Title
Regulatory T cell dynamics within chronically inflamed tissue in mouse autoimmune diabetes

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Publication Date
2016

Peer reviewed|Thesis/dissertation

REGULATORY T CELL DYNAMICS WITHIN CHRONICALLY INFLAMED TISSUE IN MOUSE AUTOIMMUNE DIABETES

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

ACKNOWLEDGEMENTS

This work would not have been possible without the important contributions of many individuals. First and foremost, I must thank Tang, my mentor. Her support, dedication, and creative approaches to science have been instrumental in shaping this week as well as helped me grow as an individual. She has not only taught me how to think like a scientist, but supported both my research activities and professional development. She has created a work environment in the lab that is welcoming and encouraging, a place where I am excited to be a part of every day.

Members of the Tang lab, surrounding labs in the Diabetes Center, Pathology department, and Anatomy Department have made substantial contributions to this work scientifically. They have also made the last few years a very enjoyable experience. A very special thanks to Ashley, Tang's first graduate student, for getting me started in the lab, always having a kind and encouraging word for me, and teaching me so many things that I needed to learn during my PhD. Annie started these projects and was a huge help in the beginning, even though these projects are unrecognizable from their original form. Nino, Karim, Vi, Joey, and Greg have been around the lab from the beginning, providing help and advice. Vinh has been in the lab since before my time, always positive, always helpful, the true foundation of the lab. I cannot give him enough thanks for everything over the years. Jeff not only managed the mouse colony, but was excited to jump on any additional projects, making working in the lab with him a great experience. Lindsay not only provided the lab with technical support, but she stepped up to every problem with a great attitude, even taking on projects and tasks that were not her own. She was my go-to person to discuss anything and everything, especially

our love of cats. Also a cat person, Asia is not only an amazing scientist, but a truly valued friend. Youmin (Jen), Janice, Vasalya, Nicole, Amy, Khalia, and Mary were interns that worked with me in the lab. They all learned how to do science in the lab, but they also taught me so much about how to mentor and support students excited about science. They have all be wonderful to work with. Susanna, Steve, Linda, Ryan, John, and Angelia have all come to the lab in recent years and have all contributed to making the lab a more intellectually stimulating and fun place to be. During my time in lab, many people have come and gone. Some past members of the transplant lab that I would also like to acknowledge for their support are Mariela, Ninnia, Ele, Shelly, and Anne.

Members of other labs at UCSF have also provided support and encouragement to me along the way, including Bluestone, Anderson, Rosenblum, and Mackenzie labs. The members of our weekly lab meetings have provided endless advice and assistance throughout my time at UCSF. Additionally, Whitney, Kelly, Sam, Mahesh, and Shen all assisted with collaborations between our labs.

I'd like to thank the members of my thesis committee, Michelle and Mike, for making committee meetings something that I look forward to having. Their much appreciated input, advice, and encouragement was essential in shaping this work.

Thank you to members of the BMS office, especially Demian, Lisa, and Monique, for taking care of some many things that I don't even know about, so I could focus on the science.

I am thankful to have made the Bay Area a second home, and for all those that have made living here such a joy. In the BMS program, Megan, Joan, Si-Han, Imran, Todd, Jillian, Melissa, Viola, and Bianca have been wonderful people to share our successes and have some good times through this process.

The biggest thank you I can give goes to my mom, for always supporting my dreams, even when I moved so far away. Thanks to Dayne and the rest of the Sterlings for all their support. My father, step-father Bill, sister Melanie, and brother Cameron have shaped me in countless ways and are so important to me. I love you all.

CONTRIBUTIONS OF CO-AUTHORS TO THE PRESENTED WORK

Chapter II of this dissertation is based on a manuscript in preparation. The co-authors on the resulting publication will be Whitney Purtha[1], Teague Sterling[3], Youmin Kim[2], Mark Anderson[1], and Qizhi Tang[2]. Whitney Purtha and Youmin Kim provided assistance with experiments. Teague Sterling assisted with data analysis. Mark Anderson and Qizhi Tang supervised the work.

Chapter III of this dissertation is based on a manuscript in preparation. The co-authors on the resulting publication will be Youmin Kim[2], Henry Garcia[4], Saul Villeda[4], and Qizhi Tang[2]. Youmin Kim, Henry Garcia, and Saul Villeda assisted with experiments. Qizhi Tang supervised the work.

Chapter IV of this dissertation is based on a manuscript in preparation. The co-authors on the resulting publication will be Janice Tam[2], Youmin Kim[2], Jeff Wang[2], Vasalya Panchumarthi[2], Joanna Klementowicz[2], and Qizhi Tang[2]. Janice Tam, Jeff Wang, and Vasalya Panchumarthi provided technical assistance. Youmin Kim and Joanna Klementowicz assisted with experiments. Qizhi Tang supervised the work.

Appendix I section titled “Targeting Treg signaling for the treatment of autoimmune diseases” is a re-print of the published review article as it appears in *Current Opinion in Immunology* in the December 2015 issue 37, pages 11-20. The co-authors of this publication are Joanna Klementowicz[2], Jeff Bluestone[1], and Qizhi Tang[2]. Joanna Klementowicz assisted with reference review, writing, and revising the manuscript. Jeff Bluestone and Qizhi Tang supervised the work.

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REGULATORY T CELL DYNAMICS WITHIN CHRONICALLY INFLAMED TISSUE IN MOUSE AUTOIMMUNE DIABETES

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ABSTRACT

Regulatory T cells (Tregs) are critical for the maintenance of immune homeostasis and prevention of autoimmunity. In the non-obese diabetic (NOD) mouse model of type 1 diabetes, Tregs accumulate in the inflamed islets of langerhans and delay or prevent the destruction of insulin-producing beta (β) cells. The regulators of Treg homeostasis and function are yet to be completely understood in the context of chronic tissue inflammation. In this work we focused on the specificity of Tregs for their cognate antigen, the dynamics of these Tregs, and influence of the cytokines IL2 and TGFβ. This was accomplished by examining the target tissue compared with peripheral lymphoid sites for different aspects of Treg biology using flow cytometry, histological analysis, and TCR sequencing. We found that islet Tregs were potent mediators of disease prevention, had most recently and strongly been exposed to their cognate antigen, and we could identify a highly activated, antigen specific subset using cell surface markers CD103, ICOS, and TIGIT. Islet Tregs were a dynamic population constantly turning over, but these Tregs were not circulating through the LNs and instead isolated to the tissue. They were specific for known autoantigens, such as insulin, but the highly activated, antigen reactive subset of Tregs were also clonally expanded due to reactivity with unknown islet antigens. After

adoptive transfer, both $CD103^+$ and $CD103^-$ Tregs prevented diabetes development and were more potent than Tregs from peripheral lymphoid organs. Their efficacy could be increased with a short treatment with IL2. When Tregs cannot respond to TGFβ signaling, their function in preventing diabetes increases, while their ability to prevent autoimmune neuropathy decreases. Overall, our findings that islet Tregs are highly localized, potent effector Tregs that can be manipulated with cytokines implicate the importance of antigen specific tissue Tregs in preventing diabetes development and the impact of Treg cytokine signaling on disease pathogenesis.

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CHAPTER I- BACKGROUND

Autoimmunity- The immune system takes aim at your own body

The immune system can be broadly divided into 2 major branches, the innate and adaptive systems. While the function of these systems is integrated during an immunological response, they each have their own unique features. Hallmarks of the innate immune system are their ability to respond rapidly to insult and their inability to generate memory cells. An adaptive immune response is much more specific to each immunological event, where the cells of interest expand due to their ability to react to that specific target. These targets are termed antigens. Adaptive immune responses are critical to proper immune function, as humans and mice lacking this compartment are severely lymphopenic and suffer from recurrent infections (Bosma and Carroll, 1991; Buckley, 2004). This disease, termed Severe Combined Immunodeficiency (SCID), is characterized by an inability to generate the T and/or B cells that comprise the adaptive arm of the immune system.

T and B cells are specialized immune cells that can respond to a wide array of antigens. Development of these cells is accomplished through a somewhat random splicing and re-joining of different sections of DNA as well as addition of nucleotides. In this fashion, a unique repertoire of cells is generated. Cells must be “educated” in specialized organs to ensure non-reactivity to self-antigens, a process called central tolerance. For T cells, this occurs in the thymus, where self-reactive cells are deleted during negative selection. However, this process is not 100% efficient, and some self-

reactive cells escape. In the periphery, T cells that escaped negative selection will be controlled through multiple redundant mechanisms, including but not limited to anergy, deletion (Xing and Hogquist, 2012), and control by regulatory T cells (Tregs) (Sakaguchi, 2004).

When central and/or peripheral tolerance mechanisms breakdown and autoreactive cells escape regulation, autoimmune disease can develop. These autoreactive cells attack and destroy target tissues. Some of the common autoimmune diseases in humans include rheumatoid arthritis, multiple sclerosis, Sjogren's syndrome (SS), and type 1 diabetes (T1D), among others. Not every patient develops every autoimmune disease, indicating unique genetic and/or environmental components influencing each disease. Therefore, understanding the pathogenesis of these diseases is of paramount importance and directly related to development of therapy for patients.

Type 1 Diabetes- Autoimmunity modeled in mice

T1D is characterized by a progressive immunological destruction of insulin-producing beta (β) cells, which reside in the Islets of Langerhans in the pancreas. Eventually, patients will no longer possess enough β cell mass to produce their own insulin, will experience chronic hyperglycemia, and will need to take exogenous insulin in the form of injections to maintain normo-glycemia for the remainder of their lives. Chronic hyperglycemia is a dangerous condition associated with extreme fatigue, weight loss, and eventual death. Management of T1D is challenging for patients, with severe complications such as cardiovascular disease (Melendez-Ramirez et al., 2010), retinopathy, nephropathy, and neuropathy that are all increased with poor glucose control (Atkinson et al., 2014). Type 1 diabetic patients account for 5-10% of all

diabetes cases in the United States, and the incidence is increasing approximately 3% per year globally depending on the geographic region (Atkinson et al., 2014). Classically, T1D has been labelled as a disease of childhood, but increasingly has been observed in adult patients. Both genetic risk factors and the environment are likely to play a major role in the development of this disease.

Access to human tissues to study this disease is severely limited, so many researchers, ourselves included, have turned to animal models to study the pathogenic mechanisms of T1D. The most commonly studied model is the non-obese diabetic (NOD) mouse, which shares many of the features of human diabetes. The NOD mouse was developed more than 30 years ago by a group in Japan (Makino et al., 1980) while they were trying to develop a cataract prone strain from outbred mice. NOD mice primarily develop T1D but also other autoimmune disease such as sialitis (Hu et al., 1992) and thyroiditis (Many et al., 1996). An initial observation upon characterization of this mouse is that NOD mice showed sex selectivity, where 80% of females but less than 20% of males developing diabetes by 30 weeks of age. Onset of diabetes occurs starting around 11 weeks of age, while peri-insulitis, which is the accumulation of immune cells around the islets, begins around 3 weeks of age in both males and females. NOD diabetes is a progressive destruction of islets. The sex specific differences as well as relatively inconsistent age of disease onset suggest regulatory mechanisms that prevent or precipitate disease occur late in this process.

Many cell types are involved in the pathogenesis of diabetes, with $CD4^+$ and $CD8^+$ T cells being important mediators of disease progression. $CD4^+$ T cells are critical players in both the early and late phases of disease progression and can directly

mediate destruction of β cells. NOD disease can be significantly impacted by alterations in cytokine signaling, strength or presence of different costimulatory molecules, and antigens recognized by the T cells (Anderson and Bluestone, 2005). Interestingly, prevention of NOD diabetes through alteration of costimulation may shift the mice towards alternative autoimmune diseases, such as peripheral neuropathy (Bour-Jordan et al., 2005), where effector T cells infiltrate and destroy the sciatic nerve.

Not all mice progress to clinical disease and in many mice disease development is delayed. Thus, there exists a balance between the destructive effector cells and the influence of regulatory cells. Tipping this balance pushes the mice in favor of disease development or prevention, respectively. Some peripheral tolerance mechanisms that are involved in controlling disease include costimulation, T helper 1 (Th1) vs T helper 2 (Th2) differentiation, and Tregs (Anderson and Bluestone, 2005). Many of these mechanisms that successfully elucidated important pathways in NOD diabetes have also been investigated in humans, but thus far there has been very little overlap between interventions successful in mice and those successful in humans (Leiter and Herrath, 2004; Roep and Atkinson, 2004). Therefore, the translation of findings in the NOD mouse to humans is an open area of investigation in the field. In this study, we utilize the NOD mouse primarily as a model to examine molecular and cellular processes that contribute to autoimmune disease. These findings will be re-evaluated in human studies to determine their contribution to this disease process.

Tregs- Enforcers of immune regulation

Another critical player in autoimmune disease prevention is $CD4^+$ T cells with suppressive capacity, the Tregs. These cells not only prevent the development of

autoimmunity but control normal immune homeostasis and play a role in cancer development. Prior to 2003, immunologists had described this population as expressing CD4 and CD25, anergic *in vitro,* and with the ability to suppress other T cells (Sakaguchi et al., 1995). CD25 is an imperfect marker, since it is expressed by many cells, including activated $CD4^+$ T cells. In 2003, this T cell subset was further defined by the discovery of Foxp3 as the master transcription factor for the Treg lineage (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). The critical function for Foxp3 in the Treg lineage is demonstrated by the development of a massive immune activation and destruction in humans and mice lacking Foxp3 (Brunkow et al., 2001; van der Vliet and Nieuwenhuis, 2007). When the 3D structure of the Foxp3 protein is altered using an N-terminal GFP-Foxp3 fusion protein, the transcriptional regulator function is changed and this results in protection against autoimmune arthritis but exacerbated NOD diabetes (Bettini et al., 2012; Darce et al., 2012), demonstrating the critical role of Foxp3 protein structure in continual maintenance of immune homeostasis.

In addition to their role in maintaining immune homeostasis, Tregs function to control inflammation (Sakaguchi, 2004). Increased Treg activity in cancer settings can prevent effector T cell function and tumor clearance. During infections, Tregs can reduce additional damage to the host by limiting ongoing inflammation. In autoimmunity, Treg number and function are linked to disease protection or development.

IL2 in Treg development, homeostasis, and function

Tregs have very high expression of CD25, the high affinity receptor for IL2, which is lacking on most naïve $CD4^+$ T cells. In the thymus, Treg development progresses in

two steps. First, the T cell receptor (TCR) is engaged in Treg precursors, resulting in the expression of CD25 and proximal IL2 signaling components. These $CD4^+CD8^-$ $Foxp3^-CD25^{hi}$ Treg precursors are poised to develop into $Foxp3^+$ Tregs without further TCR stimulation, requiring only cytokine signals from IL2 or IL15 to mediate Foxp3 induction (Lio and Hsieh, 2008b). In the periphery, there is also a requirement for IL2 to maintain the Treg population (Antony et al., 2006). Mice lacking CD25 or IL2 develop a fatal inflammatory disease similar to mice lacking Foxp3 due to this requirement for IL2 for Tregs (Malek et al., 2002; Wolf et al., 2001).

In culture, TCR, costimulation, and high levels of IL2 are sufficient to expand Tregs in large numbers that maintain Foxp3 and suppressive function (Putnam et al., 2009; Tang et al., 2004b). In mice, IL2 complexed with an anti-IL2 antibody increases the half-life of IL2. Low doses of this treatment preferentially targets Tregs due to their expression of the high affinity IL2 receptor, boosting Treg numbers and function in T1D, leading to prevention (Manirarora and Wei, 2015; Tang et al., 2008) or reversal (Grinberg-Bleyer et al., 2010). The inflamed islets likely have a decreased amount of IL2 available for Tregs, which leads to low levels of Bcl2 and reduced Treg survival as mice age (Tang et al., 2008; Tritt et al., 2008). Treatment with just a short administration of IL2/anti-IL2 complexes or expression of IL2 by beta cells in the tissue increases Bcl2 and Treg associated proteins that lead to a long lasting protection from disease (Grinberg-Bleyer et al., 2010; Johnson et al., 2013; Tang et al., 2008). At higher doses, other cell types will also be stimulated with these complexes, including effector T cells, leading to severe inflammatory disease (Tang et al., 2008).

Treg consumption of IL2 can be a mechanism that Tregs use to suppress effector cells. In the NOD.BDC2.5 TCR transgenic (tg) mouse, in which all T cells are specific for islet antigen, natural killer (NK) cells reside in and can mediate destruction of islets within 3 days in the absence of Tregs (Feuerer et al., 2009). In this model, NK cells upregulate an IL2 signature that is required for them to mediate islet destruction. Indeed, when treated with IL2/anti-IL2 complexes that target effector cells rather than Tregs (Spangler et al., 2015), NK cells upregulate IFNɣ and mice progress to diabetes within 1 week (Sitrin et al., 2013).

These and other results in mice prompted the investigation into the role of IL2 signaling on Tregs in human T1D patients. Supporting a central role for Tregs in human T1D, polymorphisms in *IL2RA* have been associated with T1D risk (Lowe et al., 2007). Early data suggested that Tregs in T1D patients have defects in IL2R signaling (Long et al., 2010; Long et al., 2011). In 2015, *Yu et al.* found IL2 responsiveness in Tregs from some T1D patients undergoing low-dose IL2 therapy, supporting the use of this therapy for enhancing Tregs in T1D. These T1D patients can be divided into 2 groups: those that respond to IL2 versus those that do not, which correlated with Treg function *in vitro* (Yu et al., 2015). Improved clinical outcomes for patients using low dose IL2 therapy has been observed in chronic graft versus host disease (GvHD) (Koreth et al., 2011; Matsuoka et al., 2013), hepatitis C virus (HCV)-induced vasculitis (Saadoun et al., 2011), and alopecia areata (Castela et al., 2014). In a T1D IL2/rapamycin combination trial, Tregs increased, but so did $CD56^{hi}$ NK cells and $CD4^{+}$ memory T cells alongside a decrease in β cell function. These two effector cell populations are the next highest IL2 responders after Tregs, highlighting the importance of dosing as well as potential side

effects of this treatment (Long et al., 2012). Thus, defining the potential of patients that have the capacity to respond to IL2 and determining the optimal dose is of upmost importance for efficacy of IL2 therapy for boosting Tregs in human T1D.

Other mechanisms of Treg function

A number of mechanisms utilized by Tregs to exert their suppressive functions have been described. Some of these mechanisms include secretion and/or activation of inhibitory cytokines, including IL10, TGFβ, and IL-35 (Vignali et al., 2008; Worthington et al., 2015). Additionally, Tregs can kill target cells using granzyme and perforin or deprive effector cells of IL2 (Yamaguchi et al., 2011). CTLA4, an inhibitory competing protein to CD28, is expressed on Tregs and essential for their maintenance of immune homeostasis (Wing et al., 2008) as well as prevention of NOD T1D (Gerold et al., 2011). Tregs can use CTLA4 to increase the activation threshold for effector T cells (Eggena et al., 2004) as well as suppress dendritic cells (DCs) through decreased IDO expression (Mayer et al., 2013) or by inhibiting their maturation (Serra et al., 2003).

In T1D, Tregs can interact with DCs in the draining pancreatic LN (pancLN) to alter antigen presentation (Tang et al., 2006) and prevent $CD8^+$ T cell activation (Serra et al., 2003). *In vitro*, DCs can increase Treg number and function (Tarbell et al., 2004). Still, as mice age the effector T cells can become less susceptible to regulation by Tregs (D'Alise et al., 2008; Gregori et al., 2003; You et al., 2005) even with an increasing number of Tregs in the pancLN and insulitic lesion (Tang et al., 2008). These Tregs, as discussed previously, have a survival defect (Tang et al., 2008) and therefore likely a reduced suppressive function (Barron et al., 2010; Fontenot et al., 2005; Malek et al., 2002). In humans, effector T cells may also become resistant to

suppression (Schneider et al., 2008). Recently, we have found that Tregs in the NOD mouse can inhibit $CD8^+$ T cells through blockade of end stage effector mechanisms such as IFNɣ as well as through the mTOR pathway (Mahne et al., 2015). Still, the critical suppressive mechanisms utilized by Tregs in T1D are not well understood.

Role of TGFβ in the Treg lineage

TGFβ is a pleotrophic cytokine involved in a wide array of cellular processes. Many types of cells can secrete it into the circulation in its latent form surrounded by the Latency Associated Protein (LAP). In order to become activated, LAP gets cleaved by the integrin αvβ8 (Travis et al., 2007; Worthington et al., 2015). In the immune system, TGFβ inhibits proliferation and activation of T cells. Specifically, TGFβ blocks proliferation during G1 phase as well as expression of IFNɣ (Kubiczkova et al., 2012; Yang et al., 2010). T cells that cannot respond to TGFβ are resistant to suppression by Tregs in adoptive transfer models of colitis (Fahlen et al., 2005; Liu et al., 2003) and experimental autoimmune encephalomyelitis (EAE) (Zhang et al., 2006). In mice with a selective loss of TGFβ signaling T cells during development, Tregs cannot control a rapid lethal inflammatory disease (Ishigame et al., 2013; Marie et al., 2006).

TGFβ secretion is part of the arsenal of immunosuppressive mechanisms utilized by Tregs. Not only can Tregs secrete TGFβ, but cleavage of the latent TGFβ by Treg expressed αvβ8 is critical for their function during an ongoing immune response. Treg homeostasis was unaffected, indicating a role for Treg-specific activation of latent TGFβ to suppress ongoing inflammation (Worthington et al., 2015).

TGFβ can play a role in Treg development, depending on the immunological context. Adding TGFβ to cultures of naïve $CD4^+$ T cells in addition to anti-CD3 and anti-CD28 stimulation can convert these cells into $Foxp3^+$ Tregs with suppressive functions (Chen et al., 2003). The intronic element conserved noncoding sequence 1 (CNS1) in the Foxp3 enhancer has binding sites for downstream TGFβ signaling molecule SMAD and is critical to the development of Tregs from naïve $CD4^+$ T cells in the peripheral lymphoid organs but not in the thymus *in vivo* (Samstein et al., 2012). Conflicting reports have suggested a role for TGFβ in the development of Tregs in the thymus (Leveen et al., 2005; Lio and Hsieh, 2008b; Lucas et al., 2000; Marie et al., 2005; Marie et al., 2006).

In addition to a role for TGFβ in Treg suppressive function and development, TGFβ may also have a role in regulating Treg proliferation. Conventional $CD4^+Foxp3^-$ T cells (Tconv) that cannot respond to TGFβ either during development using CD4-cre.TGFβRII$^{f/f}$ or in the periphery using CD4-creERt2.TGFβRII$^{f/f}$ show increased proliferation. In parallel, Tregs in these systems have also lost responsiveness to TGFβ and also exhibit increased proliferation in thymus, spleen, and LNs (Marie et al., 2006; Sledzinska et al., 2013). When the CD4-cre.TGFβRII$^{f/f}$ mouse was crossed to a BDC2.5 TCR transgenic NOD background, Treg function, development, and maintenance appeared to be unaffected (Ishigame et al., 2013). However, these mice still progressed rapidly to diabetes, which leaves a role for TGFβ in Tregs incompletely defined. TGFβ deletion in all T cells during development using the CD4-cre.TGFβRII$^{f/f}$ results in a massive lymphoproliferation which obstructs the cell intrinsic contribution of TGFβ to the increased Treg proliferation, as these Tregs may be expanding to attempt

to control the ongoing inflammation (Marie et al., 2006). If TGFβRII is deleted after the immune system has matured using a CD4-creER, no overt autoimmunity develops, but both Tconv and Treg cells do exhibit a cell intrinsic increase in proliferation (Sledzinska et al., 2013). When TGFβ expression is directed under the control of the rat insulin promoter, increased Treg proliferation occurs in the islets of NOD mice (Peng et al., 2004). Thus, the role of TGFβ in Treg proliferation is still unclear and may be distinct from its role in other T cell lineages. Alternatively, the outcome of TGFβ signaling for Treg proliferation and function may be different in the tissue versus the lymphoid organs and/or influenced by the activation state of the Tregs.

Treg function depends on location

Both the tissue and draining lymph node have been described as important sites for Tregs to exert their suppressive effects, depending on the immunological context. In 2008, *Samy et al.* found that Tregs from ovarian LNs preferentially suppressed autoimmune ovarian disease and Tregs from the lacrimal gland preferentially suppressed dacryoadenitis, supporting a model where Tregs from local LNs are positioned for control of diseases in their respective tissues (Samy et al., 2008). Experiments utilizing the Kaede photo convertible fluorescent reporter found that Tregs were more restrained in their exodus from the cervical LN than Tconv cells, supporting a more localized population of cells which are poised to exert their suppressive functions (Morton et al., 2014). Indeed, Tregs from draining LNs were 15-50 times more efficient than non-draining LN Tregs at controlling autoimmunity in ovary, prostate, and lacrimal glands (Wheeler et al., 2009). In the NOD mouse, islet antigen specific Treg therapy also prevents activation of effector cells in the draining pancLN (Tang et al., 2006).

In addition to exerting their function in the draining LN, Tregs act at the later stages of an immune response directly in the target tissue. Tregs can suppress established $CD4^+$ T mediated intestinal inflammation (Pandiyan et al., 2007) and chronic skin infection (Belkaid et al., 2002) in the tissue. In NOD mice, $CD4^+CD25^+CD69^-$ Tregs have also been found to operate directly in the autoimmune site in an ICOS dependent manner (Herman et al., 2004). In BDC2.5 TCR transgenic mice, where all $CD4^+$ T cells are reactive against islet antigen Chromogranin A (Delong et al., 2016; Stadinski et al., 2010a), the absence of Tregs did not affect LN priming, but instead T cells in the islets were immediately destructive, ascribing a critical role for Treg function in the tissue in this model (Chen et al., 2005). If Tregs are deleted after inflammation is established in BDC2.5 mice, diabetes occurs within 3 days, a phenotype that is mediated by IFNγ producing NK cells in the islets (Feuerer et al., 2009). This indicates Tregs restrain NK effector function directly at the site of inflammation. When using BDC2.5 Treg therapy, the primary effect of Treg treatment is a blockade in effector function at the site of inflammation (Mahne et al., 2015). Thus, Treg function in the tissue is an important site of Treg regulation of effector responses during autoimmunity and inflammation.

T Cell Receptor (TCR) influence on Tregs

Tregs develop in the thymus due to an enhanced, but not overly strong, reactivity to self antigen. Rather than being deleted by negative selection, thymocytes may be diverted in to the Treg lineage (Cozzo Picca et al., 2011; Jordan et al., 2001; Kawahata et al., 2002; Koonpaew et al., 2006; van Santen et al., 2004). Once these Tregs enter the circulation, they are poised to re-encounter that antigen in the draining LN or tissue and exert suppression. In contrast, naïve T cells must be primed by antigen in the

context of an immune response to exert their effects. For Tregs converted from naïve $CD4^+$ T cells in the periphery, it is likely that local antigens not found in the thymus drive conversion (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005; Paiva et al., 2013). For example, the colonic Treg repertoire may be shaped by antigens found in the gut, including the microbiota (Lathrop et al., 2011).

Recent reporters of Treg reactivity to antigen use the orphan nuclear receptor Nr4a1, or Nur77 (Moran et al., 2011; Zikherman et al., 2012). Tregs in these mice have a constitutively high expression of $Nur77^{GFP}$, which suggests continuous exposure to antigen in the periphery (Moran et al., 2011). *In vitro*, Tregs must have TCR stimulation to exert their suppressive effects (Onishi et al., 2008; Thornton and Shevach, 1998). *In vivo,* continuous TCR signaling does not appear to affect maintenance of resting Tregs, but may be critical for a subset of Tregs to control inflammation during an immune response (Arpaia et al., 2015; Levine et al., 2014; Vahl et al., 2014).

Expanding antigen specific endogenous Tregs *in vivo* may increase their efficacy in preventing autoimmunity. Oral delivery of known autoantigens in the context of anti-inflammatory cytokines such as IL10 by genetically modified bacteria (Robert et al., 2014) or Fc receptor conjugated to preproinsulin delivered to the fetus through the placenta (Culina et al., 2015) increases the frequency of islet antigen specific Tregs. Gene transfer of insulin peptide B amino acid residues 9-23 (B:9-23) or apoptotic cells delivered to APCs also induced antigen specific Tregs and prevention of T1D (Akbarpour et al., 2015; Kasagi et al., 2014).

However, not all antigens important to autoimmune disease as know. Determining the antigen specificity of Tregs involved in immune responses is of great

interest to the immunology community. Each T cell possesses a unique T cell receptor (TCR) that determines its specificity to its cognate antigen. Analysis of the TCR usage of bulk T cell populations can be used to track individual T cell clones present in the lymphoid organs and tissues. When the TCRβ chain in NOD mice was evaluated via flow cytometry, unique TCRβ usage in $CD4^+$ and $CD8^+$ T cells was found in the islets compared to peripheral lymphoid sites (Diz et al., 2012; Li et al., 2009). Sequencing the TCRβ of islet $CD4^+CD44^{hi}$ T cells found dominance of certain TCRβ chains, with comparable frequencies of Vβ gene usage between pre-diabetic and diabetic mice. Even though the islet $CD4^+CD44^{hi}$ T cells were proliferating and possessed high frequency clonotypes, there was still a large degree of diversity (Marrero et al., 2013). In young NOD mice, islet TCRβ sequencing also revealed a biased repertoire with some overlap between pancLN or gut, but not all three (Toivonen et al., 2015). At least some islet T cells do possess specificity to islet antigens (Baker et al., 2002) and their proliferation is likely driven by islet antigen encounter. When comparing between multiple mice, some clones were present in at least 2 different mice, which we call “public” clones here. However, none of these sequences were among the expanded clones in both mice (Toivonen et al., 2015). Combined, these results point to a stochastic model of TCR stimulation in the tissue in which a similar set of autoantigens stimulate a variable array of TCRs. Whether Tregs follow this same pattern of diversity and dominance of certain clonotypes in the tissue has yet to be investigated.

