would be interesting to determine whether the Th2-like pathology originally observed in aging B6 CNS1$^{-/-}$ mice is a sign of autoimmunity or a result of dysbiosis or allergic reactions. Other diseases in the NOD CNS1$^{-/-}$ model can also be studied to further clarify whether pTregs play a unique role in controlling autoimmunity.

4.3 FIGURES

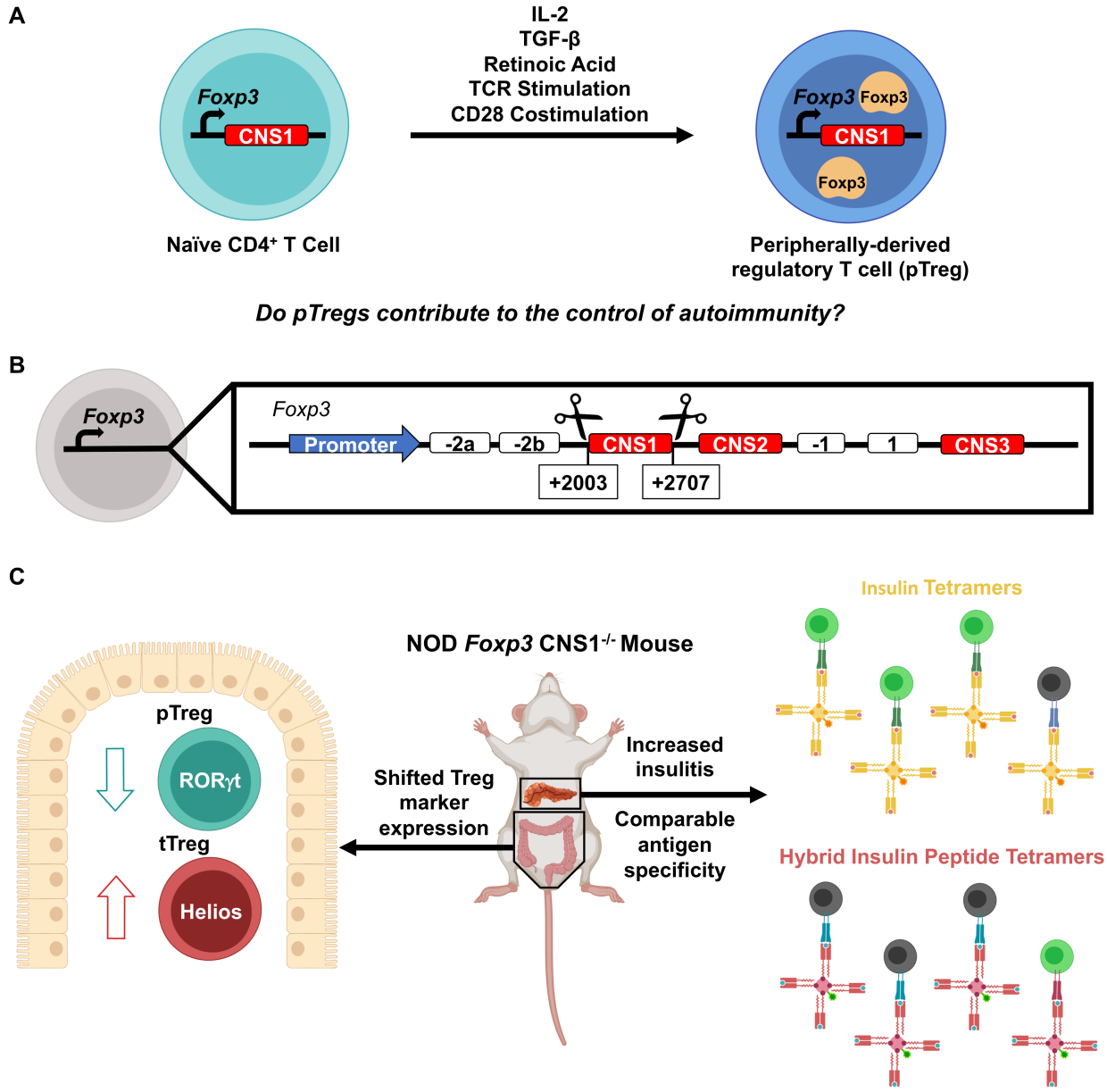

Figure 4.1. Graphical summary of the NOD *Foxp3* CNS1$^{-/-}$ mouse findings.

(A) Overview of the role of the *Foxp3* CNS1 enhancer in the differentiation of naïve CD4+ T cells into pTregs and the central question that the dissertation seeks to address (B) *Foxp3* locus showing the goal for targeted deletion of the CNS1 enhancer to generate a pTreg-deficient non-obese diabetic (NOD) mouse model (C) Summary of the key findings *in vivo* of CNS1 deletion in NOD mice, highlighting shifts in Treg populations in the large intestine lamina propria (LI LP) on the left and the observed trends in antigen specificity observed in both NOD and NOD CNS1$^{-/-}$ pancreata, suggesting that tTregs are most likely the dominant population (green cells are Tregs, black cells are Tconvs).

Created with BioRender and PowerPoint.

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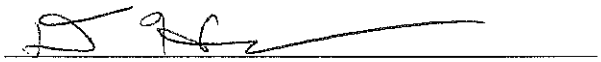

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