TCR specificity to islet antigens is important for accumulation of $CD4^+$ T cells in the islets. Using TCR transgenic cells with known reactivity, a critical role for islet antigen specificity in T cell retention in the islets was demonstrated (Calderon et al.,

2011; Lennon et al., 2009). Tregs are even less likely to circulate through lymphoid sites and the islets than effector cells (Magnuson et al., 2015), also likely due to enhanced cognate antigen recognition in the tissue. The possibility that Tregs and Tconv cells are reactive to similar tissue antigens is unknown.

Insulin as an autoantigen in T1D

Insulin is a primary driver of autoimmune diabetes for $CD4^+$ T cells (Nakayama, 2011; Nakayama et al., 2005; Unanue, 2014; Zhang et al., 2008). Pathogenic T cells can escape thymic deletion due to their recognition of post-translationally modified versions of insulin not present in the thymus (Delong et al., 2016; Mohan et al., 2010). When T cell apoptosis is prevented in Bim deficient NOD mice, Treg numbers increased, including proinsulin specific Tregs. This suggests that strong TCR signals would normally delete these cells and preventing this death results in diversion into the Treg lineage and disease prevention (Krishnamurthy et al., 2015). On a background with limited TCRβ or TCRα chains, diabetes development is prevented likely due to lack of insulin B:9-23 specific effector T cell development. However, Sjogren's syndrome (SS) still develops in these mice (Kern et al., 2014), suggesting that there is a limited range of TCR antigens responsible for diabetes development compared to SS.

Insulin specific T cells in NOD mice are capable of recognizing different amino acid sequences of insulin peptides bound to Major Histocompatibility Complex (MHC) class II. The T cell response in NOD mice to insulin is strongly directed to the β chain segment of insulin resides 9-23 (B:9-23) (Abiru et al., 2001; Abiru et al., 2000; Levisetti et al., 2007; Nakayama et al., 2007). However, this peptide binds weakly to NOD MHCII allele, $I\text{-}Ag^7$ (Hausmann et al., 1999; Levisetti et al., 2007). Using TCR

hybridomas specific for insulin, two types of $CD4^+$ T cells were identified: type “A” T cells can respond to insulin protein processed and presented by antigen presenting cells (APCs), while type “B” T cells respond only to the insulin peptide offered exogenously to the APCs. Because insulin peptide B:9-23 binds the APCs weakly, this may limit expression in the thymus and be important for type B T cell escape from negative selection. Islet DCs are capable of stimulating both types of T cells, possibly because β cells transfer peptide directly to islet DCs (Levisetti et al., 2007; Mohan et al., 2010; Mohan et al., 2011; Vomund et al., 2015).

Upon further examination of insulin B:9-23 binding to MHCII, it was discovered that the peptide can shift its position in the peptide (p) binding groove of MHCII laterally based on how well the peptide sequence accepts residues in certain anchor positions, creating different “registers” of MHCII binding. Amino acids at anchor positions p1, p4, p6, and p9 interact with the MHCII groove (McFarland and Beeson, 2002). The minimal insulin B peptide (B:12-23) can bind $I\text{-}Ag^7$ in four different registers. Most of the diabetogenic $CD4^+$ T cells target the least favorable register for stability in the MHCII pocket, register 3 (Stadinski et al., 2010b). In order to stabilize the register 3 binding to $I\text{-}Ag^7$, anchor residues p1 and p9 were mutated. Registers 1 and 2, which stimulate a minority of $CD4^+$ T cells in NOD mice, did not require anchor residue optimization to stimulate T cells (Stadinski et al., 2010b). Thus, two register 3 trapped mimotope peptides were created. In the first, the p9 residue was mutated from an arginine to a glycine while p8 remained a glutamic acid (p8E). The second mimotope was also mutated at p9 along with another mutation: that of p8 to glycine (p8G). These two mimotopes allowed for creation of a tetramer reagent to identify insulin specific T cells.

This p8 residue was found to be critical for binding of some T cells and inhibitory to others (Crawford et al., 2011). Thus, these tetramer reagents are a useful tool for studying insulin reactivity in T1D. Still, the naturally processed and presented insulin peptides in NOD mice are not well understood, but may contain a combination of these unstable peptides as well as post translationally modified peptide fusions created by β cells (Delong et al., 2016).

Use of TCR transgenic mice is yet another tool to increase our understanding of the development and function of insulin specific T cells. We can also use these mice to examine the potential of different TCRs to cause diabetes as well as facilitate Treg development. For example, on the Rag knockout background, some insulin specific TCRs do not facilitate conversion into Tregs, such as 8F10. These T cells immediately destroy the islets and mice progress rapidly to diabetes (Mohan et al., 2013). This phenotype is mirrored in BDC2.5 Chromogranin A-specific Rag deficient mice (Chen et al., 2005). Other insulin specific TCR tg mice, such as BDC12-4.1, do have the capability of forming Tregs in the periphery that are important for a partial protection from diabetes dependent on Tregs (Fousteri et al., 2012; Jasinski et al., 2006). It is not clear from these studies how the TCR structure may influence Treg development or if insulin specific Tregs are more likely to be generated in the thymus compared to the periphery. Additionally, the contribution insulin specific Tregs make to the islet T cell population in unmanipulated mice has not been defined.

Discovery of novel autoantigens both for Tregs and Tconv cells will be useful for understanding the pathogenesis of T1D. TCR sequencing of individual cells from the site of inflammation and cloning those TCRs into new vectors is currently the best

method to examine specificity, which allows one TCR at a time to be examined (Burzyn et al., 2013b; Savage et al., 2008). Since the recombination of TCR sequences in different mice is stochastic, T cells may have similar reactivity to the same antigen with different TCR sequences. In this study, one of our goals was to we examine Treg specificity within the polyclonal pool to find Treg TCRs that are responsive to tissue antigen. Once validated in humans through collaborations with groups such as nPod (Pugliese et al., 2014), these findings may assist in development of targeted therapies. Currently, isolation and expansion of polyclonal human Tregs from T1D patients that were infused back into the patient have shown no high-grade adverse events and the Tregs were still present at 1 year after transfer (Bluestone et al., 2015). The efficacy of polyclonal versus antigen specific Tregs in human T1D has yet to be investigated.

CHAPTER II: REGULATORY T CELL REPERTOIRE AND SPECIFICITY IN INFLAMED ISLETS OF DIABETES-PRONE MICE

Abstract

Regulatory T cells (Tregs) accumulate in the inflamed islets and delay the progressive destruction of insulin-producing beta cells in non-obese diabetic (NOD) mice, a mouse model of type 1 diabetes. To understand the relationship between Tregs from the inflamed tissue and peripheral lymphoid sites, we evaluated their activation state and T cell receptor (TCR) usage. Here, we demonstrate that Tregs from the inflamed islets were more effective at preventing diabetes than splenic or lymph node Tregs when transferred to Treg-deficient NOD.CD28-/- recipients. Islet Tregs had higher Nur77 and CD5 expression that correlated with higher proliferation and expression of activation markers CD103, ICOS, and TIGIT, suggesting that they had been recently activated by their cognate antigens. To better understand the efficacy of islet Tregs, we compared the repertoire of Tregs from islets and various lymphoid compartments using high throughput TCRβ sequencing. We found that islet Treg repertoires were polyclonal, highly individualized in different mice, and rarely shared with those from Tregs in peripheral lymphoid compartments. Using single-cell TCR sequencing, we isolated 8 TCRαβ pairs from islet Tregs and found 2 to be insulin specific and an additional 3 specific to unidentified islet antigens. Thus, islet antigen-specific Tregs accumulate and expand in inflamed islets and control the progression of diabetes.

Introduction

Among the multiple mechanisms involved in peripheral immune tolerance, $CD4^+$ Foxp3-exrpessing regulatory T cells (Tregs) are essential in preventing autoimmunity in mice and man (Fontenot and Rudensky, 2005; Tang and Bluestone, 2008). Numerous suppressive mechanisms are ascribed to Tregs that include secretion of inhibitory cytokines, costimulation blockade, dendritic cell (DC) modulation, IL2 deprivation of effector cells, and direct target cell killing (Shevach, 2009; Yamaguchi et al., 2011). Early *in vitro* studies demonstrate that TCR stimulation is critical for Tregs to exert their suppressive effects (Thornton and Shevach, 1998). The requirement for TCR signaling in Treg function *in vivo* is suggested by the observation that Tregs specific for antigens expressed in target organs are more effective at controlling organ specific autoimmunity such as autoimmune ovarian disease, dacryoadenitis (Samy et al., 2008), ovary, prostate, lacrimal glands (Wheeler et al., 2009), experimental allergic encephalomyelitis (McGeachy et al., 2005; Yu et al., 2005), and type 1 diabetes (T1D) (Chen et al., 2005; Herman et al., 2004). More recently, using Treg specific conditional TCR deletion, the essential role of continuous TCR signaling in Treg function during immunological challenge *in vivo* has been demonstrated (Levine et al., 2014; Vahl et al., 2014). These findings suggest that the antigen specificity and reactivity of Tregs is crucial to their function in preventing autoimmune diseases and other immunopathologies.

Tregs are thought to be predominantly specific for self-antigens. Exposure to self-antigens in the thymus during T cell development induces thymic Treg development (Cozzo Picca et al., 2011; Jordan et al., 2001; Kawahata et al., 2002; Koonpaew et al.,

2006; van Santen et al., 2004) whereas non-inflammatory exposure to self-antigens in the periphery can induce mature T cells to become peripheral Tregs (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005; Paiva et al., 2013). In the periphery, Tregs are continuously stimulated by antigens, as evidenced by their requirement for CD28 to maintain peripheral homeostasis and proliferation in steady state (Salomon et al., 2000; Tang et al., 2003). TCR signaling leads to transcriptional activation of Nur77 (Osborne et al., 1994)). Using Nur77 reporter mice that express GFP transgenes under the control of Nur77 promoter, two independent groups found that Tregs have uniformly high basal expression of $Nur77^{GFP}$, providing further evidence of continuous self-antigen stimulation of Tregs in steady state (Moran et al., 2011; Zikherman et al., 2012). However, antigen specificity of Tregs remains poorly defined. Using mice with a transgenic TCRβ chain to restrict T cell repertoire for easier identification of Treg antigen specificity, *Savage et al.* found a nearly oligoclonal expansion of Tregs in prostate tumors (Savage et al., 2008). These Tregs develop in the thymus and are specific for an unidentified prostate antigen (Leventhal et al., 2016; Malchow et al., 2013). Similarly, clonal expansion of Tregs has been reported in acutely injured muscles. *Burzyn et al.* found a single pair of TCRαβ chains repeatedly in different mice; however, the identity of the muscle antigen recognized by these Tregs remains obscure (Burzyn et al., 2013b).

NOD mice spontaneously develop inflammation in the islets of Langerhans, leading to complete destruction of insulin producing beta cells over a 2 to 6 month period (Anderson and Bluestone, 2005). Destructive inflammation is primarily driven by autoreactive $CD4^+$ and $CD8^+$ T cells that arise due to defective negative selection in the

thymus (Mohan et al., 2011; Serreze et al., 2008; Zucchelli et al., 2005). Tregs play a critical role in controlling the rate of beta cell destruction and diabetes penetrance. Tregs control this process by limiting clonal expansion in the draining lymph node (LN) (Tang et al., 2006) as well as suppressing effector T cells in the islets to prevent destruction of the beta cells (Chen et al., 2005; Feuerer et al., 2009; Herman et al., 2004; Mahne et al., 2015). Antigen specificities of diabetogenic T cells have been a focus of intensive investigation. Identifying antigens recognized by T cells cloned from inflamed islets has proven to be challenging. For example, in the non-obese diabetic mouse model of T1D, an islet antigen specific T cell clone, BDC2.5, was first isolated in 1993 and its antigen specificity remained unknown for 17 years (Katz et al., 1993; Stadinski et al., 2010a). Recent data suggests that BDC2.5 may bind most strongly to a post-translationally modified fusion peptides from islet beta cell antigens insulin and Chromogranin A (Delong et al., 2016). An alternative method for mapping antigen specificity of T cells is to start with candidate antigens. Insulin is a major protein product of islet beta cells and proven to be a key autoantigen in the NOD mice and in patients with T1D (Nakayama, 2011; Unanue, 2014; Zhang et al., 2008). The insulin B chain amino acid 9 to 23 (Ins B:9-23) is a dominant epitope recognized T cells. Insulin is expressed in the thymus in an autoimmune regulator (AIRE)-dependent manner (Anderson et al., 2002). Thus, insulin specific T cells are normally deleted during thymic development. In NOD mice, the Ins B:9-23 peptide can be presented by the sole MHC class II molecule I-Ag^7 in 4 different registers (Stadinski et al., 2010b). The binding of the peptide to I-Ag^7 in register 3 is very unstable, resulting in poor presentation of register 3 Ins B:9-23 and escape of register 3 specific T cells from thymic selection

(Crawford et al., 2011; Mohan et al., 2013; Stadinski et al., 2010b). Thus, a large number of insulin-specific diabetogenic T cells are specific for Ins B:9-23 presented in register 3.

The specificity of Tregs that oppose the progression of autoimmune attacks of the beta cells has not been studied. The use of TCR transgenic mice has shown that chromogranin A or insulin-specific Tregs can delay and even prevent diabetes (Fousteri et al., 2012; Tang et al., 2004b; Tarbell et al., 2004), however, a more comprehensive analysis of antigen specificity of the natural polyclonal pool of T cells has been lacking. Here we report our study examining the antigen specificity of Tregs in chronically inflamed islets in the NOD mouse. Our investigation revealed a local enrichment of Tregs in the islets that were highly potent in suppressing disease progression. Islet Tregs are activated and proliferate locally in the inflamed islets and are rarely found in the peripheral lymphoid organs. We further identify insulin as well as other unidentified islet autoantigens as cognate ligands for islet Tregs. These findings have important implications for understanding the balance between effector and Tregs in the pathogenesis of T1D.

Results

Tregs from inflamed islets are more effective in preventing diabetes than Tregs from peripheral lymphoid organs

We first assessed the *in vivo* capacity of Tregs from different tissues to prevent diabetes in an adoptive transfer model. As a model system, we used NOD.CD28$^{-/-}$ mice that develop diabetes at a younger age and at higher penetrance when compared to regular NOD mice due to defects in development, peripheral homeostasis, and function

of Tregs (Salomon et al., 2000; Tang et al., 2003). Majority of NOD.CD28$^{-/-}$ mice become diabetic between 8-12 weeks of age. Adoptive transfer of 50,000 islet antigen specific Tregs from the lymph nodes (LNs) of BDC2.5 TCR transgenic mice or non-TCR transgenic Tregs isolated from the islets of pre-diabetic wild-type NOD mice into NOD.CD28$^{-/-}$ mice prevented diabetes (Figure 1). On the other hand, the same dose of non-TCR transgenic Tregs sorted from spleen, pancreatic LNs (pancLN), or non-islet draining LNs (ndLNs: mesenteric LN, inguinal LN) from pre-diabetic wild-type NOD mice were unable to prevent diabetes. This data demonstrates that inflamed islets in pre-diabetic NOD mice contain potent Tregs.

T cell receptor stimulation of Tregs in inflamed islets

We hypothesized that the increased islet Treg efficacy in suppressing diabetes was due to an enrichment of islet antigen specificities. To investigate the antigen experience of islet Tregs in the NOD mice, we used a strain of Nur77^{GFP} reporter mice (Zikherman et al., 2012). T cells in Nur77^{GFP} reporter mice express GFP in response to antigen stimulation of the T cell receptor (Zikherman et al., 2012), but not acute inflammatory stimuli (Moran et al., 2011). Thus, GFP expression indicates TCR engagement. We backcrossed the Nur77^{GFP} mice for more than 10 generations with NOD mice, using speed congenics for the first four generations of backcrossing. We then further crossed the NOD.Nur77^{GFP} mice with NOD.Foxp3^{RFP} mice (Wan and Flavell, 2005; Xiang et al., 2012) to aid the analysis of Tregs. These mice had normal cellularity and proportions of T cells in peripheral lymphoid organs (data not shown) and developed diabetes at comparable kinetics and overall incidence to standard NOD mice (Supplemental Figure 1).

Tregs in these mice showed constitutively high level of GFP expression as previously reported (Moran et al., 2011; Zikherman et al., 2012). To further define the correlation between GFP expression and strength and immediacy of TCR stimulation, we analyzed activated LN cells from NOD.Nur77^{GFP}.Foxp3^{RFP} reporter mice with increasing TCR stimulation *in vitro*. CD4^{+}Foxp3^{-} conventional T cells (Tconv) and CD8^{+} T cells showed dose-dependent increases in the percentages of Nur77^{GFP+} cells and in GFP mean fluorescence intensity (MFI) (Supplemental Figure 2A, B). Tregs were nearly all GFP^{+} directly *ex vivo* prior to stimulation. Nonetheless, increasing TCR stimulation led to a dose-dependent increase in GFP MFI in Tregs (Supplemental Figure 2A, B). To determine the duration of GFP expression after TCR stimulation, we cultured purified Tconv and Treg cells with plate-bound anti-CD3 and anti-CD28 *in vitro* for 24 hours and then removed the cells from stimulation and monitored GFP levels over time. GFP MFI in both Tconv and Tregs decreased after the cessation of TCR simulation, with a half-life of approximately 3 days (Supplemental Figure 2C). GFP in purified, unstimulated Tregs decayed *in vitro* at a similar rate to *in vitro* stimulated Tregs and Tconv, suggesting that their high Nur77^{GFP} expression was due to continuous stimulation by antigens *in vivo*. Taken together, this data demonstrates that the intensity of GFP in T cells of Nur77^{GFP} reporter mice integrates both the strength of the TCR signal received and the length of time since the most recent antigen exposure.

We next examined GFP expression in Tregs from peripheral lymphoid tissues and inflamed islets of NOD.Nur77^{GFP}.Foxp3^{RFP} mice as a proxy for Treg antigen experience. We found that Tregs in islets had the highest expression of Nur77^{GFP} compared to those from spleen and pancLN (Figure 2A). Moreover, we found the

integrin αE (CD103)-expressing Tregs were enriched in inflamed islets (Figure 2B and Supplemental Figure 3A). The $CD103^+$ islet Tregs had the highest GFP expression when compared to $CD103^-$ islet Tregs and Tregs from lymphoid organs (Figure 2C). The expression of CD5, another marker of recent TCR stimulation (Azzam et al., 2001; Mandl et al., 2013), was also expressed at a higher level by islet Tregs with the highest expression on $CD103^+$ Tregs (Figure 2D). The $CD103^+$ islet Tregs co-expressed additional Treg activation markers TIGIT and ICOS (Supplemental Figure 3B), and had higher expression of Foxp3 and CTLA4 than $CD103^-$ islet Tregs (Supplemental Figure 3C). Collectively, these results support the notion that Tregs in islets, especially those expressing CD103, ICOS, and TIGIT, have been more recently and/or strongly activated by antigens.

Islet Tregs have a unique and restricted TCR repertoire

To further characterize Tregs from different tissues, we performed high throughput TCRβ sequencing analysis of $CD103^+$ and $CD103^-$ Tregs purified from spleen, pancLN, inguinal-popliteal-sciatic LNs (iLN), and islets of NOD mice. In the islets, the $CD103^-$ Tregs were further divided into Nur77 $^{GFP\text{-}hi}$ and Nur77 $^{GFP\text{-}lo}$ fractions (Figure 3A). Between 1,600 and 600,000 Tregs were analyzed from different compartments and 1,144 to 62.604 unique TCRβ sequences were identified. Polyclonality of the Treg sequences was identified in all sites, although the $CD103^+$ compartment was consistently less diverse than the $CD103^-$ Tregs (Supplemental Table 1). We found $CD103^+$ Tregs in the islets to be least diverse such that the 10 most abundant clones represented more than 30% of its repertoire (Figure 3B). They are followed by the $CD103^-Nur77^{GFP\text{-}hi}$ islet Tregs with their 10 most abundant clones

occupying 20% of the repertoire. The CD103^{-}Nur77$^{GFP\text{-}lo}$ islet Tregs and Tregs in peripheral lymphoid tissue have highest diversity with top 10 clones representing less than 10% of the repertoire (Figure 3B and C). Using another measure of diversity, termed D50, we analyzed diversity in the different Treg populations. D50 is the percent of dominant and unique clones that account for 50% of the total CDR3 sequences with values ranging from oligoclonal of 0 to a maximal diversity of 50. We found that islet Tregs are associated with lower diversity compared to peripheral lymphoid tissue Tregs, corresponding to a lower D50 value (Supplemental Table 1). The presence of high frequency clones among islet Tregs suggests their clonal expansion. Indeed, Tregs in the islets were more proliferative than those in the pancLN, and CD103^{+} Tregs were the most proliferative subset by both Ki67 staining and *in vivo* BrdU labeling (Supplemental Figure 4). Our results thus far demonstrate that islet Tregs are distinct from Tregs in peripheral lymphoid tissues in their antigen experience and the presence of dominant clones.

Previous reports have found that some tissue-derived Treg clonotypes are public, meaning they are repeatedly detected in the same tissue in different mice (Burzyn et al., 2013b; Malchow et al., 2013). Therefore, we determined if the same TCR sequences could be found in the islet tissue from multiple mice. We found a very limited sharing of TCRβ sequences from the islet CD103^{+}Nur77$^{GFP\text{-}hi}$ Tregs among multiple mice (Figure 3D). Indeed, only 1 islet TCRβ sequence was found in all 4 mice analyzed and altogether 426 sequences were found in more than one mouse among the total of 19,671 sequences identified (2.17%). A very similar result was true for the other Treg subsets (data not shown). For comparison, we analyzed peripheral lymphoid organ

Tregs. While these populations were more polyclonal than islets, they still shared very few of their unique CDR3 sequences (6-28%), albeit more than islet Tregs (0.5-2.6%) (Figure 3E and data not shown). Collectively, this data demonstrates that islet Tregs are composed of clones unique to each mouse. This further suggests that generation and expansion of islet Treg clonotypes is stochastic and not by a pre-determined mechanism commonly used in all mice.

We next analyzed the repertoire sharing among Tregs in different tissues within the same mouse. In mouse #1, less than 7% of sequences detected in islet CD103$^-$ Tregs (77, 71, and 68) were found in spleen and/or iLN, accounting for less than 1% of the total sequences in these populations, whereas 22% of those were shared between spleen and iLN (Figure 3F). This trend held true for all mice analyzed. In general, spleen, pancLN, and iLN shared 10-20% of their TCRβ sequences, while less than 1% sharing was found between CD103$^-$ Tregs in these organs and those in islets (Figure 3G). Similar observations were made when comparing CD103$^+$ Tregs in various compartments (Figure 3H). These results suggest that a fraction of Tregs in peripheral lymphoid tissue recirculate leading to repertoire sharing; whereas islet Tregs are highly localized and its repertoire is not shared with those in peripheral lymphoid organs.

Insulin-specificity among islet Tregs

Our results thus far suggest that Tregs specific to islet autoantigens are locally activated in the islets and clonally expand. Insulin is a dominant autoantigen in type 1 diabetes for CD4$^+$ T cells in both NOD mice and human patients (Nakayama et al., 2005; Zhang et al., 2008). Therefore, we asked if Tregs in the islets could also be reacting to insulin. We examined Treg and Tconv reactivity to insulin by staining T cells

from various tissues in pre-diabetic NOD mice (15-18 weeks old) with I-Ag^7 tetramer containing modified Ins B:9-23 derived peptides p8E and p8G (Crawford et al., 2011). Both p8E and p8G had been stabilized in register 3 that is frequently recognized by diabetogenic CD4$^+$ T cells. p8E possessed the natural glutamic acid at the p8 position, which was mutated to a glycine in p8G to remove the bulky side chain to mimic a truncated version of the Ins B:9-23 peptide. Insulin-specific cells can be detected among islet Treg and Tconv cells, and no signal was detected by the negative control tetramer containing a peptide from hen egg lysozyme (HEL) (Figure 4A, B). The pancLN did not contain any detectable insulin tetramer positive cells. On average, 2% of Tregs in islets were insulin-tetramer positive compared to an average of 0.5% of Tconv cells (Figure 4B). Overall, the total numbers of insulin-specific Tconv cells per islet were slightly higher than total number of insulin-specific Tregs (Figure 4C).

To further determine the ability of insulin-specific T cells to form Tregs, we created a TCR transgenic mouse line using the 4F7 TCR, which recognizes a truncated insulin B chain peptide 12:20 (Mohan et al., 2010). The majority of CD4$^+$ cells in the NOD.4F7.Rag2$^{-/-}$ mice were insulin tetramer reactive (Figure 4D). High percentages of Tregs are detected in the blood and spleen of 13-15 week old 4F7.Rag2$^{-/-}$ mice, higher than those detected in age-matched wild-type NOD mice (Figure 4E). Tregs were also detected in the islets at similar frequencies as seen in wild-type NOD mice, but very few were found in the pancLN (Figure 4E). In these mice, a partial protection from diabetes compared to other islet antigen specific TCR transgenic mice was observed (Chen et al., 2005; Mohan et al., 2013); although these mice developed diabetes starting at 6 weeks compared to wild-type NOD mice that developed disease starting at 12 weeks of

age, approximately 50-60% of the mice are diabetic by 27 weeks of age, similar to standard NOD mice (Supplemental Fig 5). Collectively, this data demonstrates that insulin-specific Tregs can be generated in NOD mice and may be partially protective in diabetes development.

Islet antigen specificity of islet Tregs

Using a more unbiased approach to determine if Tregs infiltrating islets are specific for islet antigens, we performed sequencing of paired TCR α and β chains of single-cell sorted $ICOS^+TIGIT^+$ islet Tregs. Among the 71 paired TCRαβ sequences identified, 6 TCRs were found more than once in a single mouse, indicating clonal expansion, and 2 other TCRs were found in multiple mice (Table 1). Among these 8 TCRs, 3 used the Vα chain 5D-4, a TCR chain that is frequently used by insulin-specific $CD4^+$ T cells (Table 1) (Nakayama et al., 2012). We selected these 8 TCRs for further analysis of their reactivity to islet antigens.

We expressed the TCRs using retroviral transduction in a hybridoma cell line that expresses an $NFAT^{GFP}$ reporter construct so that TCR induced calcium signaling would lead to induction of GFP expression. The 8 islet Treg-derived TCRs along with 2 control TCRs specific for HEL (PA21.14H4) or insulin (p3w17) were first screened against 2 known islet autoantigens: insulin and a peptide mimotope recognized by BDC2.5 T cells, p31. Additionally, we stimulated the hybridomas with the recently identified hybrid-insulin peptides (HIPs), including Ins-WE14, Ins-IAPP2, 2.5HIP, and 6.9HIP (Delong et al., 2016). Splenic $CD11c^+$ DCs were loaded with peptides and used to stimulate the TCR transduced hybridomas *in vitro* for 20-24 hours. None of the TCRs responded to p31 or any of the HIPs (data not shown). One TCR (521) responded to

splenic DCs loaded with the mixture of peptides that included Ins B:9-23 and modified Ins B:9-23 peptides that were locked in register 1, 2A, 3A, and 3B (Figure 5A, (Crawford et al., 2011; Stadinski et al., 2010b)). Further analysis using individual insulin peptides showed that TCR 521 was responsive only to insulin peptide trapped in register 2A and not native Ins B:9-23 (data not shown). TCR 521 was one of the three TCRs that used Vα chain 5D-4. The other two Vα 5D-4 containing TCRs (510 and 520) did not respond to insulin peptide mix (data not shown). Since 1 to 3% islet Tregs bound to register 3 trapped p8E or p8G tetramers, we also stimulated the hybridoma using immobilized p8E or p8G tetramers. TCR 510, a Vα 5D-4 containing TCR, responded to p8E but not p8G (Figure 5B). The other 5 TCRs did not respond to either splenic DCs loaded with the insulin peptide mix or insulin tetramer p8E or p8G (Table 2 and data not shown).

Next, we stimulated the hybridomas with freshly isolated islet DCs. These DCs had acquired islet antigens from beta cells nearby and could efficiently activate islet antigen specific TCR transgenic T cells from BDC2.5 and 8.3 mice (data not shown). Five out of 8 clones had measurable reactivity to antigen presented by islet DCs: 510, 518, 521, 550, and 551 (Figure 5C). We also stimulated all TCR-transduced hybridomas with splenic DC pulsed with lysate from NOD.Rag2$^{-/-}$ islets. In this assay, the same 5 clones responded (Figure 5C). TCR 550, which was found 5 times in the single-cell sort, the most highly represented clone (Table 1), very strongly reacted with islet DCs but did not react to splenic DCs without added islet antigens (Figure 5C) or with DCs from the colon (Supplemental Figure 6). We found a small yet detectable percentage of cells from TCR 521 that responded to islet DCs and islet lysate, however this was barely above the unstimulated condition (Figure 5A, C). This result suggests

that these assays are not sensitive enough to detect all islet antigen reactivity and likely underestimates the reactivity of these Treg TCRs to islet antigens. Furthermore, the amount of antigen presented in the islets will likely also play a role in determining the number of cells that can respond to TCR stimulation. In summary, out of 8 islet Treg-derived TCRs, we found 2 specific for insulin and 3 specific for unknown islet antigens (Table 2). The other 3 clones were not stimulated in any of the assays (Figure 5C and Table 2). Taken together, we have found that the clonally expanded, activated islet Tregs subset contains cells specific to both insulin and other islet antigens.

Discussion

In this study, we have analyzed the repertoire and specificity of Tregs present in chronically inflamed islets of autoimmune diabetic NOD mice. We found that Tregs from the inflamed islets were highly potent in preventing diabetes. They were polyclonal and contained cells that were activated though their TCR and had locally expanded. Islet Treg clones were rarely shared in different mice and infrequently found in peripheral lymphoid organs. Among a few high frequency clones analyzed, we found over half responded to islet antigens including insulin. Together, these findings demonstrate that Tregs specific to antigens present in inflamed tissue expand and accumulate locally to suppress local immune responses.

By combining Nur77 reporter, phenotypic analysis, and TCR sequencing, we have shown in this study that islet antigen specific Tregs are highly enriched in the inflamed islets and are rarely found in peripheral lymphoid organs. These results are consistent with previous reports that antigen-specific Tconv cells are selectively retained in the inflamed islets of NOD mice (Calderon et al., 2011; Lennon et al., 2009) and that

Treg trafficking between lymphoid organs and pancreas is less dynamic than Tconv cells in NOD mice (Magnuson et al., 2015). Islet Tregs express markers of activation, including CD103 that are expressed on tissue resident memory T cells (Mueller et al., 2014). Resident memory Tregs have also been reported for Tregs in the skin (Sanchez Rodriguez et al., 2014). A subset of Tregs has been described that reside in the spleen of normal mice with a tissue-seeking and activated phenotype (Smigiel et al., 2014) which requires TCR signal to maintain their phenotype (Levine et al., 2014). The $CD103^+$ Tregs we identified in the inflamed islets are most likely effector Tregs that are recruited by the ongoing inflammation and continuously activated by islet antigens locally.

TCR repertoire analysis revealed polyclonality of islet Tregs with clonal expansion mostly limited to the highly activated $CD103^+$ subset. Moreover, we found approximately 2% of islet Treg sequences were present in more than one mouse and only one clone out of total of 19,671 sequences identified was found in all four mice. This clone was not detected in a separate experiment using single cell TCR sequencing. The lack of public TCR among islet Tregs in the NOD mice is distinct from other reports of Tregs in tissues. For example, in MOG induced EAE, TCR clones were repeatedly identified in separate experiments for $Foxp3^-$ and $Foxp3^+$ cells (Nguyen et al., 2010). Similarly, in an acute muscle injury model, one Treg TCR appeared in the tissue in 11 out of 13 mice examined (Burzyn et al., 2013b). In the tumor setting, high frequency clones are also repeatedly found in different mice (Sainz-Perez et al., 2012). The stochasticity of islet Tregs in the NOD mice may be due to their survival defects and the chronicity of the inflammation. We have previously reported a survival defect of

islet Tregs in the NOD mice likely due to a deficit of IL-2 in the inflamed islets (Tang et al., 2008). This may lead to rapid turnover of Tregs in the islets and divergence of Treg repertoires over the course of weeks and months of asynchronous disease progression.

Although the clonotypes of the islet Tregs are distinct in individual mice, the antigens they recognize may be similar or even identical. Insulin is the most abundant protein in beta cells and a key autoantigen for $CD4^+$ and $CD8^+$ effector T cells driving the initiation and progression of autoimmune beta cell destruction (Jaeckel et al., 2004; Nakayama et al., 2005; Nakayama et al., 2007). The Ins B:9-23 peptide contains a critical epitope recognized by diabetogenic T cells. Using insulin tetramer staining, we found that insulin specific cells are present at higher frequency among islet Tregs than islet $CD4^+$ Tconv cells. The prominent presence of insulin-specific Tregs in inflamed islets is further demonstrated that 2 out of 8 islet Treg derived TCRs were specific for insulin. Recent reports show that Ins B:9-23 can potentially bind to I-Ag^7 in 4 different registers and many diabetogenic T cells are found to recognize Ins B9:23 in the weak register 3. Although insulin is expressed in the thymus to delete insulin-specific T cells, the poor binding of Ins B:9-23 in register 3 allows register 3 specific T cells to escape negative selection. 8F10 T cells are specific for Ins B:9-23 in register 3B (p8G). This TCR does not differentiate into Tregs in vivo (Mohan et al., 2013). In contrast, the 4F7 TCR specific for Ins B:9-23 in register 3A developed Tregs and delayed progression to diabetes. The lack of Treg development in the 8F10 mice maybe intrinsic to the TCRs and the way it recognizes insulin. Alternatively, it may be due to the fast progression to diabetes in these mice, not allowing time for Tregs to emerge. Interestingly, our register analysis of the two insulin-specific TCRs we single-cell cloned from islet Tregs show

that one was register 2 specific and the other was register 3 specific. More comprehensive analysis is needed to determine the register preference and thymic versus peripheral origin of insulin-specific Treg-derived TCRs.

We have previously shown that islet antigen-specific Tregs are orders of magnitude more potent than polyclonal Tregs in controlling progression of type 1 diabetes (Tang et al., 2004b). In our ongoing studies, we found that the potency of Tregs correlated with their ability to become activated by islet antigens in the islet draining lymph nodes (unpublished observations). Our results that polyclonal Tregs isolated from islets can effectively prevent diabetes demonstrate that islet antigen specific Tregs are among the natural repertoire of Tregs in diabetic prone mice and they are activated and recruited to the inflamed islets. Identifying antigens that activate these Tregs would not only help to understand the pathogenesis of the disease but also inform future therapeutic design. DCs derived from inflamed islets have naturally acquired and processed antigens from the inflamed islets. Using these DCs to measure TCR reactivity provides an approach to gauge the potency of the TCR to natural islet antigens. For example, the TCR derived from clone 550 is most strongly activated by islet DCs, but not by DCs from other tissue or DCs pulsed with high dose of insulin peptides in different registers. It would be of great interest to determine the identity of this antigen to understand specificity of islet Tregs and for future therapeutic development.

Taken together, this study shows that islet antigen specific Tregs are recruited and activated in inflamed islets to control disease progression. Some of these Tregs recognize the same epitope in insulin that is recognized by diabetogenic T cells.

Distinct TCR usage by islet Tregs in different mice suggests that these Tregs arise stochastically, or, not mutually exclusively, that the islet Treg population is highly dynamic with high turnover. This may provide an explanation for the variable penetrance and age of disease onset both in mice and potentially influence development of human T1D. Future work on determining the natural antigens for islet Tregs and factors that control the dynamics of islet Tregs will be important for understanding the pathogenic process of islet destruction and pave the way for developing therapeutic strategies to restore self-tolerance.

Materials and Methods

Mice: Female NOD/ShiLtJ, (Jackson laboratories) mice were housed and bred under specific pathogen free conditions in accordance with the UCSF (San Francisco, CA) Animal Care and Use Committee guidelines. NOD.Foxp3$^{mRFP(tm1flv)}$ (Wan and Flavell, 2005; Xiang et al., 2012), NOD.Foxp3^{eGFP} (Zhou et al., 2008), BDC2.5 (Katz et al., 1993), NOD.Rag2$^{-/-}$ (Soderstrom et al., 1996), and CD28$^{-/-}$ (Salomon et al., 2000) mice have been previously described. Nur77^{GFP} mice (Zikherman et al., 2012) were received from Art Weiss and backcrossed at least 10 generations onto the NOD background. The 4F7 insulin-specific hybridoma was received from Dr. Emil Unanue (Mohan et al., 2010). cDNA was isolated and used to amplify the TCR α (TRAV5D-4*02, TRAJ40*01) and and β (TRBV*01, TRBJ-5*01, TRBD1*01) chains by PCR. The alpha and beta chains were cloned into the pCD2 or the p428 TCR transgenic vectors, respectively (Wang et al., 2001; Zhumabekov et al., 1995). Plasmids were linearized and coinjected into NOD embryos. To genotype mice, the following primers were used: Vα forward 5'-GCAGGTGGAGCAGCTTCCTTCC-3', Cα reverse 5'-AGAGGGTGCTGTCCTGAGAC-3',

Vβ forward 5′-TCACTGATACGGAGCTGAGGC-3′, Cβ reverse 5′-GCCAAGCACACGAGGGTAGCC-3′. To confirm insulin-specificity, splenocytes were incubated with APC-conjugated tetramer containing the peptide sequence ERLYLVAGEE for 2 hours at room temperature, followed by 30 minutes at 4 C (Stadinski et al., 2010b).

Cell Transfers: $CD4^{+}Foxp3^{GFP+}$ or $Foxp3^{mRFP+}$ Tregs from NOD or BDC2.5 TCR transgenic mice were FACS sorted to >95% purity and transferred to 2-3 week old $NOD.CD28^{-/-}$ mice via intraperitoneal (i.p). injection.

Diabetes incidence: Diabetes incidence was monitored by weekly blood glucose monitoring. Mice were considered diabetic after 2 sequential blood glucose readings exceeding 300 mg/dL.

In vitro primary T cell stimulation assays: For quantification of $Nur77^{GFP}$ MFI, pooled LNs were isolated and stimulated with plate bound anti-CD3 (145-2C11) at the indicated concentrations for 16 hours. Cells were stained for CD4, CD8, fixable viability dye APC-eFluor780 (eBioscience) and analyzed on a Fortessa flow cytometer (BD Biosciences). For $Nur77^{GFP}$ decay experiments, $CD4^{+}Foxp3^{+}$ Tregs or $Foxp3^{-}$ Tconv cells were flow sorted and stimulated or not with plate bound anti-CD3 (3.05μg/mL) clone 145-2C11 and anti-CD28 (1μg/mL) clone PV1 for 24 hours in complete RPMI with 2,000 U/mL human IL2 (Treg) or 200 U/mL human IL2 (Tconv). Cells were removed from CD3/28

stimulation and grown in media and IL2 for an additional 6 days. Every 24 hours cells were analyzed for Nur77^{GFP} MFI.

Flow cytometry: Islet and lymph node single-cell suspensions were prepared as previously described (Melli et al., 2009). The following antibodies were used to stain the cells: CD103-FITC or Pacific Blue (2E7), ICOS-APC (C398.4A), Ki67-PE-Cy7 (SolA15), TIGIT-APCeFluor780 (MBSA43), CD5-APC (53-7.3), GITR-PE-Cy7 (DTA-1), Foxp3-eFluor450 (FJK-16s), (eBiosciences), anti-CD4-PE or APC (RM4-5) (eBioscience or Tonbo Biosciences). CD8-Pacific Orange (5H10) (Life Technologies), CTLA4-PE (UC10-4F10-11), CD45-APC-Cy7 (30-F11), Thy1.1-PerCP (OX-7) (BD Biosciences). For intracellular staining, cells were first fixed and permeabilized via the manufacturer's instructions (eBioscience or Tonbo Biosciences). Analyses were performed on a LSRII or Fortessa flow cytometer (BD Biosciences) with FACSDiva (BD Biosciences) and Flowjo analysis software. APC-conjugated tetramer of I-Ag^{7} bound to insulin 9:23 mimotopes p8E (HLVERLYLVCGEEG) and p8G (HLVERLYLVCGGEG) (Crawford et al., 2011) and HEL (AMKRHGLDNYRGYSL) were obtained from the National Institutes of Health tetramer facility and stained for 2 hours at 37^{o}C for pancLN and islet T cell analysis.

Bulk TCRβ analysis of Treg cells: Total RNA was extracted from flow sorted cells using ARCTURUS PicoPure RNA Isolation Kit (Life Technologies) for <100,000 cells or QIAGEN Micro RNA extraction kit (QIAGEN) for >100,000 cells. TCRβ repertoires were amplified and sequenced using Illumina MiSeq by iRepertoire Inc. (Huntsville). Data

analysis was performed using the website provided by iRepertoire Inc. (http://www.irepertoire.com).

Single cell TCRαβ primer design: All nested TCR primers were designed to amplify Vα, Vβ, Cα and Cβ, and base degeneracy was incorporated into the primers to account for TCR polymorphism and ensure amplification of all known functional regions identified in the IMGT database (http://www.imgt.org/). V-region primers were designed to target the leader sequence and near the 5' end of of each Vα, Vβ to include of the entire V(D)J region. All primers for the second PCR reaction contain the IlluminaTM common paired-end sequences, which enables further amplification with barcoding primers during the third reaction. Each primer for the third reaction consists of the appropriate Illumina adapter, an 8-nt plate ID sequence, and a 7-nt well ID sequence.

Single cell TCRαβ sort and sequencing: TCR sequence from single cells were obtained by a three rounds of PCR reactions. Cells were sorted directly into 11µl of sort buffer (8.5µl of water and 2.2µl of 5x One-step RT PCR buffer (Qiagen) and 0.3µl of Ribolock RNAse inhibitor (ThermoFisher). For the first RT-PCR reaction, reverse transcription and preamplification were performed with multiple Vα and Vβ region primers (final concentration 0.12µM each) and Cα and Cβ region primers (final concentration 0.6µM each) in a 15µl reaction. A 25 cycle RT-PCR reaction was performed according to the manufacturer's instructions. For PCR2 and PCR3 reactions, amplification was done using 2x HotStarTaq Master Mix Kit (Qiagen). In the second PCR reaction, 1µl of the RT-PCR product was added as a template to a 15µl PCR

reaction with the same final primer concentrations and the following cycling conditions: 95°C 15 min; 94°C 30 s, 68°C (TCRα) or 63°C (TCRβ) 1 min, 72°C 1 min, x 30 cycles; 72°C 5 min; 4°C. For the third PCR reaction, 1µl of the second PCR product was used as a template for a 20µl PCR reaction, which incorporates the barcode primers and enables sequencing on the IlluminaTM MiSeq platform. In the third PCR reaction, amplification was done for 36 cycles using unique plate ID barcoding primers (0.2µM), well ID barcoding primers (0.2µM), and Illumina adapter primers (1µM). The cycling conditions are as follows: 95°C 15 min; 94°C 30 s, 66°C 1 min 72°C 1 min x 36 cycles; 72°C 5 min; 4°C. The final PCR products were pooled at equal proportion by volume, followed by Ampure XP bead wash (Beckman Coulter), run on a 1% agarose gel, and a band around 450 to 500 bp was excised and gel purified using a Qiaquick gel extraction kit (Qiagen). DNA QC was determined by the Agilent 2100 Bioanalyzer and the final library was sequenced. Raw sequencing data were processed and demultiplexed according to the unique plate and well ID combination. To assign V(D)J families , data was sent to the IMGT HighV-QUEST (www.imgt.org/HighV-QUEST) database to predict germline allele usage, germline sequence recombination, and mutations relative to the germline sequence.

Hybridoma assays: TCRs of interest were cloned into the pMSCV-IRES-mCherry retroviral vector (Addgene). The hen egg lysozyme (HEL)-specific TCR, PA21.14H4 TCR, was obtained from Dr. Dario Vignali (Burton et al., 2010). Virus was generated using Phoenix-ECO packaging cells and used to transduce the TCR-deficient 58α-β-hybridoma cell line, which was modified to express GFP downstream of an NFAT

promoter (Ise et al., 2010; Letourneur and Malissen, 1989). Three days after transduction TCRβ^+mCherry$^+$ cells were sorted. For hybridoma stimulation assays, splenic or islet DCs were enriched using a CD11c$^+$ positive selection kit (STEMCELL Technologies). Together, 5x10^5 CD11c$^+$ cells and 5x10^5 hybridomas were co-cultured in round bottom 96-well plates. For cultures with splenic DCs, either commercially synthesized peptide (GenScript) at 10 μM or 1.87ug islet lysate was added to hybridoma cultures. Instead of using APCs, in some experiments, hybridomas were stimulated with 2.5 μg/mL plate bound tetramer of I-Ag^7 bound to HLVERLYLCGEEG (Insulin peptide 8E) in PBS coated 2 hours at 37^{o}C (Crawford et al., 2011). As a positive control, hybridomas were stimulated in wells coated with 3 μg/ml anti-CD3 antibody (clone 17A2, Tonbo, or 145-2C11). Cells were stimulated overnight in complete RPMI at 37^{o}C and analyzed the following day for GFP expression.

Islet lysate: Islets were resuspended in PBS and vigorously mixed. Cells were then frozen in dry ice and thawed at 37^{o}C twice. Then, lysate was mixed in a 2mL homogenizer followed by several passages through a 25 gauge needle. Protein quantification was performed with the Pierce BCA Protein Assay Kit (Thermo fisher).

Statistical analysis: Analysis was performed with Prism software (GraphPad).

Acknowledgements: We thank Bill Robinson and C. Ju for single cell sequencing analysis, J. Wang, V Dang, N. Lescano for mouse husbandry, V. Nguyen, J. Wang, JE Klementowicz, J. Tam, V. Panchumarthi for technical assistance. We would like to

thank the following individuals for generously providing us with mice or reagents: Nur77^{GFP} mice from Art Weiss, NOD.Foxp3^{mRFP} mice from Li Wen, and PA21.14H4 TCR from Dario Vignali.

FIGURES

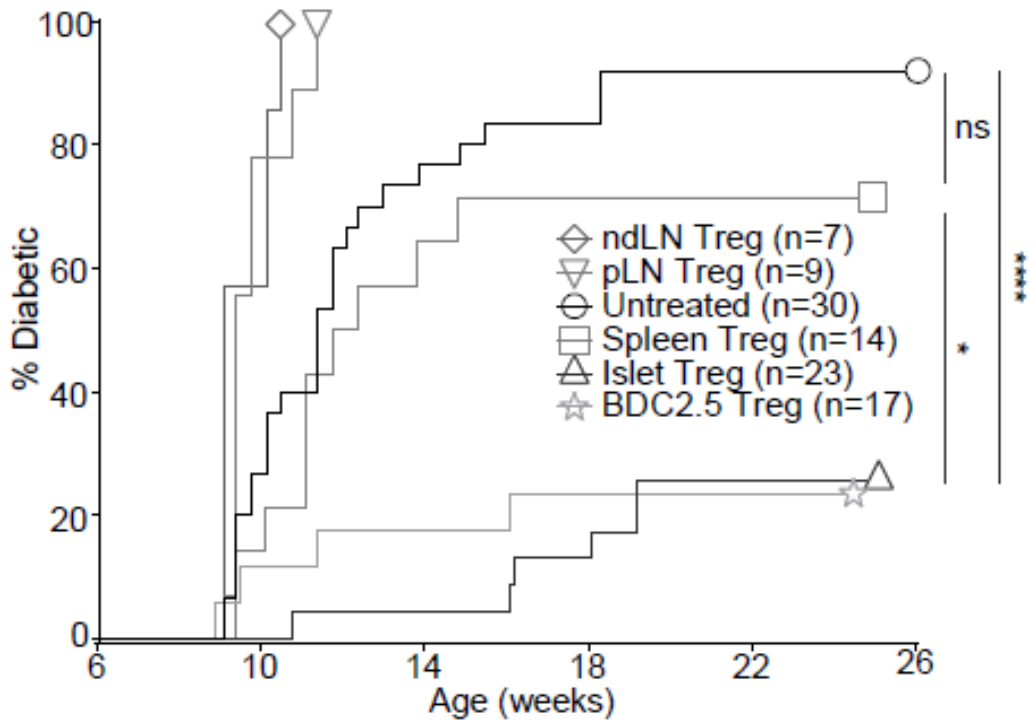


Figure 1: Polyclonal islet Tregs prevent diabetes similarly to islet antigen specific Tregs. 50K polyclonal Tregs were sorted from non-pancreas draining LN (ndLN), pLN, spleen, or islets and adoptively transferred into 2-3wk old CD28KO mice. Alternatively, BDC2.5tg+ TCRtg Tregs from LN and spleen were sorted and transferred in the same manner. Diabetes incidence was assessed.

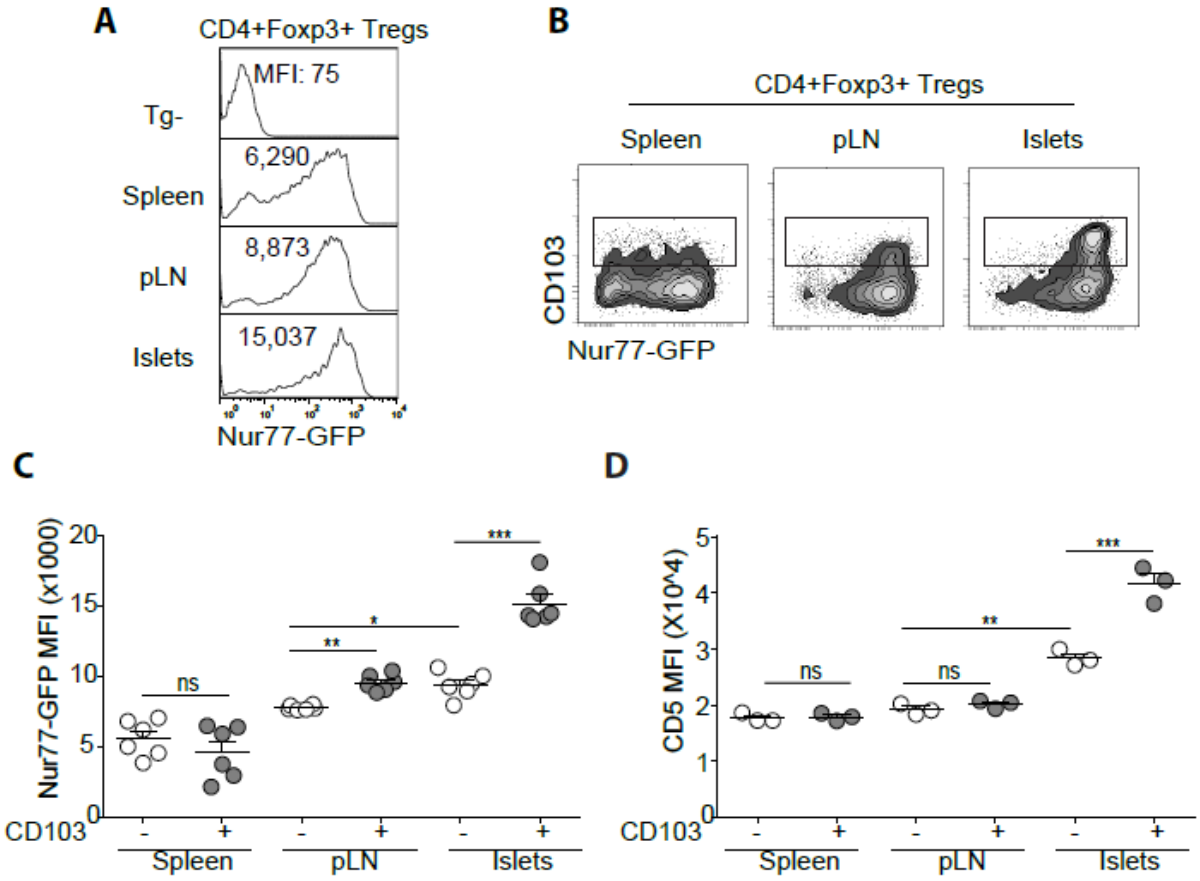


Figure 2: Evidence of antigen exposure at the site of inflammation. A. Tregs ($CD4^+Foxp3^+$) from pre-diabetic NOD mice were analyzed by flow cytometry for expression of $Nur77^{GFP}$. B. Tregs from pre-diabetic NOD mice analyzed by flow cytometry for expression of CD103 and $Nur77^{GFP}$. C. Quantification of $Nur77^{GFP}$ in $CD103^+$ or $CD103^-$ Tregs in B. D. CD5 MFI in $CD103^+$ or $CD103^-$ Tregs from pre-diabetic NOD mice.

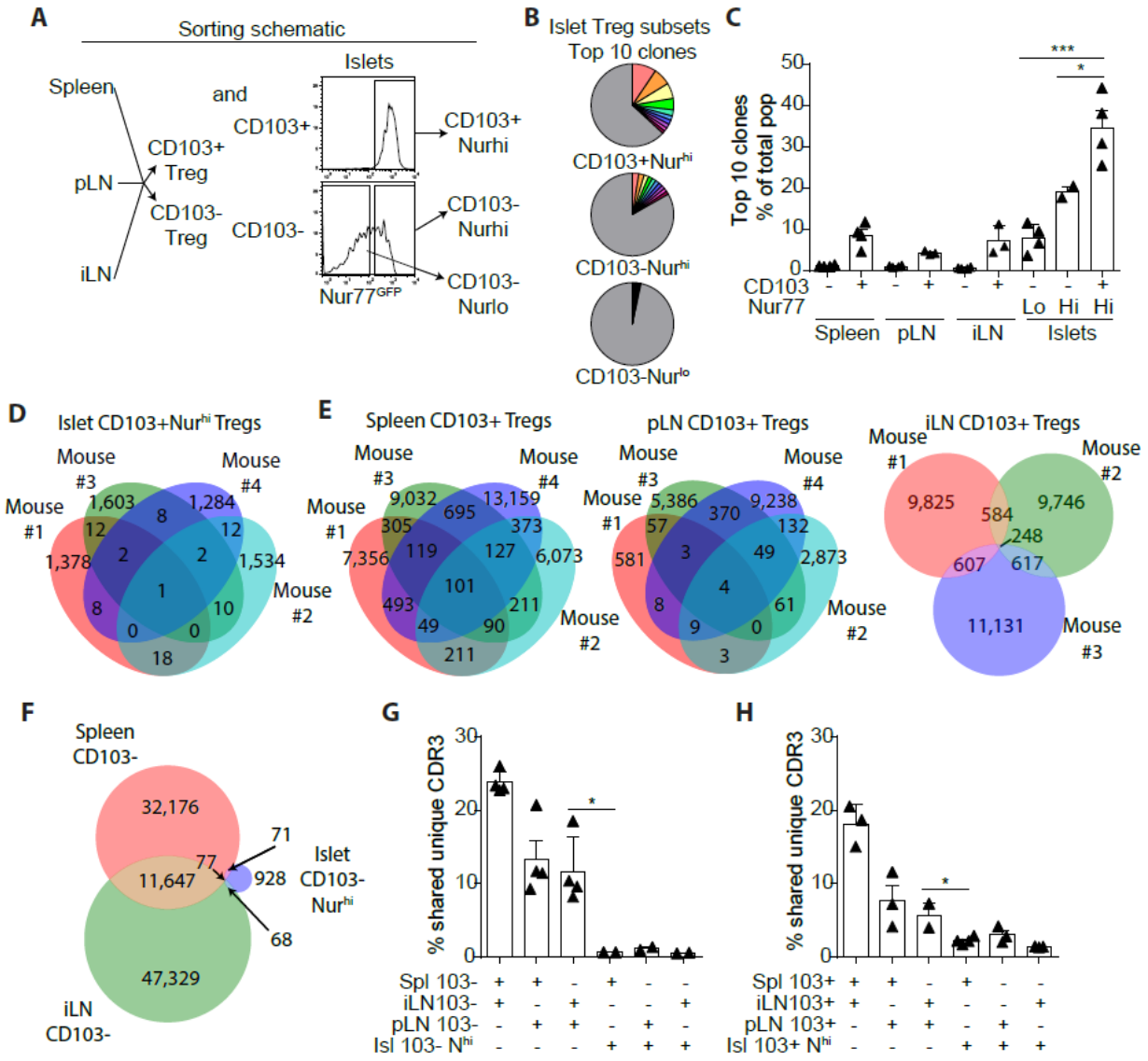


Figure 3: Islet Tregs have a restricted TCR repertoire based on antigen signaling and are not found in periphery. A. Sorting schematic spleen, pLN, ipsLN (iLN), and islets for TCRβ chain sequencing. $CD103^{+}$ and $CD103^{-}$ Treg populations were sorted from spleen, pLN, and iLN. Treg cells were additionally sorted on $Nur77^{GFP}$ expression from islets: $CD103^{+}Nur^{hi}$, $CD103^{-}Nur^{hi}$, and $CD103^{-}Nur^{lo}$. B. Frequency of the top 10 clones in different islet Treg populations. C. Quantification of the top 10 clone

percentage of the total population in each mouse. Each triangle represents a single mouse. D. Unique CDR3 sequences for 4 individual mice were compared in the islet $CD103^{+}Nur^{hi}$ Tregs. E. Unique CDR3 sequences for 3 or 4 individual mice were compared for spleen, pLN, or iLN $CD103^{+}$ Tregs. F. Unique CDR3 sequences from the same mouse were compared for Spleen $CD103^{-}$, iLN $CD103^{-}$, and islet $CD103^{-}Nur^{hi}$ Tregs. G. Quantification of unique CDR3 overlap between $CD103^{-}$ Tregs from spleen, pLN, iLN, and islet $CD103^{-}Nur^{hi}$. Each triangle represents a single mouse. H. Same analysis as in G for $CD103^{+}$ Tregs.

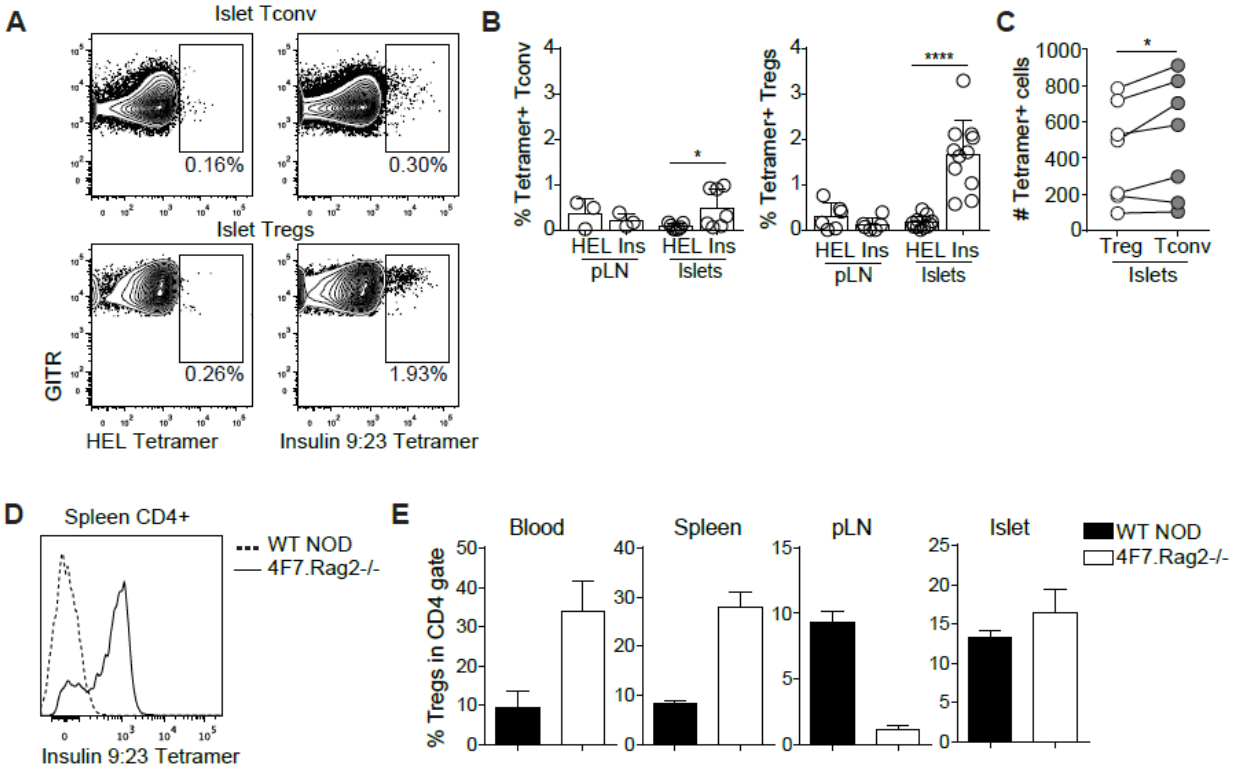


Figure 4: Insulin specific Tregs are found in islets. A. Insulin peptide 9:23 tetramer staining versus HEL tetramer control in Islet Tconv and Tregs. Cells were gated as $CD45^{+}DAPI^{-}Thy1.1^{+}CD8^{-}GITR^{hi}Foxp3^{+/-}$. B. Quantification of HEL and Insulin tetramer staining in pLN and Islet Tconv and Tregs. C. Total number of Insulin tetramer+ Tregs and Tconv from islets. D. Insulin tetramer staining of $4F7.Rag^{-/-}$ mice versus WT NOD mice. E. Percentage $Foxp3^{+}$ Tregs in the CD4 gate in Blood, Spleen, pLN, and Islets of 13-15wk old NOD and 4F7.RagKO mice.

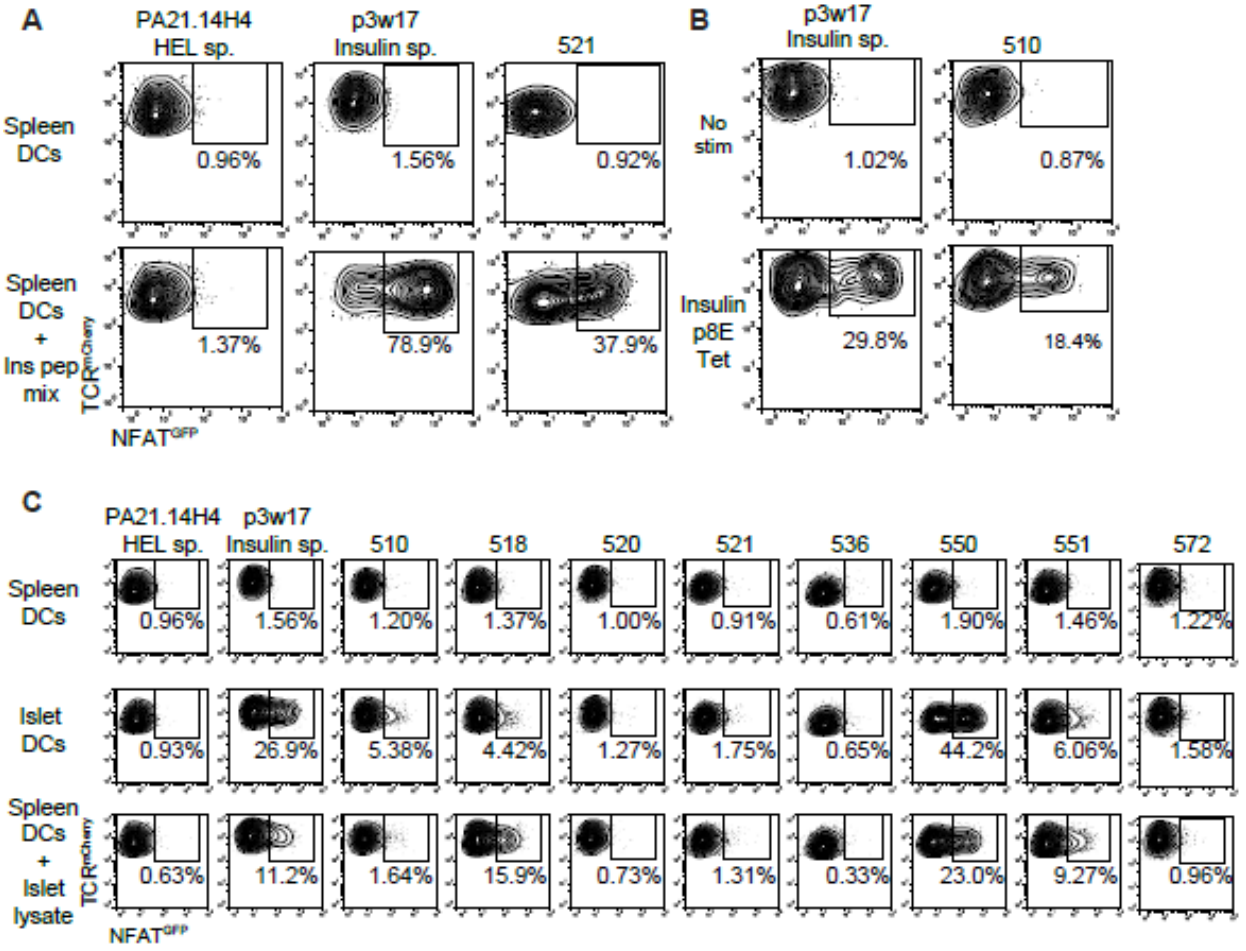
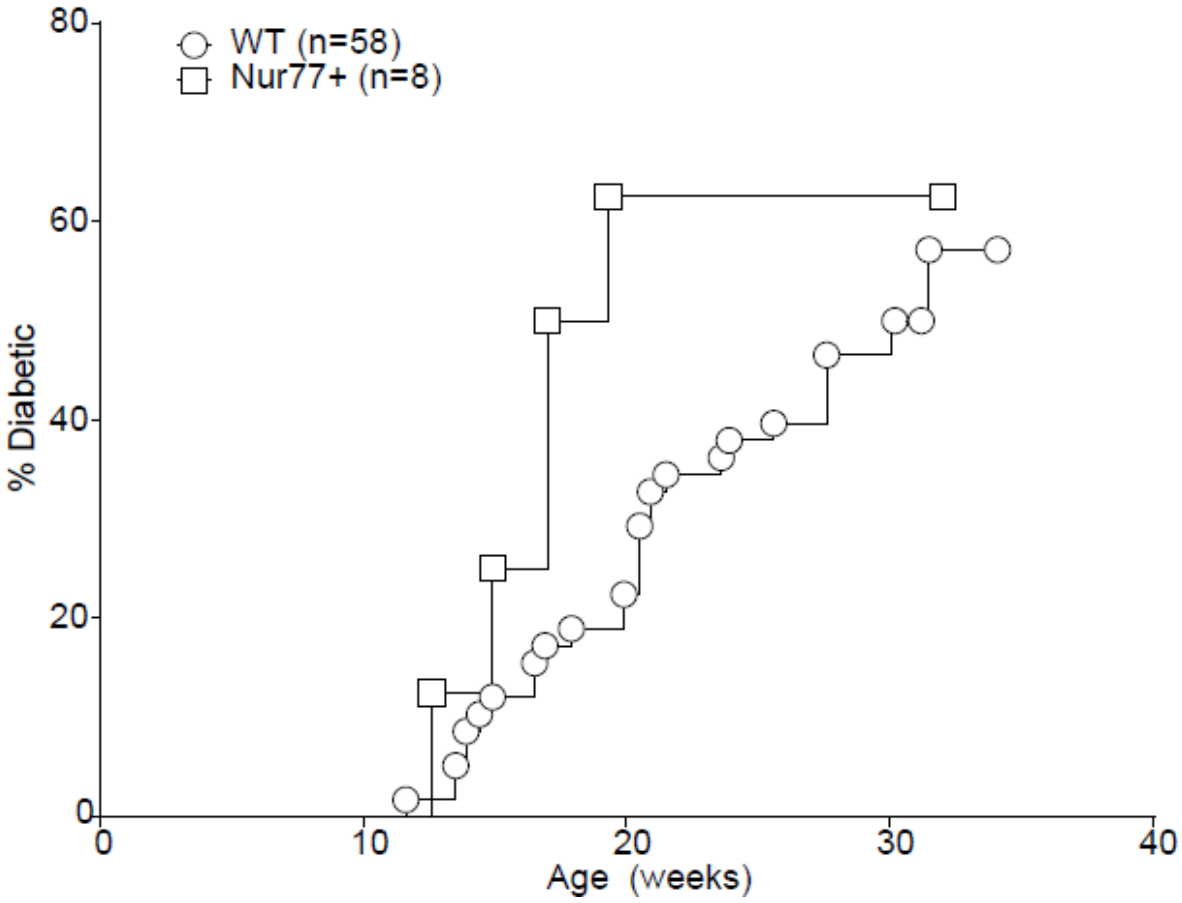
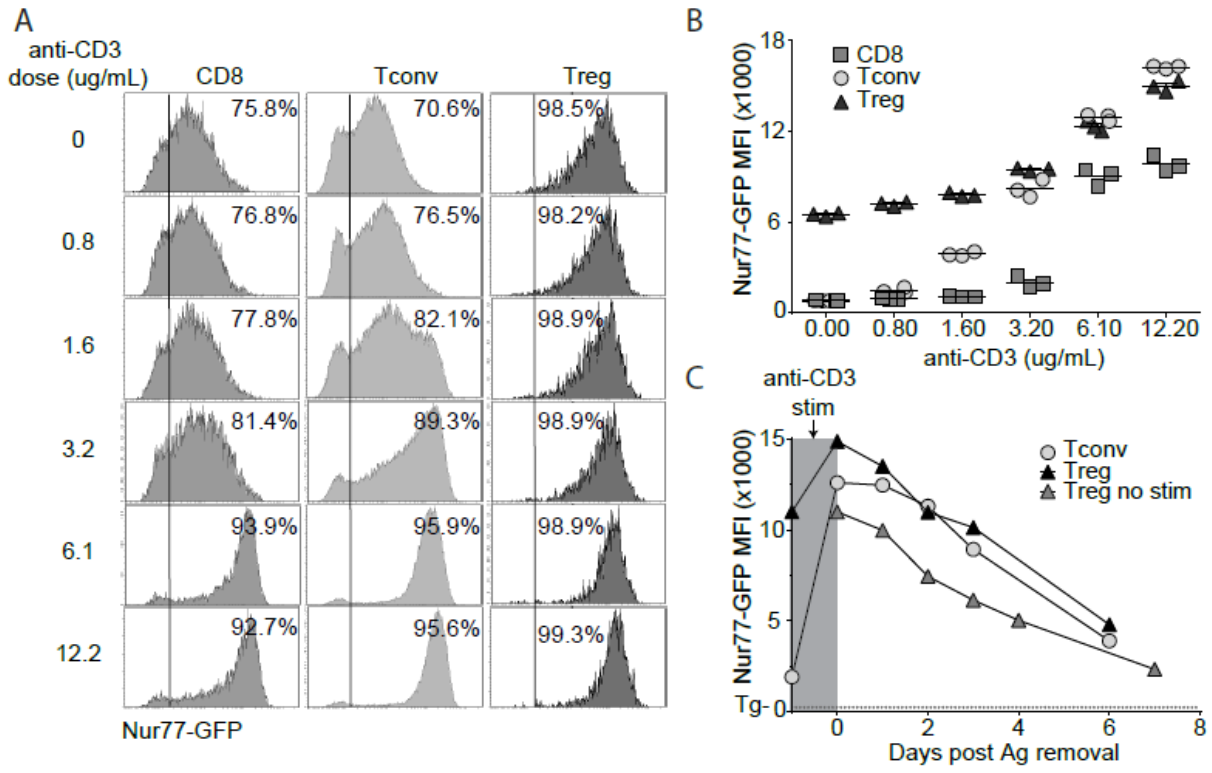


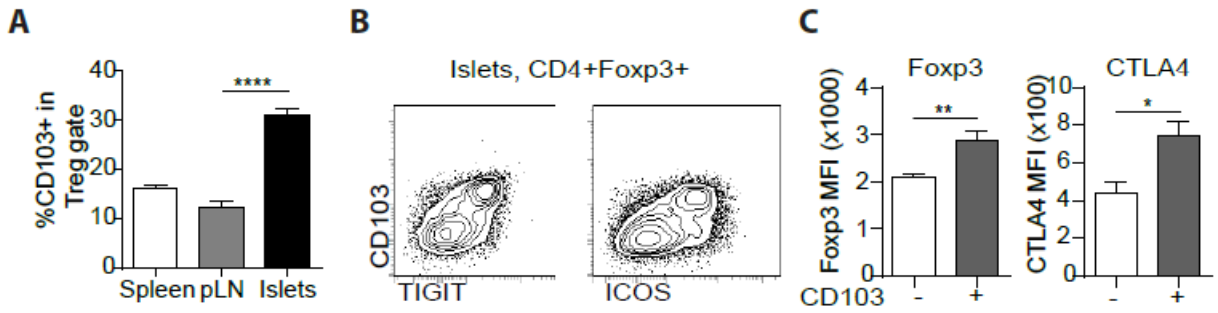
Figure 5: Islet antigen reactivity of activated islet Tregs. A. Selected hybridomas were stimulated for 20-24 hours with primary CD11c^{+} splenic DCs presenting a mix of insulin peptides. TCR signaling was measured by upregulation of an NFAT^{GFP} reporter. B. Selected hybridomas were stimulated with plate bound insulin tetramer p8E and reactivity was assessed as in A. C. Hybridomas were stimulated with splenic DCs, islet DCs, or splenic DCs presenting islet lysate and reactivity was assessed as in A.



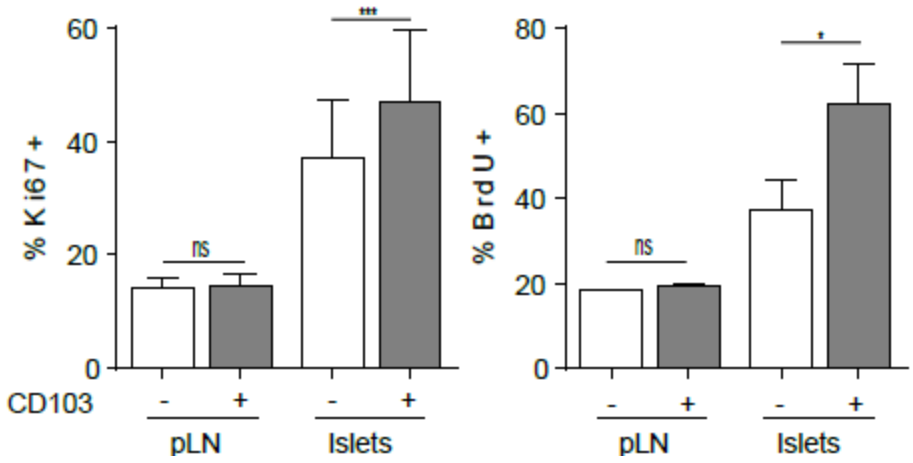
Supplemental Figure 1: Nur77^{GFP} reporter mice develop diabetes similarly to WT NOD mice. Wild type NOD and Nur77^{GFP} mice followed for >30 weeks for incidence of diabetes.



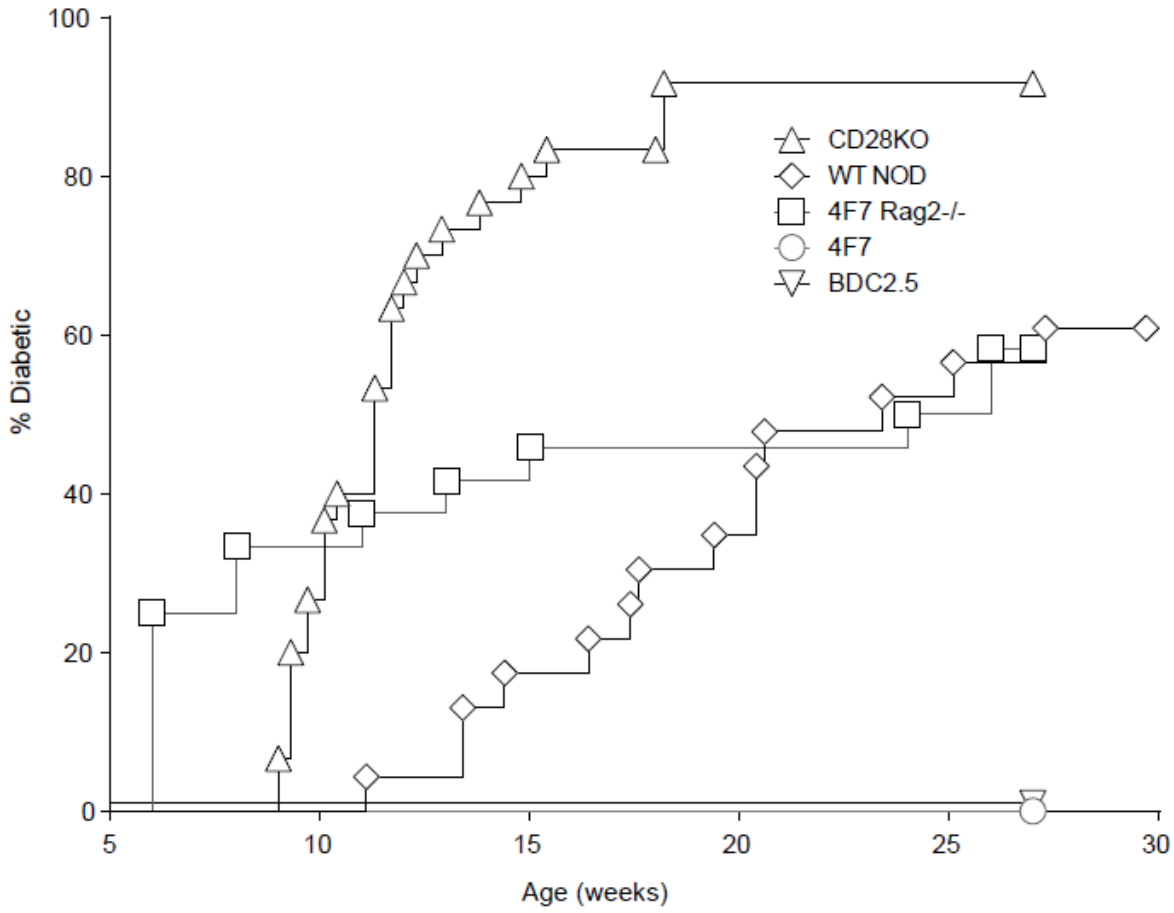
Supplemental Figure 2: Nur77^{GFP} reporter measures strength of and duration of TCR signaling. A. Pooled LNs were stimulated for 16 hours with plate bound anti-CD3 at concentrations listed. The percentage of Nur77^{GFP+} cells was measured for each population. B. Quantification of Nur77^{GFP} MFI of the cells stimulated in A. C. Pooled LNs were sorted for CD4+Foxp3+ (Tregs) or CD4+Foxp3- (Tconv) and then stimulated or not with plate bound anti-CD3 and anti-CD28 for 24 hours. Cells were removed from stimulation and Nur77^{GFP} MFI was measured for 6 days.



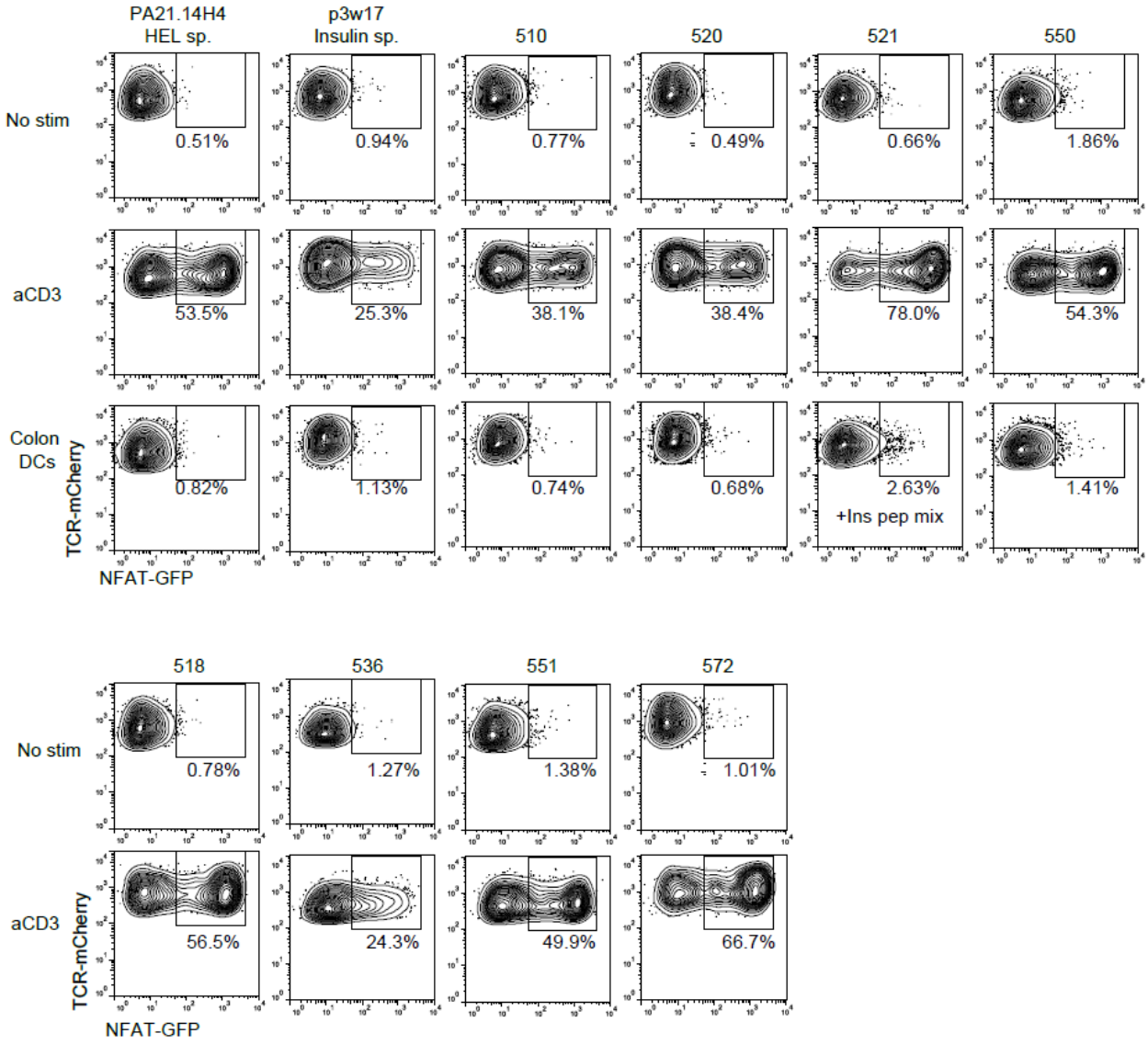
Supplemental Figure 3: CD103+ Tregs are more activated than CD103- Tregs and are present primarily at the site of inflammation. A. Percentage of Tregs expressing CD103 in different organs of pre-diabetic NOD mice. B. Co-staining of islet Tregs for CD103, TIGIT, and ICOS. C. Foxp3 and CTLA4 MFI in Treg subsets from pre-diabetic NOD mice.



Supplemental Figure 4: Proliferation of Islet Treg subsets. Ki67 and BrdU staining in pre-diabetic NOD mice. BrdU was administered continuously for 1 week prior to analysis.



Supplemental Figure 5: 4F7.RagKO mice have similar diabetes incidence to WT NOD mice. Wild type NOD, BDC2.5, 4F7, CD28KO, and 4F7.RagKO mice were followed for 27 weeks for incidence of diabetes.



Supplemental Figure 6: TCR hybridomas respond to non-specific TCR stimulation but not other tissue DCs. Hybridomas were left un-stimulated, stimulated with anti-CD3, or stimulated with colon DCs with or without Insulin peptide mix and reactivity was assessed as described in Figure 5.

TABLES

ICOS+TIGIT+ Treg single cell sorted TCRs

Sequence ID	# Sequences found	Frequency (%)	Found in >1 mouse?	Vα	CDR3α	Vβ	CDR3β
510	1	1.41	Yes	5D-4	CAASVSGGSNYKLTF	5	CASSQGTGGTEVFF
518	2	2.82	No	16	CAMRGVNSGGSNYKLTF	13-3	CASSDLGAYEQYF
520	1	1.41	Yes	5D-4	CAASATGNTRKLIF	19	CASTGVQNTLYF
521	2	2.82	No	5D-4	CAASATGSGGKLTL	13-1	CASSGDSGNTLYF
536	2	2.82	No	7N-4	CAVRNSGGSNAKLTF	3	CASSLNRDEQYF
550	4	5.63	No	10	CAASRTGNYKYVF	2	CASSQGLGGLEQYF
551	2	2.82	No	16	CAMRQGTGSKLSF	15	CASSLDRAGNTLYF
572	2	2.82	No	9-1	CAVSYNNNRIFF	1	CTCSAIGGAHEQYF
Total	16	22.55					

Known TCRs and Controls

Name	Specificity	Hybridoma control?	Vα	CDR3α	Vβ	CDR3β
4F7	Insulin	No	5D-4	CAASRNYAQGLT	1	CTCSADQNQAPLF
p3w17	Insulin	Yes	5D-4	CAASKGGSALGRLH	1	CTCSADGGGAQEQYF
PA21.14H4	HEL	Yes	5D-4	CAASEQGTGSKLSF	19	CASSIGGTGGYEQYF

Table 1: Single cell sorting activated islet Tregs finds sequences similar to insulin specific T cells or clonal expansion. Tregs were sorted CD4+Foxp3+ICOS+TIGIT+ and TCRα and β chains were sequenced.

	Reactivity to:				
Sequence ID	Insulin	Islet DC	Islet lysate	Islet Ag specific?	Islet Antigen?
510	+	+	+	Yes	Insulin
518	-	+	+	Yes	Unknown
520	-	-	-	Unknown	N/A
521	+	+	+	Yes	Insulin
536	-	-	-	Unknown	N/A
550	-	+	+	Yes	Unknown
551	-	+	+	Yes	Unknown
572	-	-	-	Unknown	N/A
PA21.14H4 (HEL)	-	-	-	No	N/A
p3w17 (Insulin)	+	+	+	Yes	Insulin

Table 2: Treg hybridoma reactivity to islet antigen.

Mouse #	Organ	Treg Population	Cell #	Unique TCRβ sequences	D50 (Diversity)
1	Spleen	$CD103^{+}$	40,000	8,724	7
2	Spleen	$CD103^{+}$	29,000	7,235	8.6
3	Spleen	$CD103^{+}$	110,000	10,639	5.5
4	Spleen	$CD103^{+}$	89,000	15,157	4.9
1	Spleen	$CD103^{-}$	250,000	43,968	27.1
2	Spleen	$CD103^{-}$	235,000	52,167	25.1
3	Spleen	$CD103^{-}$	604,000	62,607	20.6
4	Spleen	$CD103^{-}$	436,000	54,959	23.4
1	pLN	$CD103^{+}$	12,000	695	2.4
2	pLN	$CD103^{+}$	15,000	3,131	8.9
3	pLN	$CD103^{+}$	22,000	5,969	11.1
4	pLN	$CD103^{+}$	36,000	9,804	11.4
1	pLN	$CD103^{-}$	47,000	13,097	19.4
2	pLN	$CD103^{-}$	54,000	13,722	20.5
3	pLN	$CD103^{-}$	90,000	9,485	13.3
4	pLN	$CD103^{-}$	107,000	35,612	27.5
1	ipsLN	$CD103^{+}$	60,000	11,264	10.5
2	ipsLN	$CD103^{+}$	56,000	11,195	11.8
3	ipsLN	$CD103^{+}$	55,000	12,603	6.4
1	ipsLN	$CD103^{-}$	335,000	59,124	30.1
2	ipsLN	$CD103^{-}$	373,000	57,429	30.6
3	ipsLN	$CD103^{-}$	262,000	57,629	26.5
4	ipsLN	$CD103^{-}$	245,000	45,376	29.6
1	Islet	$CD103^{+}Nur77^{GFP\text{-}hi}$	3,800	1,419	2.6
2	Islet	$CD103^{+}Nur77^{GFP\text{-}hi}$	8,600	1,577	1.5
3	Islet	$CD103^{+}Nur77^{GFP\text{-}hi}$	6,400	1,638	0.9
4	Islet	$CD103^{+}Nur77^{GFP\text{-}hi}$	3,500	1,317	2.4
1	Islet	$CD103^{-}Nur77^{GFP\text{-}hi}$	1,600	1,144	3.3
2	Islet	$CD103^{-}Nur77^{GFP\text{-}hi}$	3,600	1,261	3.7
1	Islet	$CD103^{-}Nur77^{GFP\text{-}lo}$	5,000	1,626	5.5
2	Islet	$CD103^{-}Nur77^{GFP\text{-}lo}$	12,000	5,225	10.2
3	Islet	$CD103^{-}Nur77^{GFP\text{-}lo}$	8,000	2,863	6.3
4	Islet	$CD103^{-}Nur77^{GFP\text{-}lo}$	5,900	1,601	4.2

Supplemental Table 1: Summary of TCRβ sequencing results. Sorted populations described in Fig 3a showing the number of cells and unique TCRβ sequences for each

population within each organ for up to 4 individual mice. The D50 measure of diversity is the percent of dominant and unique T cell clones that account for the cumulative 50% of the total CDR3s counted in the sample. More diversity brings the number closer to 50, less diversity brings the number closer to 0.

CHAPTER III: TREGS IN THE PRE-DIABETIC LESION ARE ACTIVELY TURNING OVER BASED ON ANTIGEN ACTIVATION AND IL2 DEFICIENCY

Abstract

In the non-obese diabetic (NOD) mouse model of type 1 diabetes regulatory T cells (Tregs) with suppressive capacity accumulate in the inflamed tissue and delay destruction of insulin-producing beta cells. Tregs isolated from the insulitic lesion were more activated and proliferative than Tregs from spleen and pancreatic LN (pancLN). These activated islet Tregs could be identified using the cell surface markers integrin αE (CD103), ICOS, and TIGIT and had higher expression of additional Treg molecules such as Foxp3, CTLA4, and Nur77^{GFP}. Islet Tregs were a dynamic population: they had higher turnover than Tregs from non-inflamed tissues and in the tissue the CD103^{-} Tregs could acquire CD103 expression. The CD103^{+} Treg accumulation in the tissue reached a plateau between 10-12 weeks of age, limited by poor survival in this population, despite having the highest sensitivity to IL2. CD103^{-} Tregs, on the other hand, are more sensitive to IL7 and have increased capacity for survival. Both subsets of Tregs were effective when used as cellular therapy, and even more potent when pre-treated with IL2 prior to transfer. Thus, antigen activated islet Tregs are effective for diabetes prevention but are prone to death due likely to limited IL2 in the tissue.

Introduction

Tregs are critical players in the prevention of autoimmune diseases. Local Treg regulation of their respective target organs is important for controlling organ specific

autoimmune diseases such as of the ovary, prostate, lacrimal gland (Wheeler et al., 2009), and pancreas (Chen et al., 2005; Herman et al., 2004). In the NOD mouse, $CD4^+$ and $CD8^+$ effector T cells are the primary mediators of beta cell destruction. Tregs control disease progression both in the draining LN (Tang et al., 2006), and inflamed tissue (Chen et al., 2005; Herman et al., 2004; Mahne et al., 2015; Wheeler et al., 2009). Tregs in the tissue represent a final suppressive mechanism to limit the destructive function of effector cells (Chen et al., 2005; Feuerer et al., 2009; Mahne et al., 2015). Thus, maintenance of Tregs present in the tissue is critical to prevent onset of disease.

Tregs present in spleen that are poised to infiltrate tissues have been described as $CD44^{hi}CD62L^{lo}$ "effector" Tregs (eTregs), while $CD44^{lo}CD62L^{hi}$ Tregs that reside primarily in the spleen are referred to as "central" Tregs (cTregs). Included in the eTreg phenotype is expression of the integrin αE (CD103) (Smigiel et al., 2014; Toomer et al., 2016). CD103 expression has been described on highly suppressive Tregs *in vitro* and *in vivo* in graft versus host disease (GvHD), arthritis and colitis models (Huehn et al., 2004; Lehmann et al., 2002; Zhao et al., 2008). Still, the contribution of eTregs to inflammatory disease and the maintenance of this phenotype after tissue entry remain unclear.

IL2 signaling is crucial for Treg development, homeostasis, and function. Early data suggested that patients with T1D have defects in IL2 receptor signaling in Tregs (Long et al., 2010; Long et al., 2011). These patients may still respond to *in vivo* IL2 therapy, which correlates to *in vitro* Treg function (Yu et al., 2015). In parallel, when the NOD mouse insulin-dependent diabetes gene loci 3 (IDD3), which maps close to the IL2

gene (Denny et al., 1997), is mutated, NOD mice are protected from disease development (Wicker et al., 1994). Inflamed islets likely have a limited availability of IL2, which influences expression of prosurvival factor Bcl2 and decreases Treg survival, contributing to disease progression (Tang et al., 2008; Tritt et al., 2008). Exogenous administration of low doses of IL2/anti-IL2 complexes preferentially bind CD25 expressing cells, with Tregs being the main targets, and can prevent or reverse diabetes in NOD mice (Grinberg-Bleyer et al., 2010; Manirarora and Wei, 2015; Tang et al., 2008). Thus, effectively targeting Tregs with IL2 can prevent T1D in the NOD mouse and may have efficacy in human patients.

Survival and function of Tregs is critical to disease prevention in the NOD mice, however the dynamics of Tregs at the site of inflammation is not well characterized. While increased Treg localization to the inflamed tissue has been observed (Magnuson et al., 2015), low Bcl2 in islet Tregs (Grinberg-Bleyer et al., 2010; Tang et al., 2008) will suggest that these Tregs may have altered survival. In this study we investigated the dynamics and cytokine responsiveness of islet Tregs. In doing so, we found reduced survival particularly in the $CD103^+$ Tregs as well as differential cytokine sensitivities for different subsets of islet Tregs. While many of the cell populations are found in similar proportions and activation states at different points in the disease process, the composition of the inflammatory infiltrate is constantly changing.

Results

Proliferation and activation of Tregs at the site of inflammation

We first examined the proliferation of Tregs in spleen, pancLN, and islets. Tregs were most proliferative in the islets using a 1 week continuous BrdU labelling (Figure 1A), Ki67 (Figure 1B), or a 16hr BrdU pulse (Figure 1C). When we analyzed activation markers on Tregs, we found CD103, TIGIT, and ICOS upregulation on islet Tregs compared to spleen and pancLN (Figure 1D). These three markers were coexpressed on islet Tregs (data not shown). We found CD103 expression described a more highly activated subset of islet Tregs based on Foxp3, CTLA4, and Nur77^{GFP} expression (Figure 1E). Nur77^{GFP} measures recent TCR signaling (Zikherman et al., 2012). Tregs from different organs had a demethylated Foxp3 Treg-specific demethylated region (TSDR) in their conserved noncoding sequence 2 (CNS2) region of their promoter regardless of Nur77^{GFP} or islet CD103 expression (Figure 1F). Thus, we find that islet Tregs expanding in the tissue and that expression of CD103 identifies the most activated, antigen reactive subset.

Dynamics of islet Tregs

Next, we asked if islet Tregs went through a step-wise progression to acquire CD103 and an activated phenotype, similar to the cTreg to eTreg transition observed in spleen (Smigiel et al., 2014; Toomer et al., 2016). CD103^{+} islet Tregs were purified and transferred into 2-3 week old NOD.CD28$^{-/-}$ mice. NOD.CD28$^{-/-}$ mice were chosen as recipients because of their paucity of Tregs and the synchronicity of inflammation in their islets as compared with wild type NOD mice (Salomon et al., 2000; Tang et al.,

2003). Donor cells, identified by the congenic marker Thy1.1, were analyzed 3-6 weeks post-transfer. In both the spleen and pancLN, $CD103^+$ Tregs did not maintain either CD103 or Foxp3 expression in the majority of transferred cells. However, at the site of inflammation, 80% of transferred $CD103^+$ Tregs maintained CD103 and Foxp3 expression (Figure 2A). When $CD103^-$ islet resident Tregs were transferred and analyzed in the same manner, a minor population of cells acquired CD103 expression in the spleen and pancLN, while approximately 40% of the cells turned on CD103 at the inflammation site (Figure 2B).

TCRβ CDR3 sequences were compared between islet Treg populations $CD103^+Nur77^{GFP\text{-}hi}$, $CD103^-Nur77^{GFP\text{-}hi}$, and $CD103^-Nur77^{GFP\text{-}lo}$ to determine the extent that the repertoire overlaps between populations. The only populations that overlapped were $CD103^-$ and $CD103^+$ $Nur77^{GFP\text{-}hi}$. The $CD103^-Nur77^{GFP\text{-}lo}$ population did not share repertoire with either $Nur77^{GFP\text{-}hi}$ Treg population (Figure 2C). This result is consistent with a model in which antigen recognition at the site of inflammation by $CD103^-$ Tregs precedes expression of CD103.

Treg turnover in the inflamed tissue

We next examined whether these locally activated, antigen reactive cells built up in the islets overtime. The total number of Tregs per islet increased with age until 20-30 weeks in pre-diabetic mice (Figure 3A). When we analyzed the $CD103^+$ Tregs specifically, we found an accumulation of these Tregs until 10-12 weeks of age, when they held flat at approximately 30% of the total Treg population (Figure 3B). Collectively, this result suggests that Tregs in the islets are constantly in flux but the proportion of $CD103^+$ Tregs is maintained at a steady level after 10-12 weeks of age.

To ask if islet Tregs are long-term residents in the tissue, we parabiosed age-matched congenic mice for 4 weeks to determine the ability of tissue Tregs to enter circulation or vice versa. Tregs from circulation populated the islets of the partner mouse to a greater extent than Tregs in non-inflamed tissues skin and colon. In these two tissues, approximately 70% of the Tregs were resident to the host mouse, indicating they did not enter circulation and traffic to the partner mouse or have a large influx of Tregs from circulation. Tregs in the blood represent the maximum circulation between hosts. Islet Tregs were more localized to their respective host compared to Tregs in the blood (Figure 3C). Mice were paired between 9-25 weeks of age with no major differences observed in cell trafficking based on age of parabiosis (data not shown). This result suggests an intermediate capability of Tregs to traffic through circulation to their partner mouse's islets compared with colon and skin.

CD103^{+} Tregs have a survival defect

Our data thus far show that Tregs are activated locally to acquire CD103 expression, the CD103+ Tregs proliferate at higher rate, and Tregs continuously enter the inflamed islets from the circulation, but paradoxically, CD103+ Treg proportions remain stable over time after 6 weeks of age. We therefore reasoned that CD103^{+} Tregs must either be exiting the islet or dying in the tissue. To examine the possibility that Tregs exit the islets and populate the lymphoid organs, individual Treg clones were examined for sharing of unique TCRβ CDR3 sequences between lymphoid organs and islets. 5-15% of islet Treg sequences were found in either spleen, pancLN or inguinal-popliteal-sciatic LNs (iLN), which accounted for <1% of total sequences in these populations. Tregs from spleen, pancLN, or iLN shared 10-20% of their unique CDR3

repertoire (Figure 4A), suggesting that islet Treg clones are primarily restricted to the tissue.

To examine the possibility that islet Tregs may be dying in the tissue, we first examined the expression of the prosurvival molecule Bcl2. While islet Treg expression is overall lower than pancLN (Tang et al., 2008), we additionally found that islet CD103$^+$ Tregs have the lowest level of Bcl2 expression (Figure 4B). After adoptive transfer of the Tregs described in Fig 2A, B, recovery of CD103$^+$ Tregs was significantly reduced compared to CD103$^-$ Tregs in spleen, pancLN, and islets (Figure 4C). Thus, although we cannot rule out the exit of a small percentage of islet Tregs, this data suggests that CD103$^+$ Tregs are prone to death.

CD103$^+$ Tregs are highly responsive to IL2 while CD103$^-$ Tregs can respond to IL7

Due to the requirement for IL2 to maintain islet Tregs (Tang et al., 2008), we next examined CD25 and IL2 signaling in islet Treg subsets. CD25 expression is higher on CD103$^+$ islet Tregs than CD103$^-$ islet Tregs, Tconv, and CD8$^+$ T cells (Figure 5A). Sensitivity to IL2 stimulation by phosphorylation of STAT5 is also greater in the CD103$^+$ population in a dose dependent manner (Figure 5B). Conversely, CD103$^-$ Tregs express higher levels of the IL7 receptor CD127, albeit still less that Tconv and CD8$^+$ T cells (Figure 5C). Correlating with receptor expression, Tconv and CD8$^+$ T cells are more sensitive to IL7 stimulation at the lower doses, while CD103$^-$ Tregs can phosphorylate STAT5 to a similar extent at high doses of IL7. Still, CD103$^+$ Tregs are least sensitive to IL7, even at high doses (Figure 5D). Collectively, we find a dichotomy between IL2 and IL7 receptor expression and sensitivity to cytokine stimulation between the CD103$^+$ and CD103$^-$ islet Treg subsets.

Islet CD103^{+} and CD103^{-} Tregs are functional therapeutic Tregs

We next assessed the *in vivo* capacity of islet Treg subsets to prevent diabetes using an adoptive transfer model. Diabetes development in NOD.CD28$^{-/-}$ mice occurs at a younger age and with higher penetrance than regular NOD mice due to defects in Treg development, homeostasis and function (Salomon et al., 2000; Tang et al., 2003). The majority of NOD.CD28$^{-/-}$ mice become diabetic between 8-12 weeks of age (Figure 6A). 50,000 CD103^{+} or CD103^{-} Tregs purified from islets and transferred into 2-3 week old NOD.CD28$^{-/-}$ mice were able to prevent diabetes with similar efficacy, while Tregs sorted from pancLN, non-draining LN (ndLN: inguinal and mesenteric LNs), or spleen were not effective (Figure 6A). The efficacy of CD103^{+} Tregs despite their survival disadvantages suggests that these Tregs are more potent on a per cell basis that CD103^{-} Tregs.

We next assessed the ability of pre-treating the Tregs with IL2 to improve protection. 10,000 islet CD103^{+} or CD103^{-} Tregs were unable to prevent diabetes progression (data not shown). However, pre-treating the Tregs with IL2 before transfer improved the ability of islet Tregs to prevent diabetes at this dose (Figure 6B), however, no statistically significant differences were observed between CD103+ and CD103- subsets. Thus, *ex vivo* IL2 stimulation prior to adoptive transfer increases the potency of islet Tregs at least 5-fold.

Discussion

In this study we have characterized the homeostasis and survival of islet Tregs. We found that islet Tregs are highly proliferative yet contained in the tissue. A subset of

Tregs expressing cell surface markers CD103, ICOS, and TIGIT enriched for activated antigen reactive Tregs. These Tregs are a dynamic population where the cells continue to turnover as well as acquire CD103 expression based on antigen recognition in the tissue. $CD103^+$ Tregs are very sensitive to IL2 stimulation and the most prone to death. On the other hand, $CD103^-$ Tregs are more sensitive to IL7 than $CD103^+$ Tregs and less prone to death. Still, both $CD103^+$ and $CD103^-$ Tregs are potent as therapeutic Tregs and their efficacy is increased with exogenous IL2 treatment prior to transfer.

The highly activated phenotype of $CD103^+$ Tregs combined with their low expression of Bcl2 suggests these are functional effector Tregs that may be prone to apoptosis. $CD103^+$ Tregs have been previously described as the most effective Treg in different models of inflammation such as GvHD, arthritis, and colitis (Huehn et al., 2004; Lehmann et al., 2002; Zhao et al., 2008), as well as *in vitro* (Lehmann et al., 2002). Yet we did not find that $CD103^+$ Tregs were more effective than $CD103^-$ Tregs as a therapeutic treatment in NOD.$CD28^{-/-}$ diabetes. This may be due to the reduced survival of these Tregs post-transfer. By 3-6 weeks after transfer, approximately 1% of the transferred $CD103^+$ Tregs were recovered from the islets, which is 10 time less than the recovery of $CD103^-$ Tregs. Thus, the ability of $CD103^+$ Tregs to prevent diabetes and at a similar dose to $CD103^-$ Tregs suggests that on a per cell basis these Tregs are much more potent. This coupled with our finding that nearly half of $CD103^-$ Tregs upregulate CD103 3-6 weeks after transfer will also suggest that the potency of the $CD103^-$ transferred Tregs is further increased over their efficacy at the time of transfer. Thus, CD103 expression after antigen exposure is likely identifying a terminally differentiated state for islet Tregs. The upregulation of activation and suppressive

molecules makes them good candidates for Treg therapy. However, their increased susceptibility to death makes them less tempting to use as therapeutic Tregs.

Improving the survival of this Treg subset may play a role in disease prevention. Since islet Tregs have decreased survival in the tissue secondary to lack of IL2 (Grinberg-Bleyer et al., 2010; Tang et al., 2008), an increased sensitivity to IL7 in the $CD103^-$ population may provide a new avenue to improve islet Treg survival. Tregs in the periphery do rely on IL7 for their survival, paralleling naïve T cells (Kim et al., 2012; Mazzucchelli et al., 2008; Simonetta et al., 2012). A reliance on IL7 in memory Tregs in the tissue after resolution of inflammation has been suggested in a different model (Gratz et al., 2013), so it will be interesting to determine a role for IL7 in islet Treg survival. Also of interest is the apparent swap of sensitivity in the islet Treg populations, which correlates with receptor expression. This result suggests that $CD103^+$ Tregs may be lacking sufficient IL2 stimulation *in vivo* or require a larger amount of IL2 to be maintained, while $CD103^-$ Tregs may be maintained by IL7 in the tissue. It remains to be determined if the intracellular wiring of $CD103^+$ and $CD103^-$ Tregs is shifted to regulate cytokine sensitivity and receptor expression as well as the role this may play in the disease process.

Measuring the trafficking of islet Tregs revealed an intermediate capacity of these cells to migrate into the tissue compared to non-inflamed tissues skin and colon and circulation through the blood. The high proliferation observed in the islet compared to LNs and spleen alongside the consistent Treg numbers in the islets would support a constant turnover of these cells. This is possibly due to reduced IL2 availability in the tissue (Tang et al., 2008). This data supports a model where tissue Tregs require IL2 to

sustain their survival in the tissue. In the absence of the required amounts of IL2, more Tregs can enter the tissue, become activated, and suppress inflammation. Not all NOD mice may be able to sustain this continual replenishment of Tregs, which could contribute to disease progression.

Our data support a role for antigen in upregulation of CD103, TIGIT, and ICOS expression in the tissue. However, $Nur77^{GFP}$ expression in the islets also finds a population of $Nur77^{GFP\text{-}hi}$ Tregs that lack expression of these molecules. Perhaps TCR signaling precedes expression of this high activation phenotype, however the exact conditions that result in CD103, TIGIT, and ICOS upregulation in the tissue are still unclear. It is possible that only certain types of antigens are able to induce their expression or that induction of this phenotype requires a very strong TCR signal. Since CD103 expression is known to be regulated by TGFβ signaling it is possible that at least some of these Tregs are converted from naïve T cells directly into the $CD103^{+}$ population, bypassing a $CD103^{-}$ state. Simply adding TCR stimulation with IL2 *in vitro* is not enough to induce CD103 expression in Tregs. However, current Treg expansion protocols are completed in media containing high levels of IL2 (Putnam et al., 2009) and our data will suggest that this method is the most optimal for enhancing Treg function after infusing into patients.

Taken together, this study highlights the dynamic nature of Tregs present within the inflammatory infiltrate of the islet of Langerhans. The most highly activated, antigen specific subset is the most sensitive to IL2 and also the most prone to death. Future work on determining how to regulate the survival of these cells and on the TCR specificities that may influence upregulation of this activation phenotype will be

important for understanding fundamental aspects of Treg homeostasis and activation and pave the way for development of therapeutic strategies for treatment of autoimmunity.

Materials and Methods

Mice: Female NOD/ShiLtJ, (Jackson laboratories) mice were housed and bred under specific pathogen free conditions in accordance with the UCSF (San Francisco, CA) Animal Care and Use Committee guidelines. NOD.Foxp3$^{Cre/eGFP}$ (Zhou et al., 2008) and CD28KO (Salomon et al., 2000) mice have been previously described. NOD.Foxp3$^{mRFP(tm1flv)}$ (Wan and Flavell, 2005) were generously provided by Li Wen. Nur77^{GFP} mice (Zikherman et al., 2012) were received from Art Weiss and backcrossed at least 10 generations onto the NOD background.

Cell Transfers: CD4^{+}Foxp3^{GFP+} or Foxp3^{mRFP+} Tregs from NOD mice were FACS sorted to >95% purity and transferred to 2-3 week old NOD.CD28KO mice via intraperitoneal (i.p). injection.

Flow cytometry: Islet, spleen, and lymph node single-cell suspensions were prepared as previously described (Melli et al., 2009). The following antibodies were used to stain the cells: CD103-FITC or Pacific Blue (2E7), ICOS-APC (C398.4A), Ki67-PE-Cy7 (SolA15), TIGIT-APCeFluor780 (MBSA43), CD25-PE (PC61.5), CD127 (A7R34), Foxp3-eFluor450 (FJK-16s) (eBiosciences), anti-CD4-PE or APC (RM4-5) (eBioscience or Tonbo Biosciences), CD8-Pacific Orange (5H10) (Life Technologies), CD45-APC-Cy7 (30-F11), CTLA4-PE (UC10-4F10-11), Thy1.1-APC or PE-Cy7 (OX-7), BrdU-FITC, Bcl2-PE (100), pSTAT5 (47) (BD Biosciences), Thy1.2-AL700 (30H12), (UCSF

Hybridoma Core). For intracellular staining, cells were first fixed and permeabilized via the manufacturer's instructions (eBioscience or Tonbo Biosciences). Analyses were performed on a LSRII or Fortessa flow cytometer (BD Biosciences) with FACSDiva (BD Biosciences) and Flowjo analysis software.

Treg methylation analysis: Genomic DNA was extracted using Section I of the EZ Methylation-Direct Kit (Zymo Research) and submitted for Bisulfite modification and pyrosequencing of the Treg-specific demethylated region (TSDR; CNS2) performed by EpigenDx (Hopkinton, MA) as previously described (Zheng et al., 2010).

Phospho-flow for STAT5: For staining with pSTAT5 following IL2 or IL7 stimulation, cells were stained for 15 minutes at room temperature, washed with PBS and fixed in 0.5% paraformaldehyde for 3-5 minutes. Cells were washed again with PBS and permeabilized with 90% ice cold methanol for 25 minutes at 4^{o}C. Cells were washed with perm buffer (eBioscience or Tonbo Biosciences) and stained with secondary antibodies.

Bulk TCRβ analysis of Treg cells: Total RNA was extracted from flow sorted cells using ARCTURUS PicoPure RNA Isolation Kit (Life Technologies) for <100,000 cells or QIAGEN Micro RNA extraction kit (QIAGEN) for >100,000 cells. TCRβ repertoires were amplified and sequenced using Illumina MiSeq by iRepertoire Inc. (Huntsville). Data analysis was performed using the website provided by iRepertoire Inc. (http://www.irepertoire.com).

Diabetes incidence: Diabetes incidence was monitored by weekly blood glucose monitoring. Mice were considered diabetic after 2 sequential blood glucose readings exceeding 300 mg/dL.

IL2 treatment of islet Tregs: During islet isolation as previously described (Melli et al., 2009), 20,000U/mL human IL2 was added to all incubations as well as final resuspension. All subsequent steps were performed with human IL2 at 2,000U/mL added. After sorting, cells were rested for 30 minutes at 37 degrees with 2,000U/mL IL2 and then transferred.

Statistical analysis: Analysis was performed with Prism software (GraphPad).

Acknowledgements

We thank J. Wang, V. Dang, N. Lescano, and R. Guerrero-Moreno for mouse husbandry, V. Nguyen for islet isolations and lab support, L. Bates, S. Cheng, and A. Wiederanders for lab support, JE Klementowicz, K. Remedios, M. Lowe, J. Wang, J. Tam, and V. Panchumarthi for technical assistance, Art Weiss for providing the Nur77^{GFP} mice, and Li Wen for providing us with the NOD.Foxp3$^{RFP(tm1flv)}$ mice.

FIGURES

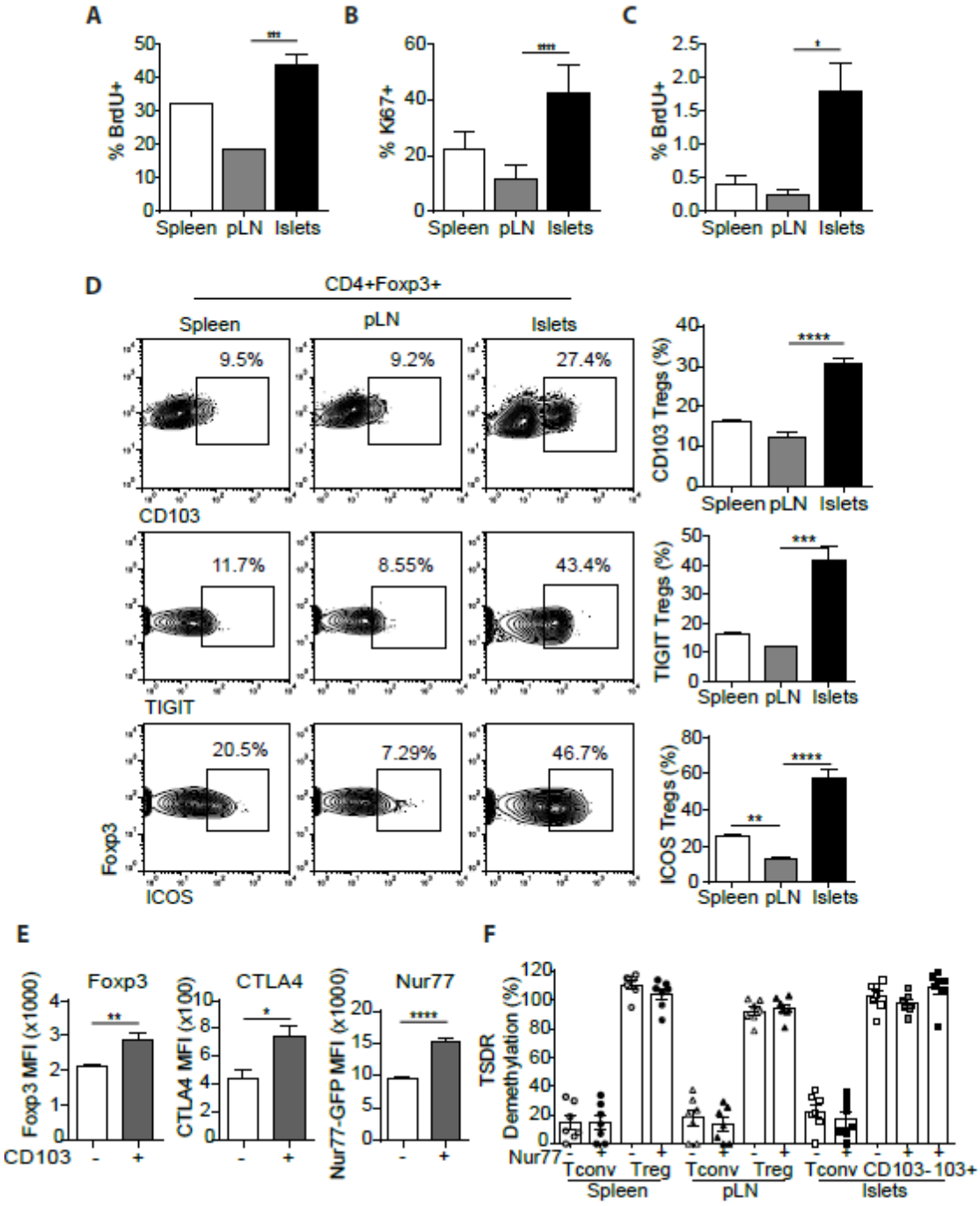


Figure 1: Proliferation and activation of Tregs at the site of inflammation. A. Pre-diabetic NOD mice were given BrdU in their drinking water for 1 week prior to analysis

for Treg (CD4+Foxp3+) proliferation. B. Pre-diabetic NOD mice were analyzed ex vivo for expression of Ki67 in Tregs. C. Pre-diabetic NOD mice were given a pulse of BrdU and Tregs were analyzed for proliferation 2 hours later. D. Percentage of CD103, TIGIT, or ICOS+ Tregs in different organs from pre-diabetic NOD mice. E. MFI of different molecules in islet Treg CD103+ and CD103- subsets from pre-diabetic NOD mice. F. TSDR Demethylation of the Foxp3 CNS2 in CD4+Foxp3+ Tregs and Foxp3- Tconv. Cells were additionally sorted on Nur77-GFP expression.

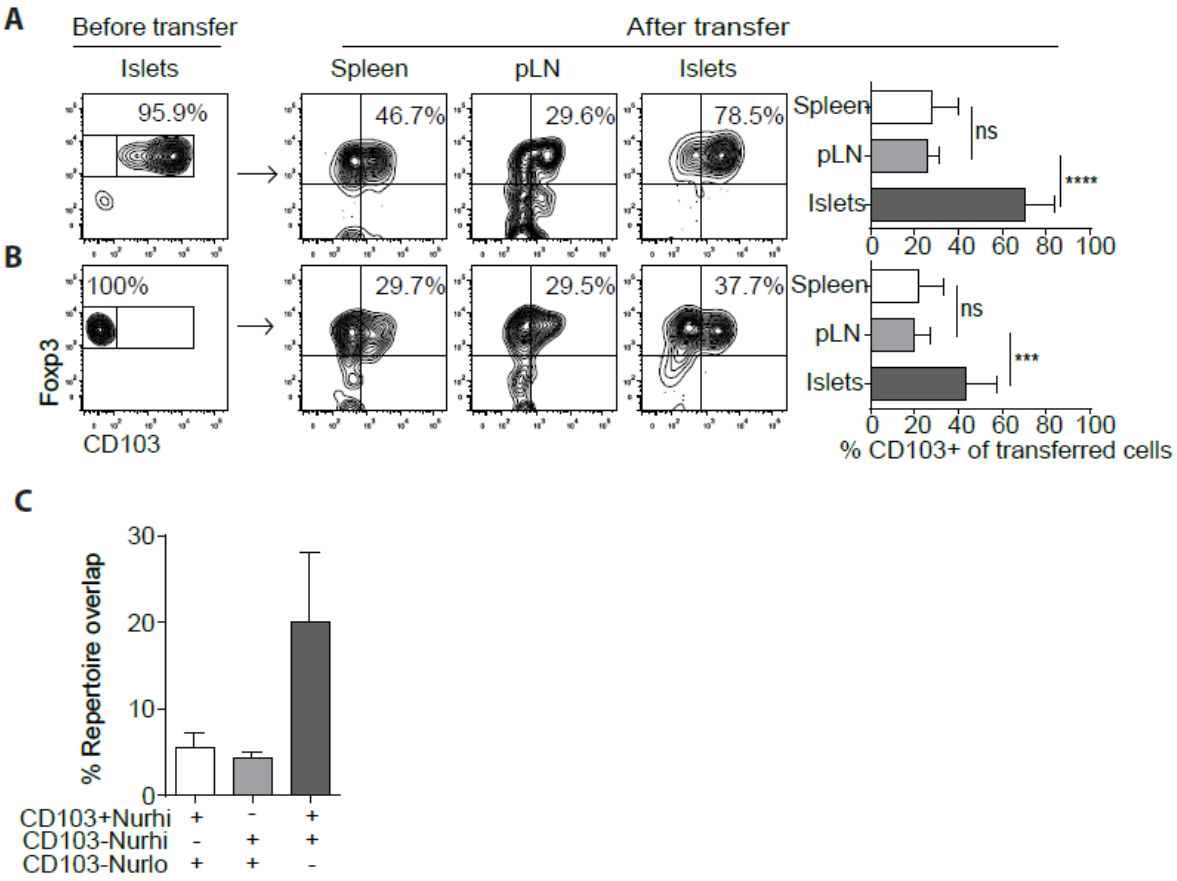


Figure 2: Dynamics of islet Tregs. A/B. Islet resident CD103^{+} or CD103^{-} Tregs were sorted from pre-diabetic NOD mice and transferred to 2-3wk old CD28$^{-/-}$ mice. Phenotype of transferred cells was analyzed 3-6wks later. C. TCRβ repertoire sharing between islet Treg populations.

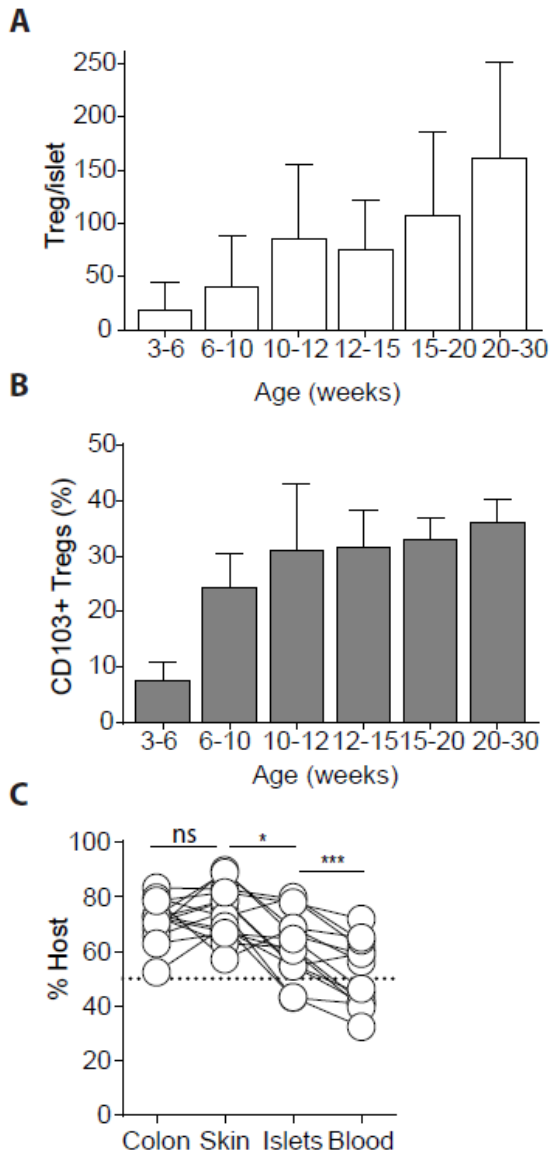


Figure 3: Treg turnover in the inflamed tissue. A. The number of Foxp3^{+} Tregs per islet was calculated for different ages of pre-diabetic NOD mice. B. Percentage of CD103^{+} Tregs among the total islet Treg population was calculated for different ages of pre-diabetic NOD mice. C. Two congenic mice were parabiosed to share blood supply for 4 weeks. Then, trafficking of Treg cells from one host to the other was measured.

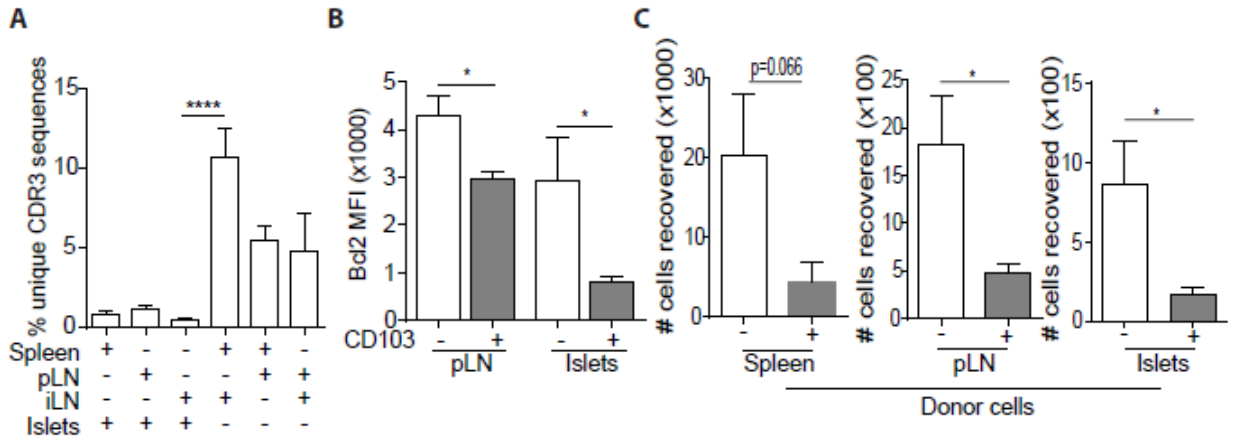


Figure 4: CD103⁺ Tregs have a survival defect. A. Quantification of unique CDR3 TCRβ sequence overlap between Tregs from spleen, pLN, iLN, and islets. B. *Ex vivo* flow cytometric analysis of Bcl2. C. Transferred cell recovery using same experimental setup as in Figure 3A/B.

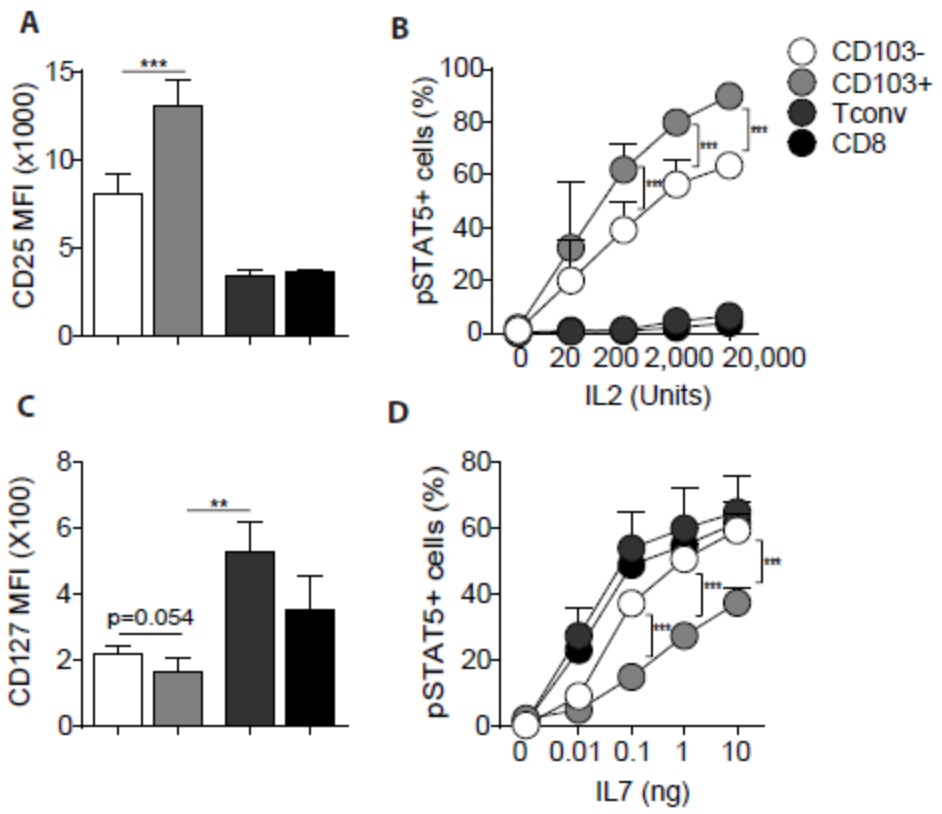


Figure 5: CD103^{+} Tregs are highly responsive to IL2 while CD103^{-} Tregs can respond to IL7. A. *Ex vivo* CD25 protein expression in inflamed islets. B. Islets stimulated for 30 minutes in vitro with different IL2 doses and analyzed for STAT5 phosphorylation. C. *Ex vivo* CD127 protein expression in inflamed islets. D. Islets stimulated as in B with IL7.

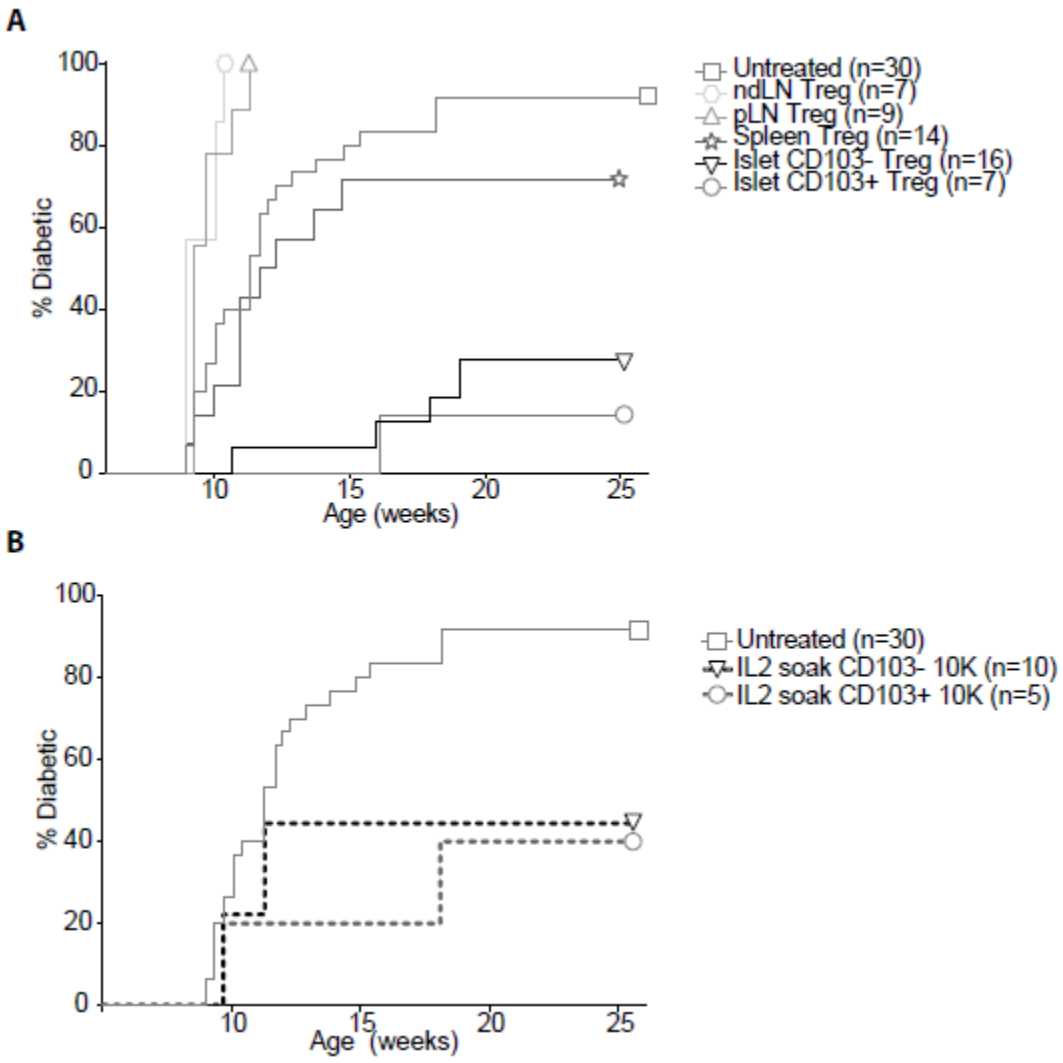


Figure 6: Islet CD103^{+} and CD103^{-} Tregs are functional therapeutic Tregs. A. 50,000 polyclonal Tregs (CD4^{+}Foxp3^{+}) sorted from non-pancreas draining LN (ndLN), pLN, or Spleen, or islet (CD103^{+} or CD103^{-}) were adoptively transferred into 2-3wk old CD28KO mice and diabetes incidence was assessed. B. islet CD103^{+} or CD103^{-} Tregs were soaked in IL-2 prior to adoptive transfer. 10,000 Tregs were transferred as in A and diabetes incidence was assessed.

CHAPTER IV: INFLUENCE OF TGFβ SIGNALIING ON TREG HOMEOSTASIS AND FUNCTION

Abstract

Regulatory T cells (Tregs) are critical players in the prevention of autoimmunity in the non-obese diabetic (NOD) mouse model of type 1 diabetes. In this study we investigated the role of TGFβ in Tregs in the NOD mouse. We found that although inflammation was still present in the islets, the mice were protected from diabetes development. Tregs were phenotypically similar to WT NOD mice except for decreased expression of CD103, CD25, and Bcl2 and were present at comparable proportions with wild type (WT) NOD mice in peripheral lymphoid organs. However, in the thymus and islets, Treg proportions were increased. This accompanied a decrease in effector T cells in the tissue, primarily in the $CD8^+$ T cell subset. Unexpectedly, these mice developed peripheral neuropathy associated with excision of the TGFβRII in effector T cells. These findings suggest opposing roles for TGFβ signaling in Tregs- it is critical to prevention of neuropathy but detrimental to diabetes prevention in NOD mice.

Introduction

Tregs play a critical role in preventing tissue specific autoimmunity. In the non-obese diabetic (NOD) mouse model of type 1 diabetes, Treg control of autoimmunity at the site of inflammation, the islet of Langerhans, is critical to prevent effector function of T and NK cells (Feuerer et al., 2009; Mahne et al., 2015). Regulating this balance of Tregs and effector cells is important to maintain non-destructive insulitis. Shifting this

balance by altering costimulation in a B7-2 deficient NOD mouse results in prevention of diabetes mediated by Tregs (Bour-Jordan et al., 2004). However, in this model, prevention of diabetes is accompanied by autoimmunity mediated by $CD4^+$ T cells infiltrating the peripheral nerves (Bour-Jordan et al., 2005; Salomon et al., 2001). Thus, the pathogenesis of autoimmunity targeting different tissues in NOD mice are distinct.

In this study, we have examined the role TGFβ signaling in the Treg compartment plays in this balance between effector and regulatory mechanisms. TGFβ is inhibitory to effector T cell proliferation and activation (Ishigame et al., 2013; Marie et al., 2006). For Tregs, TGFβ also inhibits proliferation in the peripheral sites (Sledzinska et al., 2013) but may increase Treg proliferation in the islets (Peng et al., 2004). We have crossed the $TGF\beta RII^{f/f}$ allele with $Foxp3^{Cre}$ on the NOD background and assessed homeostasis of Tregs and development of autoimmunity. Our results point to a differential role for TGFβ signaling in Tregs which affects their function in different autoimmune diseases.

Results

Diabetes incidence in $Foxp3^{Cre}$ x $TGF\beta RII^{f/f}$ NOD mice

We first crossed the NOD.$TGF\beta RII^{f/f}$ allele (Chytil et al., 2002) with NOD.$Foxp3^{Cre/GFP}$ expressing mice (Zhou et al., 2008) and assessed incidence of diabetes. $Foxp3^{Cre}$ x $TGF\beta RII^{f/f}$ (hereafter referred to as "Flox") mice had protection from diabetes development compared to WT NOD mice, with approximately 15% of the mice progressing to clinical disease (Figure 1A). When we examined the islet inflammation by H&E staining we found similar insulitis levels between both mice

(Figure 1B). Thus, Flox mice are protected from diabetes but their islets are still inflamed.

Treg and effector cell percentages in Foxp3^{Cre} x TGRβRII$^{f/f}$ NOD mice

We next examined the overall Treg proportions in lymphoid organs in WT and Flox mice. Tregs in the thymus of Flox mice represented nearly double the proportions of CD4^{+}CD8^{-}Foxp3^{+} in WT mice (Figure 2A). In spleen, pancreatic LN (pancLN), inguinal LN (iLN), and mesenteric LN (mLN), Tregs proportions in WT and Flox mice were similar (Figure 2B). In the islets, Tregs represented approximately 40% of the total CD4^{+} T cells compared with 20% in WT NOD mice (Figure 2C), while the total number of Tregs per islet was unaffected (Figure 2D).

Total cellularity in spleen, pLN, iLN, and thymus was unaffected by the loss of TGFβ signaling in Tregs (Figure 3A). In the peripheral lymphoid organs spleen and mLN there was a mild shift in the CD4/CD8 ratio, indicating a reduction of CD8^{+} T cells relative to CD4^{+} T cells, while pLN and iLN CD4/CD8 ratios were similar between WT and Flox (Figure 3B). In the tissue, the CD4^{+}Foxp3^{-} (Tconv):CD8^{+} T cell ratio was skewed such that the CD8^{+} T cell population was reduced relative to the Tconv cells (Figure 3C), even though the proportion of both populations were reduced per islet (Figure 3D, E). Collectively, this data shows that a loss in Treg responsiveness to TGFβ signaling in Flox mice results in effective control of the effector T cell numbers in the islets.

Tregs in Foxp3^{Cre} x TGFβRII$^{f/f}$ NOD mice are phenotypically similar to WT except a possible survival defect

We next examined protein expression of different Treg-associated markers on WT and Flox mice. Expression of Foxp3, ICOS, TIGIT, CD5, and CD62L were identical between WT and Flox mice in the spleen, pancLN, and islets (Figure 4A). In contrast to previous reports using a $CD4^{cre}$ (Marie et al., 2006) or $CD4^{creER}$ (Sledzinska et al., 2013), we found no major changes in proliferation of Flox Tregs compared to WT Tregs beyond a slight increase in proliferation in the pancLN (Figure 4B). We measured proliferation using a short pulse of BrdU for 16 hours (Figure 4B, left), a 1 week continuous BrdU label (Figure 4B, middle), and by Ki67 staining (Figure 4B, right). As expected, there was very little CD103 expression on Tregs from Flox mice (Figure 4C), since CD103 is a TGFβ responsive integrin in other T cell populations (El-Asady et al., 2005). The only major differences observed phenotypically between WT and Flox was a decrease in CD25 and Bcl2 expression (Figure 4D), suggesting a possible cytokine response and survival defect in these Tregs.

Development of peripheral neuropathy in $Foxp3^{Cre}$ x $TGFbRII^{f/f}$ NOD mice

In Flox mice protected from diabetes development, we observed a decrease in salivary gland inflammation and an increase in thyroid inflammation (Supplemental Fig 1A, B) as well as a progressive paralysis and loss of hind limb function, which we call peripheral neuropathy, starting around 20 weeks of age that affected approximately 80% of the mice by 37 weeks of age (Figure 5A). This hind limb paralysis was associated with inflammation of the sciatic nerve, which was uninflamed in WT NOD mice (Figure 5B, C). The composition of the leukocytes infiltrating the sciatic nerves included $CD4^+$, $CD8^+$, and Treg cells as well as B cells (Figure 5D). These sites were dominated by a few expanded T cell clones, as indicated by analysis of TCRβ

sequencing. Both islet and nerve Tregs had expanded clones, with the top 10 clones in each population representing more than 20% of the whole repertoire (Figure 5E). In the peripheral lymphoid compartment, the top 10 clones represented less than 10% of the total population, indicating more diversity in these populations (Figure 5F). $CD4^{+}Foxp3^{-}$ Tconv cells were also expanded in the nerve compared to islet Tconv or peripheral Tregs (Figure 5F). Less than 1% of the unique CDR3 TCRβ clones overlapped between islet and nerve Tregs or islet Tregs and Tconv, with a bit higher overlap between nerve Treg and Tconv cells and islet and nerve Tconv cells (Figure 5G). Taken together, neuropathy observed in Flox mice is likely to be caused by infiltrating lymphocytes that are distinct from islet effector cells.

Possible mechanism for neuropathy development in $Foxp3^{Cre}$ x $TGF\beta RII^{f/f}$ NOD mice

Since effector cells are pathogenic in the $CD4^{cre}$ x $TGF\beta RII^{f/f}$ mice (Marie et al., 2006), a deletion of TGFβRII in effector T cells in this system may also be responsible for development of peripheral neuropathy. Thus, we utilized quantitative PCR to determine amount of TGFβRII DNA excision in different populations and different organs. To accomplish this, we normalized the input of DNA based on expression of genes that we know should have two copies, including Rosa26 and ST2. To determine excision of the loxp sites at the TGFβRII locus, we utilized primers that create a 241bp product only when TGFβRII exon 2 is deleted by the Cre protein (Chytil et al., 2002). Tregs purified from LNs of Flox mice were considered 100% efficient at excision of TGFβRII, so all other samples were compared to this standard across multiple experiments. WT Tconv cells had no excision of TGFβRII as well as naïve $CD44^{lo}CD62L^{hi}$ Tconv cells and $CD8^{+}$ T cells from LNs of Flox mice (Figure 6). 30-40%

of activated $CD44^{hi}CD62L^{lo}$ Tconv cells in LN showed a deletion of TGFβRII. Tconv cells from islets and nerves both had excision of TGFβRII in 40-60% of the cells. All B cells in the tissues as well as $CD8^+$ T cells from nerves had no deletion of TGFβRII. Approximately 30% of $CD8^+$ T cells in the islets had deletion of TGFβRII. To determine if deletion in Tconv cells was activation induced, we expanded Tconv cells from the islets *in vitro* with anti-CD3 and anti-CD28 for 9 days and then assessed their TGFβRII DNA. Only 10% of the cells lost TGFβRII expression, less than *ex vivo* sorted Tconv, (Figure 6). Thus, exacerbation of neuropathy may be related to the loss of TGFβ sensitivity in Tconv cells.

Discussion/Future Directions

In this study, we have found that deletion of TGFβRII in Tregs resulted in a protection from diabetes development without prevention of islet inflammation. Treg proportions in the peripheral lymphoid organs were comparable to WT mice, with the only increases in Treg proportions seen in the thymus and islets. Total cellularity of the primary and secondary lymphoid organs was normal. The major effector population altered in these mice were $CD8^+$ T cells in the islets, which were significantly reduced. A milder but still significant decrease in Tconv cells was also observed in the islets. Phenotypically, Tregs were similar to WT in all organs and all markers examined except CD103, CD25, and Bcl2. All three were reduced, and the reducing in CD25 and Bcl2 suggests reduced cytokine sensitivity and survival in Flox mice. Additionally, mice that did not develop diabetes instead developed peripheral neuropathy starting around 20 weeks of age, which was associated with infiltrating T and B cells in the sciatic nerves. Both Tregs and Tconv cells were clonally expanded in the nerves compared to the

islets, with some overlap in the TCRβ seen between Tconv and Tregs in the nerves as well as between islet and nerve Tconv cells. Lastly, when we examined the deletion of TGFβRII in effector cells, we found 30-60% of Tconv cells in the islets or nerves had deletion.

Previous studies have found that deletion of TGFβRII in all T cells using a $CD4^{cre}$ results in spontaneous autoimmunity that cannot be controlled by Tregs (Marie et al., 2006). However, when using a $CD4^{creER}$, a different group found that spontaneous autoimmunity did not result except when the host was lymphopenic (Sledzinska et al., 2013). Therefore, the loss of TGFβRII in effector cells may not be playing a major role in development of peripheral neuropathy. If the loss of TGFβRII increases the activation and proliferation phenotype of the cells to the extent observed in the $CD4^{cre}$ model, we would expect that the mice would develop both diabetes and neuropathy, rather than only 1 of these two autoimmune diseases. Additionally, this experiment does not describe which cells have deleted 1 or both copies of TGFβRII, only describing the total DNA in the population has a 30-60% incidence of TGFβRII deletion. Thus, the possibility remains that many of the cells only excised 1 copy of the TGFβRII gene, which would not be expected to increase their activation and proliferation state. Alternatively, a lower percentage of the cells may be excising both copies and be the pathogenic effector cells in neuropathy. A measurement of the actual mRNA and/or TGFβRII protein levels in this populations would also be useful for determining the amount of deletion observed in these different populations. Thus, the mechanism responsible for the dichotomy between prevention of diabetes and precipitation of neuropathy is still unclear. Alternate models of peripheral neuropathy using the $B7\text{-}2^{-/-}$

mice found that this disease was driven by $CD4^+$ effector T cells that required IFNɣ (Bour-Jordan et al., 2005; Salomon et al., 2001). Assessment of this requirement for CD4 versus CD8 T cells and IFNɣ is ongoing.

The TCRβ sequencing data shows a clonal expansion of only a few TCRs in the nerves in both Tregs and Tconv cells. This suggests that a limited number of antigens may be responsible for initiating this disease. The increased TCRβ sharing between nerve Treg and Tconv versus islet Treg and Tconv may indicate a conversion to and/or from the Treg phenotype. We may hypothesize that Tregs may lose their expression of Foxp3 in the nerves and convert to an effector phenotype that mediates nerve destruction. Cell fate tracking experiments are ongoing in the lab to address this question.

TCRβ sharing between islet and nerve Tconv may indicate a similar antigen recognition in the nerves and islets. One hypothesis is that TGFβ signaling is inhibitory to the function of islet Tregs but required for the function of Tregs responsible for prevention of nerve inflammation. If this is the case, effector T cell recognition of antigens shared between islet and nerves are controlled by TGFβ-dependent Tregs in WT NOD mice. In Flox mice, effector T cells would be restrained by TGFβ-independent islet Tregs but left unregulated because either these cells did not develop initially or do not maintain stable expression of Foxp3 and suppressive function. The small TCRβ overlap between islet and nerve Tregs would support this hypothesis.

CD103 is a TGFβ responsive integrin and we have previously found the $CD103^+$ Tregs to be the most highly antigen activated subset in the inflamed islets (Chapter II and III). Expression of CD103 was greatly reduced in Flox mice, which raises the

possibility that these islets lack the highly activated effector subset of Tregs. However, not only were mice protected from diabetes, but both ICOS and TIGIT expression were unaffected by the loss of TGFβ signaling, which we have found to be co-expressed on this population of Tregs (Chapter II). Thus, loss of CD103 expression in islet Tregs in Flox mice may only indicate a loss of the marker expression; this population may still be present in the islets.

Previous studies have suggested that Tregs in CD4 specific deletion of TGFβRII have increased proliferation, paralleling effector T cells (Marie et al., 2006; Sledzinska et al., 2013). However, in our study we did not see any increased proliferation, suggesting the increase observed in other systems may be a result of the uncontrolled expansion of effector T cells that Tregs are attempting to control or that this expansion occurs immediately after the excision of TGFβRII.

In summary, we have some preliminary data and a phenotype whose mechanism we do not yet fully understand. Elucidating the mechanism behind this dichotomy between diabetes and neuropathy development will provide significant insight into the role of TGFβ in the Treg population during a spontaneous autoimmune disease. Since TGFβ is such an important player in many immunological process, including autoimmunity and cancer, any additional insight into the mechanism of action of different populations of immune cells will be of great interest to both the Treg basic biology community and for developing targeted therapies for disease treatment. Some future steps include detailed analysis of all tissues of Flox mice to determine any additional inflammatory diseases these mice may develop as well as determining the

exact mechanism of disease development and the important players in this disease process.

Materials and Methods

Mice: Female NOD/ShiLtJ, (Jackson laboratories) mice were housed and bred under specific pathogen free conditions in accordance with the UCSF (San Francisco, CA) Animal Care and Use Committee guidelines. NOD.Foxp3$^{Cre/GFP}$ (Zhou et al., 2008), TGFβRII$^{f/f}$ (Chytil et al., 2002) were previously described.

Diabetes incidence: Diabetes incidence was monitored by weekly blood glucose monitoring. Mice were considered diabetic after 2 sequential blood glucose readings exceeding 300 mg/dL.

Histological staining and section scoring: Hematoxylin and eosin staining was performed using paraffin-embedded pancreas, sciatic nerve, salivary and thyroid glands fixed in 4% paraformaldehyde. For islets, score 0 indicates absence of insulitis, score 1 indicates one layer of infiltration, score 2 indicates infiltrating representing <50% of the islet area, and score 3 indicates infiltration representing >50% of the islet area. For nerves, salivary and thyroid glands, score 0 indicates absences of infiltration, score 1 indicates light infiltration, and score 2 indicates heavy infiltration.

Flow cytometry: Islet, spleen, and lymph node single-cell suspensions were prepared as previously described (Melli et al., 2009). The following antibodies were used to stain the cells: CD103-FITC or Pacific Blue (2E7), ICOS-APC (C398.4A), Ki67-PE-Cy7 (SolA15), TIGIT-APCeFluor780 (MBSA43), CD25-PE (PC61.5) CD5-APC (53-7.3), PD-1-PE-Cy7 (J43), CD62L-PE-Cy7 (MEL14), Foxp3-eFluor450 (FJK-16s) (eBiosciences),

anti-CD4-PE or APC (RM4-5) (eBioscience or Tonbo Biosciences). CD8-Pacific Orange (5H10) (Life Technologies), CD45-APC-Cy7 (30-F11), Thy1.1-PerCP (OX-7), BrdU-FITC, Bcl2-PE (100), B220-PE-Cy7 (RA3-6B2), CD44-APC (IM7) (BD Biosciences). For intracellular staining, cells were first fixed and permeabilized via the manufacturer's instructions (eBioscience or Tonbo Biosciences). Analyses were performed on a LSRII or Fortessa flow cytometer (BD Biosciences) with FACSDiva (BD Biosciences) and Flowjo analysis software.

Nerve dissociation: Sciatic nerves were cut into small fragments and incubated in RPMI 1640 with collagenase IV (2mg/mL), DNaseI (250µg/mL), and dispase (1mg/mL) at 37 degrees shaking 50rpm for 1 hour. Every 15 minutes, nerves were mixed and filtered through a 100µM MACS Smart Strainer (Miltenyi) and fresh media was added. Cells were washed with PBS containing 3% serum, and resuspended in staining buffer (PBS, 3% serum, 0.02% azide) for flow cytometric staining.

Bulk TCRβ analysis of Treg cells: Total RNA was extracted from flow sorted cells using ARCTURUS PicoPure RNA Isolation Kit (Life Technologies) for <100,000 cells or QIAGEN Micro RNA extraction kit (QIAGEN) for >100,000 cells. TCRβ repertoires were amplified and sequenced using Illumina MiSeq by iRepertoire Inc. (Huntsville). Data analysis was performed using the website provided by iRepertoire Inc. (http://www.irepertoire.com).

Quantitative PCR of TGFβRII DNA excision: DNA was prepared using QIAamp DNA Micro Kit (Qiagen). Primers recognizing the deletion product after cre-mediated excision of TGFβRII were previously described (Chytil et al., 2002). For measurement of ST2, we used the primers 5'-TAA CAT ACG AAA CAG AAG CCC A and 5'- CAG

ATG AGG CAC CTA GAG TC. For Rosa26, we used primers as previously described (Srinivas et al., 2001). Real-time PCr was conducted using the SYBR Green PCR Master Mix (Applied Biosystems) using a standard temperature profile. DNA amount was normalized with respect to levels of ST2 and Rosa26 expression and compared to control levels of TGFβRII excised DNA.

Statistical analysis: Analysis was performed with Prism software (GraphPad).

Acknowledgements

We thank J. Wang, V. Dang, N. Lescano, and R. Guerrero-Moreno for mouse husbandry, V. Nguyen for islet isolations and lab support, L. Bates, S. Cheng, and A. Wiederanders for lab support. The UCSF Pathology core and UCSF Diabetes center processed and stained of paraffin sections, and Li Wen generously provided us with the NOD.TGFβRII$f^{/f}$ mice.

FIGURES

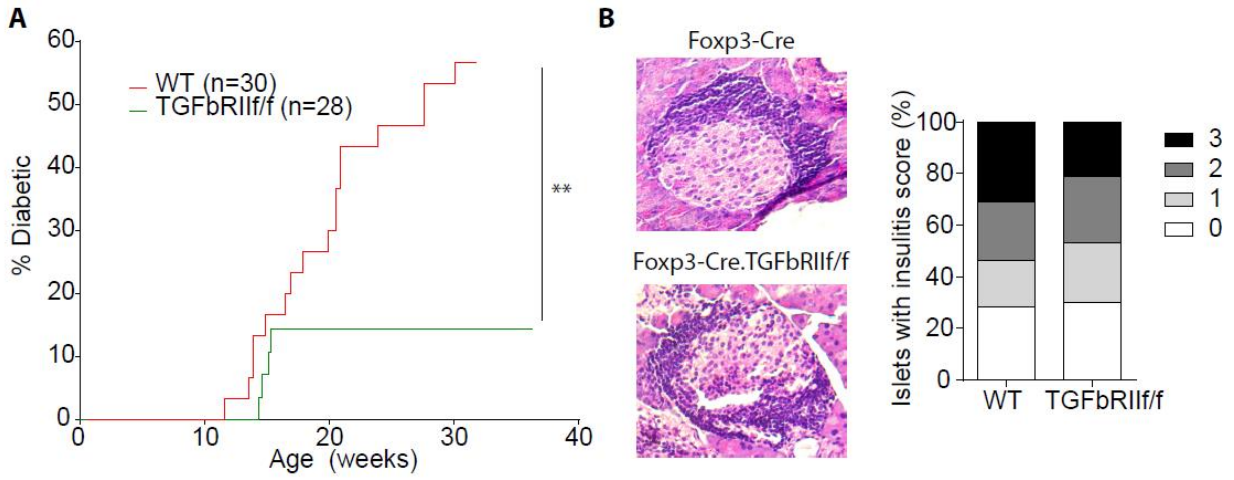


Figure 1: Diabetes incidence in Foxp3^{Cre} x TGFbRII$^{f/f}$ NOD mice. A. Diabetes incidence in WT NOD mice compared to Foxp3^{Cre} x TGFβRII$^{f/f}$ (Flox) mice. B. H&E staining of islets in WT and Flox mice (left) and insulitis quantification (right).

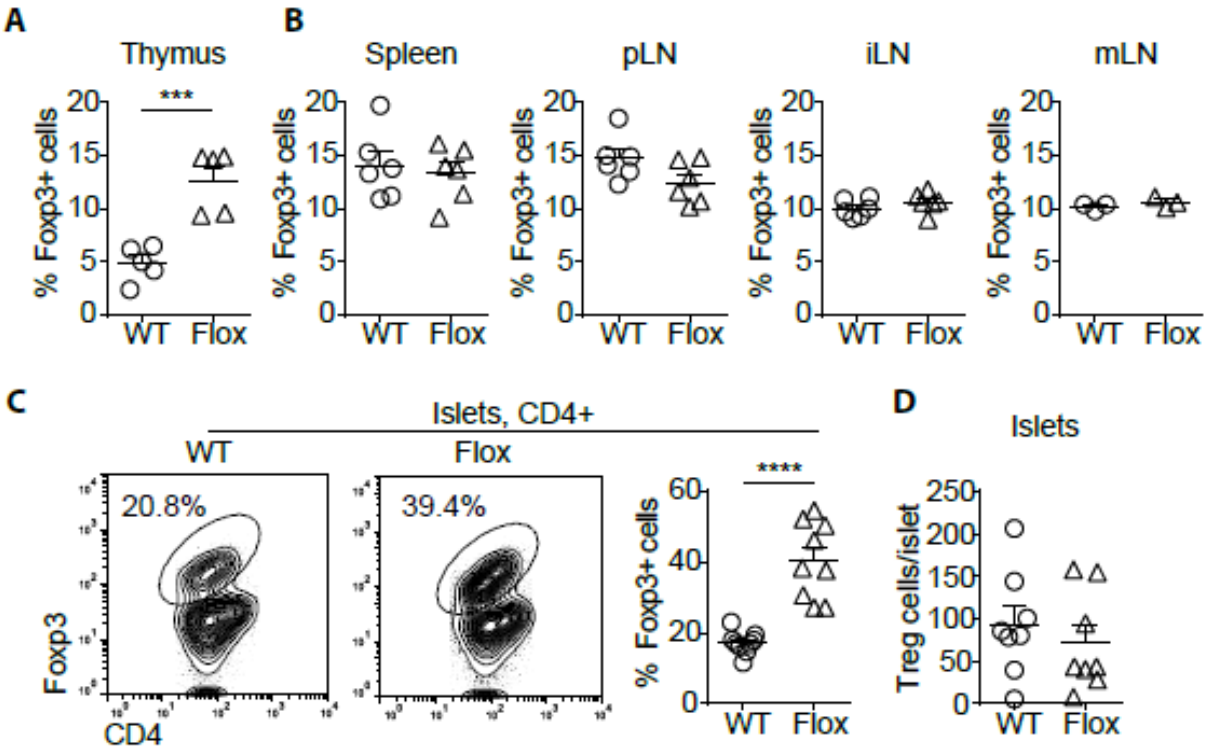


Figure 2: Treg percentages in Foxp3^{Cre} x TGRbRII$^{f/f}$ NOD mice. A. Proportion of Foxp3^{+} Tregs among total CD4SP cells in the thymus of pre-diabetic NOD WT and Flox mice. Thymic Tregs were defined as CD4^{+}CD8^{-}Foxp3^{+}. B. Proportion of Foxp3^{+} Tregs among total CD4^{+} cells in different peripheral lymphoid organs of pre-diabetic NOD WT and Flox mice. C. Flow cytometric staining of CD4^{+} T cells in islets (left) and quantification (right) of pre-diabetic NOD WT and Flox mice. D. Total Treg cells per islet in pre-diabetic NOD WT and Flox mice.

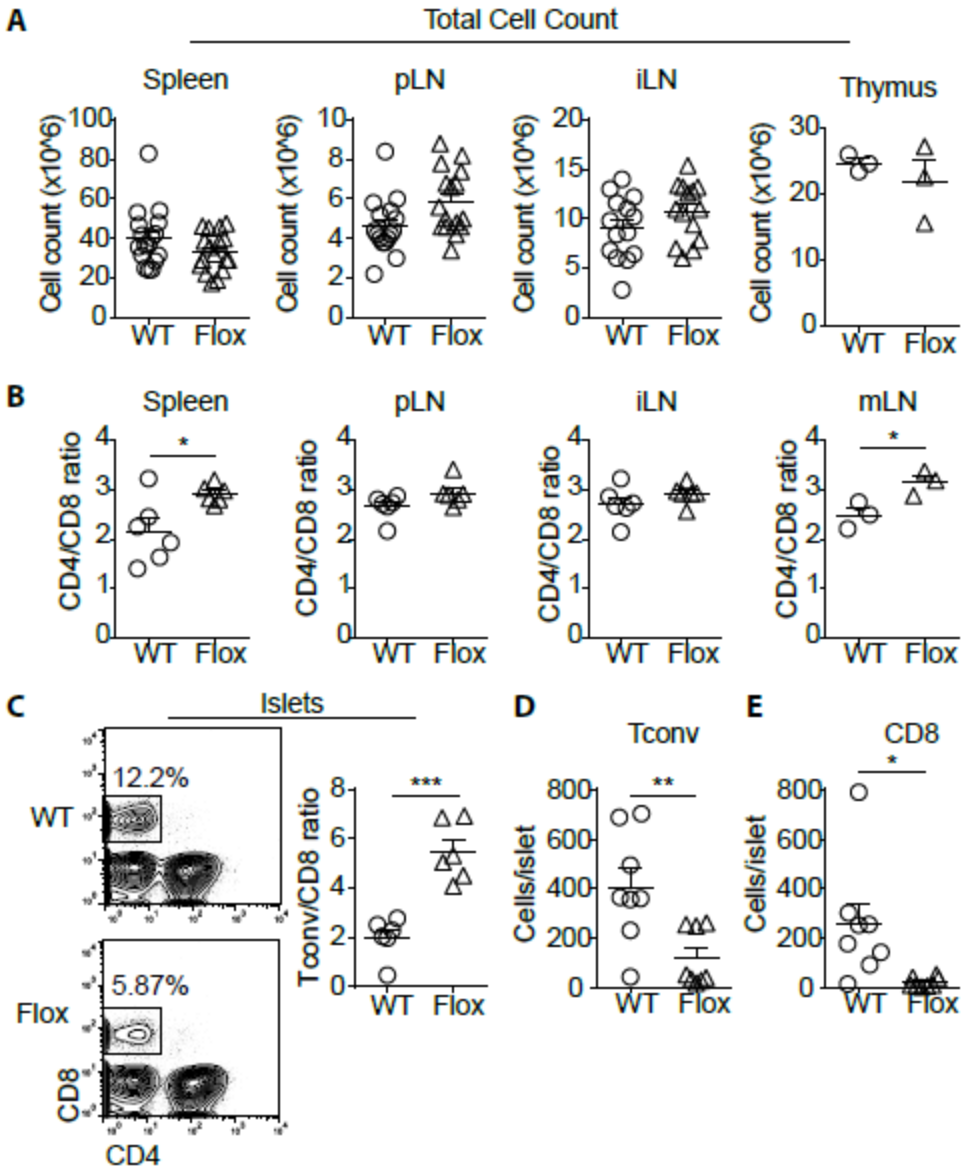


Figure 3: Reduced effector T cells in Foxp3^{Cre} x TGRbRII$^{f/f}$ NOD mice. A. Total number of cells present in lymphoid organs in pre-diabetic WT and Flox mice. B. Ratio of total CD4:CD8 T cells in lymphoid organs in pre-diabetic WT and Flox mice. C. Flow cytometric analysis of lymphocytes in islets (left). Ratio of CD4^{+}Foxp3^{-} Tconv cells to

$CD8^+$ T cells in islets (right). D. Total Tconv cells per islet in WT and Flox mice. E. $CD8^+$ T cells per islet in WT and Flox mice.

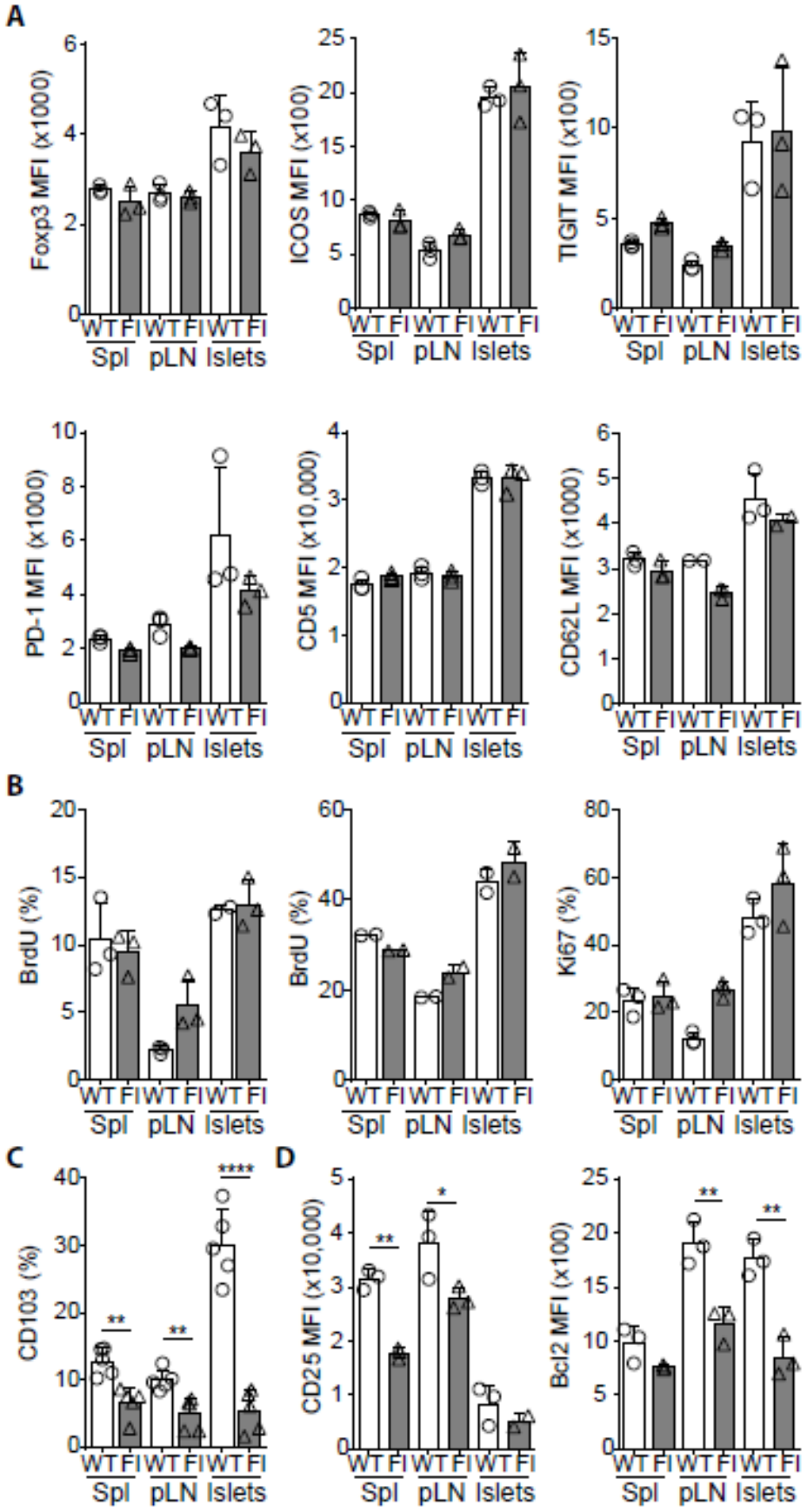

A
Foxp3 MFI (x1000)
ICOS MFI (x100)
TIGIT MFI (x100)
PD-1 MFI (x1000)
CD5 MFI (x10,000)
CD62L MFI (x1000)
B
BrdU (%)
BrdU (%)
Ki67 (%)
C
CD103 (%)
D
CD25 MFI (x10,000)
Bcl2 MFI (x100)
WT FI WT FI WT FI
Spl pLN Islets

Figure 4: Tregs in Foxp3^{Cre} x TGFbRII$^{f/f}$ NOD mice are phenotypically similar to WT except a possible survival defect. A. Protein expression of Foxp3, ICOS, TIGIT, PD-1, CD5, and CD62L in spleen, pancLN, and Islets of pre-diabetic WT and Flox NOD Tregs. B. 16hr BrdU (left), 1wk continuous BrdU (middle), and Ki67 expression in spleen, pancLN, and islets of pre-diabetic WT and Flox NOD Tregs. C. CD103 expression in spleen, pancLN, and islets of pre-diabetic WT and Flox NOD Tregs. D. CD25 and Bcl2 expression in spleen, pancLN, and islets of pre-diabetic WT and Flox NOD Tregs.

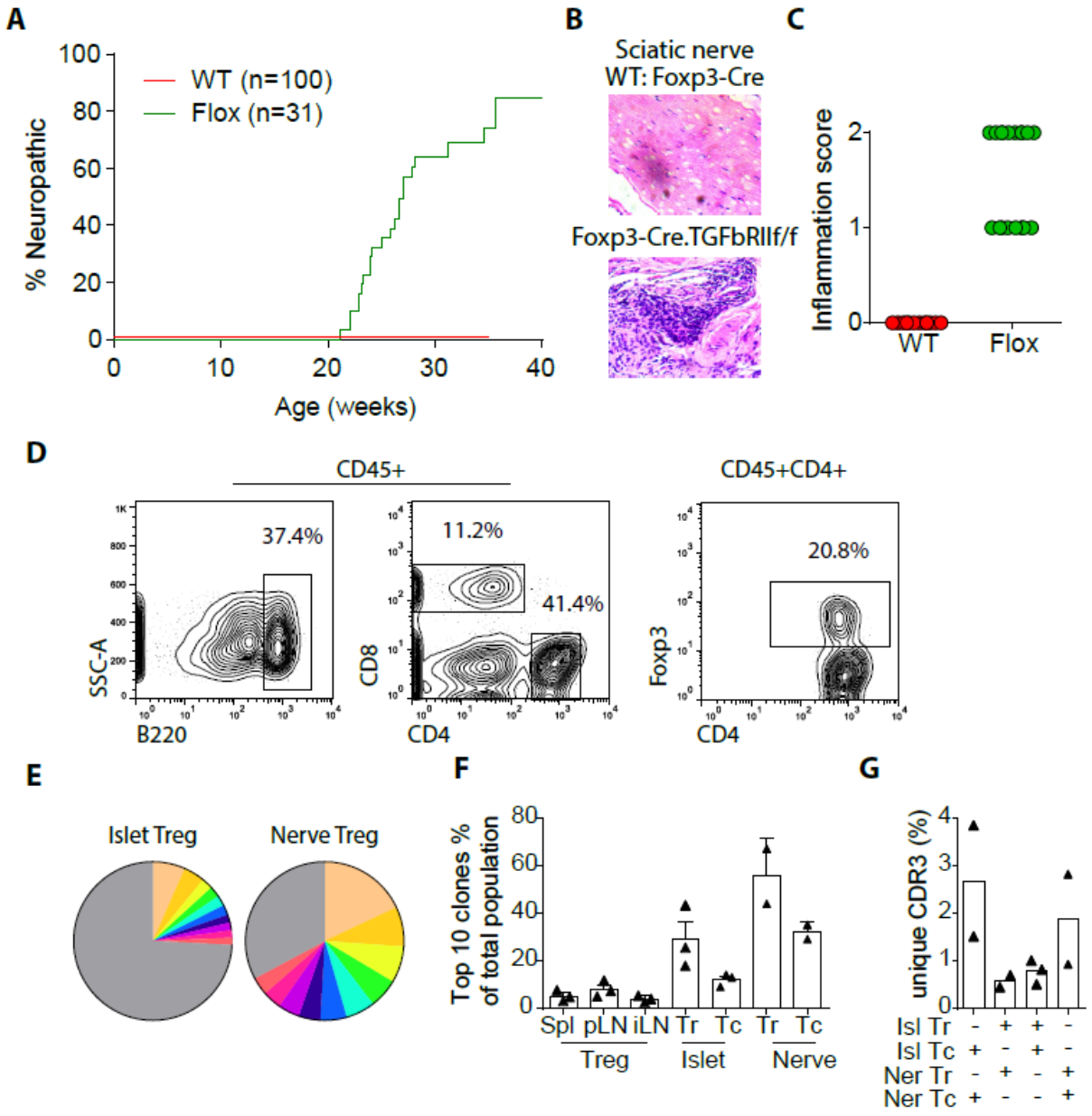


Figure 5: Development of peripheral neuropathy in Foxp3^{Cre} x TGFbRII$^{f/f}$ NOD mice. A. Incidence of neuropathy in WT and Flox mice. B. H&E staining of sciatic nerves in WT and neuropathic Flox mice. C. Histology inflammation score of aged WT and neuropathic Flox mice. D. Flow cytometric analysis of the CD45+ cells infiltrating

the nerves of Flox mice. E. Top 10 TCRβ clones in islet and nerve Tregs (colored) out of the total population. F. Proportion the top 10 TCRβ clones in each population represents out of the total population. G. Unique CDR3 sequence overlap between islets, nerves, Tregs, and Tconv cells.

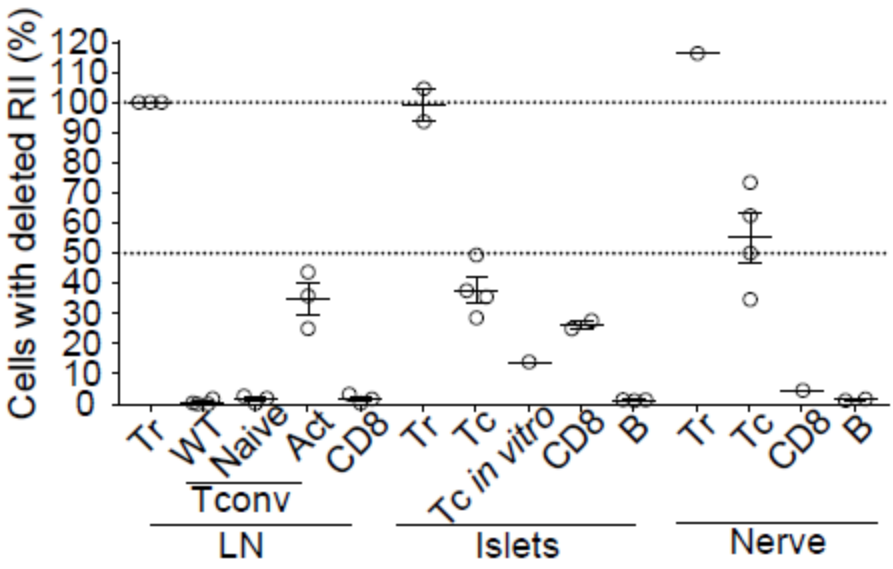
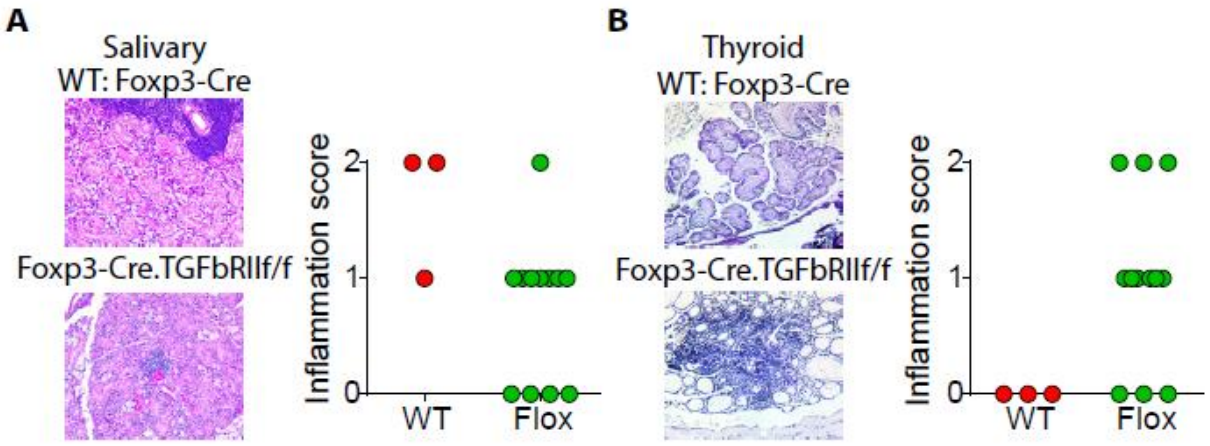


Figure 6: Possible mechanism for neuropathy development in Foxp3^{Cre} x TGFβRII$^{f/f}$ NOD mice. Quantitative PCR of the TGFβRII DNA excision in different cell populations in LN, islet, and sciatic nerves. Input was normalized to Rosa26 and ST2 genes.



Supplemental Figure 1: Salivary and Thyroid inflammation in Foxp3^{Cre} x TGFbRII$^{f/f}$ NOD mice. A. H&E staining (left) and inflammation scoring (right) of the submandibular portion of the salivary gland in WT and neuropathic Flox mice. B. H&E staining (left) and inflammation scoring (right) of the Thyroid in WT and neuropathic Flox mice.

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

Summary

This work has highlighted many facets of Treg biology that underlie their efficacy during ongoing immune responses. We have sought to characterize the dynamics of the Treg compartment comprising and dampening the immunological infiltrate in the islet of the Langerhans. Prior to this study, the antigen specificity of Tregs at the site of chronic inflammation was unknown as well as the impact of decreased IL2 presence in the tissue on Treg turnover. The role of TGFβ in islet Tregs has not been investigated in polyclonal mice. There has also been debate surrounding the percentage of Tregs that are islet antigen specific present in the tissue. We found that Tregs reactive to their cognate antigen were indeed present in the inflammatory infiltrate, where they have most recently and strongly been stimulated through their TCR compared to Tregs in peripheral lymphoid sites. This increase in TCR signaling, as measured by $Nur77^{GFP}$, correlated with increased therapeutic function over the peripheral lymphoid organs, including the draining LN. Islet Tregs were unique to the tissue, where they proliferated, gained expression of activation molecules such as ICOS, TIGIT, and CD103, and died due to an IL2 deficiency. Treg subsets expressing CD103 were the most sensitive subset to IL2, while $CD103^-$ Tregs were most sensitive to IL7, suggesting an intracellular wiring switch between these two populations. Highly activated islet Tregs do react with known drivers of autoimmunity, such as insulin, but also have novel and unknown islet antigen specificity. There is no requirement for TGFβ signaling to maintain the phenotype and function of islet Tregs, except a possible a role for TGFβ in

the survival of Tregs in NOD mice. This alteration in the Treg's ability to respond to TGFβ resulted in protection from diabetes but precipitated the onset of peripheral neuropathy in the majority of mice.

Antigen specificity of Tregs: Can TCR sequence be used to predict TCR structure?

In investigating the TCR reactivity of islet Tregs, we discovered a high Nur77^{GFP} MFI compared to peripheral lymphoid sites, where the primary increase in GFP signal was contributed by the CD103^{+} Treg subset. CD103 expression defined this highly antigen reactive Treg population only in the tissue, where its expression was increased over peripheral lymphoid organs. When we sequenced the TCRβ chain from different Treg populations in different sites, the islet Tregs, whether CD103^{+} or CD103^{-}, contained a few highly expanded clones rarely found across multiple mice, particularly in the tissue. This suggests some stochastic generation of TCR sequence selection between different mice. The unique nature of the tissue Treg TCRβ sequences would suggest that expanded Tregs in the tissue are not circulating through the lymphoid compartments, but instead remain localized at the site of inflammation, similar to total CD4^{+} T cells in NOD mice (Diz et al., 2012; Li et al., 2009). To attempt to identify the antigen specificity of these islet Tregs, we first sorted the highly activated ICOS^{+}TIGIT^{+} subset for single cells and sequenced both the TCRα and TCRβ within the same cell. Tetramer analysis identified around 2% of islet Tregs as reactive to insulin B:9-23, so we first compared our single cell sequencing to known insulin reactive clones. For example, T cells that use the alpha chain TRAV5D-4 have been described as specifying

insulin specific T cell clones (Nakayama et al., 2012), and we found 3 clones out of 71 that were either expanded (found more than once), or public clones (found in at least one additional mouse). In this case, 2 out of the 3 clones were indeed insulin specific, and the third one inconclusive but no reactivity to insulin or other islet antigens were detected. Previous studies have found that insulin is a key autoantigen (Nakayama, 2011; Nakayama et al., 2005; Unanue, 2014; Zhang et al., 2008) and we have described it as a cognate antigen for Tregs as well. In some of our clones a reactivity to unknown islet antigens was discovered, which prompts us to further investigate the identity of their cognate antigen. The number of antigens involved in NOD diabetes is currently unknown, but may be more limited than other autoimmune diseases such as Sjogren's syndrome (Kern et al., 2014). Thus, the private nature of the TCR sequence in different mice would support a model where these different sequences specify a similar 3D structure that recognizes similar antigens. Development of a model to identify and predict similarities in 3D TCR conformations will greatly aid in the identification of dominant T cell clones that can be utilized to find cognate autoantigens. These autoantigens will likely be found in multiple mice as well as patients. Discovery of these autoantigens will be instrumental in the development of targeted Treg therapies in the clinic.

Islet Treg relationship and homeostasis

During our investigation of islet Treg homeostasis, we discovered that islet Tregs are unique to their tissue, yet have a high rate of turnover and are relatively stable in their subset proportions over time. Coupled with an IL2 deficiency in the pancreas

(Tang et al., 2008), this data supports a model where these Tregs enter the tissue, recognize cognate antigen, proliferate, acquire CD103 expression and increased effector function, and finally die without additional IL2 to maintain their survival. The $CD103^-$ Treg sensitivity to IL7 may sustain their survival in the IL2 poor environment of this tissue prior to acquisition of CD103 expression. This $CD103^+$ phenotype is associated with increased activation, higher levels of CD25, IL2 sensitivity, decreased Bcl2, and decreased survival after adoptive transfer. Taken together, this data fits a model in which cTregs in the periphery convert to eTregs as previously described (Smigiel et al., 2014; Toomer et al., 2016). These eTregs further differentiate to $CD103^+$ terminally differentiated Tregs with a reduced lifespan and increased suppressive function.

Possibility of two types of Tregs: TGFβ independent and TGFβ dependent that have distinct functions in autoimmunity

Although many of these findings are still preliminary and lacking in fully defined mechanism, we find that mice with a conditional deletion of the TGFβRII in Tregs using $Foxp3^{cre}$ are protected from diabetes development. This is likely due to an increased proportion of Tregs and decreased effector cells, particularly $CD8^+$ T cells, at the site of inflammation, suggesting enhanced Treg function in this model. The phenotype of these Tregs is similar to Tregs found in wild type (WT) NOD mice, except a decrease in CD25 and Bcl2 expression. This phenotype may suggest reduced survival of these Tregs. Unexpectedly, the majority of these mice develop a rapidly progressing inflammation of the sciatic nerve between 20-30 weeks of age, which results in limited

use of the hind limbs and severe weight loss accompanying infiltration by T cells into the sciatic nerve. Some of the other autoimmune sites, such as the thyroid and salivary glands, have either exacerbated or partial protection from inflammation. So we could hypothesize that Tregs important for control of diabetes are actually less effective when TGFβ signaling is intact, whereas Tregs important for control of neuropathy require TGFβ for their function. We currently do not know if the role of TGFβ is to support stability of the Treg phenotype or if response to TGFβ enhances some suppressive mechanism critical for preventing neuropathy. The lack of TGFβ sequence sharing between islet and nerve Tregs would suggest that these Tregs may be reacting to different antigens. Another non-exclusive possibility is that these Tregs develop entirely independently of islet Tregs. An increased TGFβ sequence overlap between islet and nerve Tconv, on the other hand, might suggest that these T cells are reactive to similar antigens and migrate between islet and nerve. Further exploration of the disease mechanism in these systems is required, but these preliminary results support a novel role for TGFβ in supporting or inhibiting Treg function. One of the main targets of Treg function in diabetes are the $CD8^+$ T cells (Mahne et al., 2015), and in this model we also find the $CD8^+$ T cells are the most significantly reduced T cell subset. Alternatively, in a different model of peripheral neuropathy using a B7-$2^{-/-}$ host, the main effector T cell population are $CD4^+$ T cells (Bour-Jordan et al., 2005). Thus, due to different pathogenic mechanisms of disease development, a different function of Tregs may be required. These Treg subtypes may be lost upon TGFβ signaling ablation.

Outlook

As Treg cellular therapy enters the clinic, much remains to be understood about their biology and how best to target them to either expand and or inhibit their function and survival. The work presented as the subject of this dissertation contributes to our understanding of Treg biology by examining the antigen specificity and homeostasis of Tregs present in the chronically inflamed tissue. This study highlights the important phenotypic and functional differences between islet Tregs and Tregs in the periphery, which provides another reminder of the differences between studying the peripheral blood of human patients and supports the use of tissues collected by organizations such as nPOD to gain more insight into the human T1D. Our data will highlight the importance of antigen specificity in development of Tregs for T1D therapy in humans. While polyclonal populations of Tregs may not have adverse effects in GvHD (Brunstein et al., 2010; Trzonkowski et al., 2013) and T1D (Bluestone et al., 2015; Marek-Trzonkowska et al., 2012), antigen specific Tregs may be required for effective control of disease. The cytokine requirements for maintaining homeostasis and survival may be linked to the reactivity of the TCR in the tissue. New methods of delivering cells to their destination with cargo using nanoparticles (Cruz et al., 2012; Shao et al., 2015) may have applications for Tregs by encapsulating cytokines or other molecules to increase Treg survival and function. Alternatively, pre-treatment with cytokines such as IL2 appears to be efficacious for increasing Treg function. How well these findings will translate into clinical therapies remains to be seen, but we will continue to push forward defining the mechanisms of Treg survival and function that will reveal new insights into immune regulation.

APPENDIX I: TARGETING TREG SIGNALING FOR THE TREATMENT OF AUTOIMMUNE DISEASES

Published in *Curr Opin Immunol.* 2015 Dec;37:11-20

Abstract

Regulatory T (Treg) cells are critical players in the prevention of autoimmunity. Treg lineage commitment and functional stability are influenced by selected extracellular signals from the local environment, shaped by distinctive intracellular signaling network, and secured by their unique epigenetic profile. Recent advances in our understanding of the complex processes of Treg lineage differentiation, maintenance, and function has paved the way for developing strategies to manipulate these important cells for therapeutic benefit in many diseases. In this review, we will summarize recent advances in our understanding of Treg biology as well as Treg-targeted therapies in the context of autoimmune disease.

Introduction

Forkhead box P3 (Foxp3)-expressing regulatory T cells (Tregs) are a small subset of $CD4^+$ T cells that are vital to immune homeostasis and prevention of autoimmunity in mice and man (Sakaguchi et al., 2010). Expression of the transcription factor Foxp3 in these cells is essential for their development, maintenance, and function. Treg potency lies in their ability to deploy various immunosuppressive mechanisms depending on the immunological context as well as extending their influence through the process of infectious tolerance (Tang and Bluestone, 2008). An

emerging concept is that Tregs not only control immune responses, but also promote tissue homeostasis by suppressing inflammation and aiding in tissue repair (Burzyn et al., 2013a). Moreover, this system is exploited by tumor cells to evade immune surveillance (Nishikawa and Sakaguchi, 2014). Thus, changes in Treg number and function underlie many illnesses of the immune system and beyond.

Manipulating Tregs is a new therapeutic strategy for treating various diseases including autoimmunity, transplant rejection, and cancer (Tang and Bluestone, 2013; von Boehmer and Daniel, 2013). Elucidating factors influencing Treg homeostasis and function has important implications in understanding disease pathogenesis and identifying therapeutic opportunities. This review will focus on recent advances in how Tregs integrate extracellular and intracellular signaling to control their survival and stability. We will discuss how these new insights can be utilized for the development of new approaches to promote and stabilize Tregs in autoimmunity and transplantation.

TCR, CD28, and IL-2: the essential triad for Treg lineage specification and maintenance

Thymic Treg (tTreg) development is initiated by T cell receptor (TCR) signaling followed by sequential activation of CD25 expression, IL-2 signaling, and then Foxp3 expression (Lio and Hsieh, 2008a; Weissler and Caton, 2014). tTreg development can be enhanced through the constitutive activation of signal transducer and activator of transcription 5 (STAT5), which is downstream of the IL-2 receptor and directly binds cis elements in the Foxp3 promoter and enhancer to stabilize Foxp3 expression (Burchill et al., 2008). Indeed, the level of IL-2 in the circulation dictates the size of the thymic Treg compartment (Tai et al., 2013; Weist et al., 2015; Yi et al., 2014). In addition to

induction of CD25, TCR and CD28 signaling also contribute to establishing and stabilizing the Treg lineage commitment in the thymus by inducing epigenetic and differentiation events in Tregs (Franckaert et al., 2015; Salomon et al., 2000; Tai et al., 2013; Zhang et al., 2013). Thus, antigen and IL-2 signaling transmitted via TCR, CD28, and CD25 are essential for Treg lineage specification in the thymus.

In the periphery, mature Tregs continue to depend on TCR, CD28, and CD25 for their homeostasis and function, but their roles appear to be distinct from those in the thymus. Tregs proliferate more than conventional $CD4^+$ T cells in steady state in a CD28 dependent fashion, suggesting that Tregs are constantly seeing antigens that drive their cell cycle progression (Tang et al., 2003; Walker et al., 2003). Recently, analysis of Treg subsets in the periphery found that the $CD62L^{lo}CD44^{hi}$ effector Tregs (eTregs) were relatively more responsive to TCR stimulation and less IL-2 dependent than $CD62L^{hi}$ $CD44^{lo}$ central Tregs (cTregs) (Smigiel et al., 2014). Consistent with the idea that eTregs are TCR dependent, deletion of the TCR specifically in mature Tregs led to a selective loss of $CD62L^{lo}CD44^{hi}$ eTregs as soon as 9 days after excision of the TCR gene. This suggests that constant stimulation through the TCR is required to maintain this population (Levine et al., 2014; Vahl et al., 2014). These TCR-deficient Tregs proliferated less and expressed fewer eTreg molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), IL-10, Ebi3, and, correspondingly, conventional T cells became activated to express cytokines. However, this immune activation profile is fairly mild, which is dramatically different from the catastrophic systemic autoimmunity observed after Treg depletion (Kim et al., 2007). This is likely because the frequency of Tregs remained normal after TCR deletion and Tregs

maintained their responsiveness to IL-2, high levels of Foxp3 expression, and Treg-specific epigenetic profile. Through these observations, it is suggested that the role of TCR signaling in mature Tregs is mainly to activate their proliferation and effector functions, but not for lineage maintenance.

Proliferating Tregs have a tendency to lose their Foxp3 expression and lineage stability in vitro and in vivo in lymphopenic hosts (Hoffmann et al., 2006; Rubtsov et al., 2010; Zhou et al., 2009). The conserved noncoding sequence 2 (CNS2) enhancer element, also known as Treg-specific demethylation region, is critical for safeguarding lineage stability of proliferating Tregs (Feng et al., 2014; Li et al., 2014b). However, stimulation via TCR with limited IL-2 leads to a loss of Foxp3 expression in Tregs even in wild type cells with intact CNS2. CNS2 has binding sites for both the TCR-triggered transcription factor nuclear factor of activated T-cells (NFAT) and IL-2-induced transcription factor STAT5, providing a transcriptional basis for Treg stability by coordinating TCR and IL-2 signaling. Interestingly, forced expression of constitutively active STAT5 prevented the loss of Foxp3 in CNS2 deleted Tregs, demonstrating that STAT5 can stabilize Foxp3 expression independent of CNS2 (Feng et al., 2014). This may be explained by the NFAT-mediated looping between CNS2 and the Foxp3 promoter, which also has binding sites for NFAT and STAT5 (Li et al., 2014b). In conclusion, TCR-mediated signals are important for mature Treg function but pose a threat to their stability unless they are balanced by IL-2 signaling.

PI3K-Akt-mTOR: a critical signaling node for Treg development and homeostasis

Phosphatidylinositide 3 kinase (PI3K), protein kinase B (Akt), mammalian target of rapamycin (mTOR) form an intracellular signaling hub common to the TCR, CD28,

and IL-2 receptor. PI3K is directly activated when these receptors are engaged, leading to initial activation of Akt by the PH-domain containing protein PDK1 through phosphorylation of threonine 308. Akt is fully activated by additional phosphorylation on serine 473 by the mTOR complex 2 (mTORC2). Akt has many cellular targets, but the Forkhead box O (Foxo) transcription factors and mTORC1 are most relevant to Treg biology. Foxo family transcription factors are critical for Treg lineage specification (Harada et al., 2010; Ouyang et al., 2012; Samstein et al., 2012) and are inhibited by Akt. mTORC1 coordinates anabolic activities in cells and inactivates mTORC2, thus limiting further Akt activation. In the thymus, Treg development is enhanced by mutating the p110d catalytic subunit of PI3K (Patton et al., 2006) and it is repressed by forced expression of a constitutively active Akt (Haxhinasto et al., 2008), demonstrating a negative role of the PI3K axis on tTreg development. However, deletion of mTOR (thus inactivating both mTORC1 and 2) or individual deletion of mTORC1 or 2 in T cells does not alter thymic development (Delgoffe et al., 2009), suggesting that the negative effect of PI3K and Akt on tTreg development is mTOR independent and mainly due to their role in Foxo1 inactivation.

In the periphery, this axis controls peripheral Treg (pTreg) generation. Similar to the thymus, Foxp3 induction is favored after T cell activation in the presence of pharmacological inhibitors of PI3K (Sauer et al., 2008). However, distinct from tTregs, pTreg generation is significantly impacted by mTOR signaling. mTOR-deficient T cells exhibited mild proliferative defects, failed to express effector cytokines, and defaulted to Foxp3 induction after TCR activation. Inhibition of both mTORC1 and 2 was required for this effect (Delgoffe et al., 2011). Activation of PI3K is naturally antagonized by

phosphatase and tensin homolog (PTEN). PTEN expression is progressively inhibited by stronger TCR stimulation, permitting efficient T cell activation and effector differentiation, an effect mediated by interleukin-2-inducible T-cell kinase (Itk) (Gomez-Rodriguez et al., 2014). Thus, T cells with Itk deficiency fail to down regulate PTEN after activation and favor Foxp3 induction over Th17 differentiation. Similarly, loss of tuberous sclerosis 1 (TSC1), an inhibitor of mTORC1, results in excessive IL-17 production, defective pTreg induction, and severe chemical induced colitis (Jin et al., 2013). Lastly, CD5 was found to block PI3K during pTreg induction, making pTregs refractory to destabilization (Henderson et al., 2015). Together, these data support the notion that PI3K and mTOR activity in mature T cells critically controls the bifurcation between effector verses pTreg cell fates.

In committed Tregs, the PI3K-Akt-mTOR signaling axis continues to be repressed by high expression of PTEN. tTregs constitutively express high level of neuropilin (Weiss et al., 2012; Yadav et al., 2012), which directly binds PTEN and blocks Akt activation during immunological challenge (Delgoffe et al., 2013). Treg specific deletion of PTEN disrupted Treg homeostasis, function, and stability (Huynh et al., 2015; Shrestha et al., 2015). These PTEN-deficient Tregs lost both Foxp3 and CD25 expression but had a significant increase of mTORC2, but not mTORC1 activities. Additional deletion of mTORC2 in Tregs largely rescues the phenotype in mice with Treg-specific deletion of PTEN, demonstrating the normal function of PTEN in mature Tregs is to keep mTORC2 in check. In fact, intact mTORC1 function is required for Treg function because mice with selective deletion of mTORC1 in Tregs die of multi-organ autoimmune diseases similar to Foxp3-deficient mice (Zeng et al., 2013).

Mechanistically, mTOR is found to control Treg function in part by regulating metabolic programming. T cells rely on mitochondrial oxidative phosphorylation at rest and switch to glycolysis after activation, a process essential for effector T cell differentiation (Wang and Green, 2012). In contrast, Tregs preferentially use oxidative metabolism even after activation. An emerging concept is that metabolic input can also dictate T cell fate decision (Wang and Green, 2012). PTEN-deficient Tregs show exaggerated glycolysis that is thought to contribute to Treg instability (Huynh et al., 2015; Shrestha et al., 2015). Additionally, functional defects in mTORC1-deficient Tregs are associated with disrupted lipid biosynthesis (Zeng et al., 2013). Thus, the impact of PI3K-Akt-mTOR axis on mature Treg function is far from black and white, while excessive activation of this pathway is clearly detrimental to Treg function as seen in PTEN-deficient Tregs, complete blockade of PI3K impairs Treg function as well (Patton et al., 2006; Patton et al., 2011).

Epigenome: a foundation for Treg stability

Treg lineage commitment and maintenance is ultimately secured by their epigenetic traits, which are governed by three complementary elements: histone modification, DNA methylation, and transcription factor binding (Huehn and Beyer, 2015; Morikawa and Sakaguchi, 2014). Foxp3 binds to many histone-modifying proteins such as TIP60, Histone deacetylases (HDACs), p300, and Enhancer of zeste homolog 2 (Ezh2) to maintain epigenome stability. It is worth noting that Foxp3-mediated epigenetic changes lead to mostly gene repression, rather than activation, which is dependent on histone methyltransferase Ezh2 (Arvey et al., 2014; DuPage et al., 2015). This genome wide repression is especially important for maintaining the

Treg lineage under inflammatory conditions when activation of effector molecules normally expressed by conventional T cell need to be repressed in Tregs (Morikawa et al., 2014). While Ezh2-deficient Tregs are phenotypically normal and have unaltered suppressive function *in vitro*, they lose Foxp3 expression after activation and are unable to control immune responses *in vivo*. Thus, antigen activation poses a threat to Treg stability and Tregs have intrinsic signaling and epigenetic mechanisms to safeguard their lineage stability.

Manipulating Tregs to treat autoimmune diseases

Elucidating the basic mechanisms underlying Treg biology is the key to manipulating these cells for therapeutic benefit. Changing the balance between effector cells and Tregs is a promising avenue to restore immune homeostasis and treat autoimmune diseases. Experimentally, all the critical elements in Treg biology described above have been targeted for the purpose of manipulating the balance between Tregs and effector cells and some of these approaches are being actively evaluated in the clinic.

Targeting TCR, CD28, and IL-2 triad

Although both Tregs and effector T cells express TCR and the associated CD3 complex, monoclonal antibodies to CD3 can tip the balance in favor of Tregs and induce long-lasting remission of type 1 diabetes in mouse models (Chatenoud et al., 1994). This change of Treg to effector T cell balance is due to higher resistance of Tregs to anti-CD3 induced cell death as well as increased induction of pTregs in the periphery (Belghith et al., 2003; Penaranda et al., 2011). Interestingly, delayed treatment with

anti-CD3 reduced effector T cells and increased the proportion of Tregs in a mouse model of heart transplantation, resulting in long-term graft survival (Goto et al., 2013). In humans, anti-CD3 antibodies induce the outgrowth of $FOXP3^{+}CD8^{+}$ Tregs *in vitro* and increase IL-10 in the serum *in vivo* (Bisikirska et al., 2005). These encouraging preclinical findings have led to clinical trials with promising results (Herold et al., 2013; Herold et al., 2002; Keymeulen et al., 2005; Lebastchi et al., 2013; Vudattu and Herold, 2014). In type 1 diabetes, anti-CD3 treatment improves control of the disease and beta cell function during the first year after onset (Herold et al., 2002; Keymeulen et al., 2005). However, this therapy does not have efficacy for all patients (Herold et al., 2013) or in patients with long-standing disease (Lebastchi et al., 2013).

Targeting CD28 using CTLA4Ig is also effective in changing the Treg to effector T cell balance to prevent immune activation. Although Treg development and peripheral homeostasis depend on CD28, effector T cell differentiation is more sensitive to CTLA4Ig-mediated CD28 blockade; thus, a low dose of CTLA4Ig can block effector differentiation with minimal impact on Tregs (Tang et al., 2004a). This is also observed in kidney transplant patients treated with belatacept, a high affinity variant of humanized CTLA4Ig (Bluestone et al., 2008). Currently, CTLA4Ig has been approved by the Food and Drug Administration for the treatment of rheumatoid arthritis and for the prevention of kidney transplant rejection (Bluestone et al., 2006; Wojciechowski and Vincenti, 2012). Selectively targeting pathogenic effector cells may be particularly effective for restoring immune tolerance, especially when the pathology arises as a consequence of effector resistance to regulation (Schneider et al., 2008). In this regard, a CD2-targetting fusion protein, alefacept, has been recently shown to deplete effector T cells

while preserving Tregs in type 1 diabetes patients (Rigby et al., 2015). It is worth mentioning that a form of agonist anti-CD28 was shown to selectively increase Tregs and prevent experimental allergic encephalitis, a model of multiple sclerosis (MS) (Beyersdorf et al., 2005). When evaluated in a phase 1 clinical trial, TGN1412, the humanized agonist anti-CD28 induced pan T cell activation and severe cytokine storm in healthy volunteers (Suntharalingam et al., 2006). Therefore, the potential impact on effector cells should be carefully considered when developing drugs that stimulate TCR and CD28. Alternatively, antagonistic antibodies may selectively preserve Tregs depending on the dosing (Haanstra et al., 2015).

Owning to their constitutive expression of the high affinity IL-2 receptor and distinct biochemical wiring, Tregs preferentially respond to low-dose IL-2 therapy. This therapy is effective in preventing and reversing type 1 diabetes in mouse models (Grinberg-Bleyer et al., 2010; Tang et al., 2008). Low-dose IL-2 therapy has been effective in increasing Tregs in type 1 diabetes (Hartemann et al., 2013; Yu et al., 2015), GvHD (Matsuoka et al., 2013), and alopecia areata (Castela et al., 2014). In HCV-induced vasculitis, Tregs were induced by IL-2 therapy and 8 out of 10 patients showed clinical improvement (Saadoun et al., 2011). Thus, IL-2 therapy is a promising avenue for increasing Tregs and improving clinical outcomes for patients with autoimmune disease.

However, since many cell types can respond to IL-2, one concern with IL-2 therapy is its Treg selectivity. For example, eosinophilia was observed in patients on IL-2 therapy, and in mouse models, it was found to be mediated by the CD25-expressing type 2 innate-lymphoid cells (Van Gool et al., 2014). Increasing IL-2 dose in a mouse

model of type 1 diabetes led to an increase of Natural Killer (NK) cell and cytotoxic CD8 T cells and exacerbation of diabetes (Tang et al., 2008). Quantitative measurement of IL-2 sensitivity of various cell types in human blood showed that Tregs were most responsive followed by $CD56^{hi}$ NK cells and memory T cells (Tang, 2015; Yu et al., 2015). Acute Treg depletion in mice (Gasteiger et al., 2013a; Gasteiger et al., 2013b; Sitrin et al., 2013) led to an increase in NK cells expressing cytotoxic effector molecules. Interestingly, this did not lead to an increase in NK killing of autologous cells, suggesting that NK activation does not contribute to the fatal autoimmunity after Treg depletion (Gasteiger et al., 2013b). Similarly, anti-CD25 therapy led to a reduction of Tregs in patients with MS, which corresponded with increases of serum IL-2 and $CD56^{hi}$ NK cells, but dramatic disease protection (Gold et al., 2013; Wynn et al., 2010). *In vitro* analysis suggests that the $CD56^{hi}$ NK cells may substitute the function of Tregs and suppress immune responses by killing activated effector cells (Jiang et al., 2011). Thus, the rise of $CD56^{hi}$ NK cells after IL-2 therapy may actually be beneficial rather than problematic. Nonetheless, ongoing efforts are devoted to improving the safety of IL-2 therapy. One approach to more selectively target Tregs is to mutate the IL-2 molecule to make its binding to its receptor CD25 dependent, which has shown efficacy in a Lewis rat model of MS (Weishaupt et al., 2015).

Targeting PI3K-Akt-mTOR axis

A myriad of inhibitors have been developed to target PI3K-Akt-mTOR pathways with the goal of inducing immunosuppression and as therapies for cancer. The most extensively studied inhibitor in the context of Tregs is rapamycin. Initially, rapamycin was thought to be a specific mTORC1 inhibitor but was later found to inhibit both

mTORC1 and 2 when used at high concentrations or with prolonged exposure. As discussed above, ablation of both mTORC1 and 2 are required for the preferential induction of pTregs, and mature Treg function critically depends on mTORC1. In culture, Tregs are more resistant to rapamycin-mediated growth inhibition, thus, rapamycin has been a favored additive to Treg expansion cultures to increase their purity (Battaglia et al., 2005). However, rapamycin does not expand Tregs and has clearly been shown to retard the growth of Tregs *in vitro* and *in vivo* (Hippen et al., 2011; Wang et al., 2011). In the clinic, rapamycin has been used in transplant recipients as an alternative immunosuppressive agent to the widely used calcineurin inhibitors (CNI). Converting patients from CNI to rapamycin or its analogs has been associated with a rise of Tregs in circulation. However, it is not clear if this effect is mainly a result of decreased use of CNI, which are clearly inhibitory to Tregs, or a direct effect of rapamycin. In type 1 diabetes, mouse studies found that rapamycin and IL-2 combination therapy prevented diabetes (Rabinovitch et al., 2002). In patients, however, this treatment led to a transient worsening of beta cell function and increased NK cells and eosinophils despite the dramatic rise in Tregs (Long et al., 2012). The negative impact of this regimen in patients was attributed to a direct effect of rapamycin on beta cells. Thus, the effect of rapamycin can be seen on multiple immune and non-immune cells, and its utility in autoimmune diseases is yet to be determined. Additionally, findings from genetic ablation studies in mice suggest that the selective targeting of mTORC2 would be more effective for tipping the balance towards Tregs.

Targeting epigenetic regulation

Although epigenetic programming is important for safeguarding Treg lineage identity, it is also dynamically regulated, providing an opportunity for pharmacological manipulations. Histone acetylation contributes epigenetic regulation and the process is balanced by the histone acetyltransferases (HATs) and HDACs. HDAC inhibitors have been extensively explored as anti-inflammatory and immunosuppressive agents. Particularly, inhibition of certain HDACs has been shown to selectively enhance Tregs, although these effects are likely more complicated than just histone acetylation because HDACs have many other cellular targets (Hancock et al., 2012). Ezh2-mediated repression is essential for Treg stability during antigenic challenge, suggesting that preserving and enhancing Ezh2 function would have an impact for promoting tolerance in the face of autoimmune diseases and inflammation. Much of the pharmacological development targeting Ezh2 focuses on inhibiting the enzyme in cancer cells with the added benefit of destabilizing Tregs. The activity of Ezh2 is naturally opposed by the histone demethylase Jmjd3 and UTX. Ablation of Jmjd3 in T cells inhibits Th1 and Th17 differentiation and preserves Tregs under Th1 polarizing conditions (Li et al., 2014a; Liu et al., 2015). Thus targeting Jmjd3/UTX pathway may be effective for promoting Treg stability.

Achieving antigen-specific tolerance

Research in animal models shows that antigen-specific Tregs are more effective for controlling organ-specific autoimmune diseases and transplantation rejection when compared to polyclonal Tregs (Lee et al., 2014; Tang et al., 2004b; Tarbell et al., 2004). A long-term global increase in Tregs may impair effective immune surveillance against

infections and malignancies; therefore, antigen-specific therapies are more effective and safer for organ-specific autoimmune diseases. Self-antigens coupled to killed splenocytes or erythrocytes via chemical crosslinking can inactivate self-reactive effector cells and induce expansion of antigen-specific Tregs in mouse models of MS, type 1 diabetes, and transplant rejection (Fife and Bluestone, 2008; Luo et al., 2008; Miller et al., 2007). These pioneering studies are just beginning to be translated into the clinic (Lutterotti et al., 2013). Various newer experimental approaches have been explored to increase antigen-specific Tregs. For example, apoptotic cells pulsed with peptide have been described to have therapeutic effect in both experimental allergic encephalitis and type 1 diabetes by producing TGFβ and inducing antigen-specific pTregs *in vivo* (Kasagi et al., 2014). Additionally, CD45 ligation on Tregs resulted in increased antigen-specific Treg-DC interactions and selective expansion of antigen-specific Tregs (Camirand et al., 2014). Synthetic nanoparticles represent an exciting new therapeutic platform to achieve antigen-specific manipulation of the immune system (Irvine et al., 2015).

Treg cell therapy

Infusion of Tregs is a direct approach to selectively increase Tregs. Several phase 1 clinical trials of Treg cell therapy for the prevention of GvHD (Brunstein et al., 2010; Di Ianni et al., 2011; Martelli et al., 2014) and one trial in type 1 diabetes have been reported (Marek-Trzonkowska et al., 2012). Currently, more than a dozen Treg cell therapy trials are registered on clinicaltrials.gov website, mostly in GvHD and solid organ transplantations using polyclonal Tregs. While it is feasible to produce large numbers of alloantigen-reactive Tregs by selective stimulation using allogeneic antigen

presenting cells (Putnam et al., 2013), large-scale manufacturing of tissue antigen-specific Tregs for autoimmunity is far more challenging because of their low precursor frequency and the tendency of Tregs to destabilize with repeated *in vitro* stimulation in an attempt to expand them (Hippen et al., 2011; Hoffmann et al., 2006). New technology using chimeric antigen receptor (CAR) engineered T cells is promising for cancer immunotherapy (Themeli et al., 2015), and may have applications for engineering antigen-specific Tregs to combat autoimmune disease. Indeed, engineered Tregs do have utility in mouse models of autoimmunity (Elinav et al., 2009; Elinav et al., 2008; Fransson et al., 2012; Wright et al., 2009). In addition to therapeutic development, CAR-engineering of Tregs also offers an opportunity for investigating fundamental biology of Tregs by defining the optimal CAR design to preserve Treg stability and function.

Conclusion and future prospects

In the past several years, we have gained deeper mechanistic understanding of the molecular control of Treg development, maintenance, and function thanks to genetic tools in mouse models. These discoveries are instrumental for the development of better targeted therapies for alternating the balance between Tregs and effector cells in various disease settings. It is clear that no specific molecule or pathway is uniquely utilized by Tregs and the distinction between Tregs and effector T cells may be quantitative. Tregs may preferentially use a combination of pathways; therefore, combination therapies may be able to more specifically target Tregs with lower and less toxic doses of drugs. Additionally, drugs that are not Treg-specific may be used for *ex vivo* manipulation of purified Tregs to increase their number while preserving their

stability for therapeutic use. In the future, advanced tools for faster and more specific genetic manipulation of human cells (Schumann et al., 2015) will allow us to more directly investigate the critical molecular pathways of human Tregs, such as engineering better CAR Tregs for achieving antigen specific tolerance.

Acknowledgements

The text of the portion entitled "**Targeting Treg signaling for the treatment of autoimmune diseases"** is a reprint of the material as it appears in Current Opinion in Immunology. This work was supported by grants from the National Institutes of Health R01 DK08231 (Q.T.), JDRF grants # 17-2013-549, # 2-SRA-2014-150 and 17-2011-661 and R01 AI046643 (J.B.). The authors would like to thank members of the Tang and Bluestone labs for their helpful discussions and Susanna Cheng for editing of this manuscript.

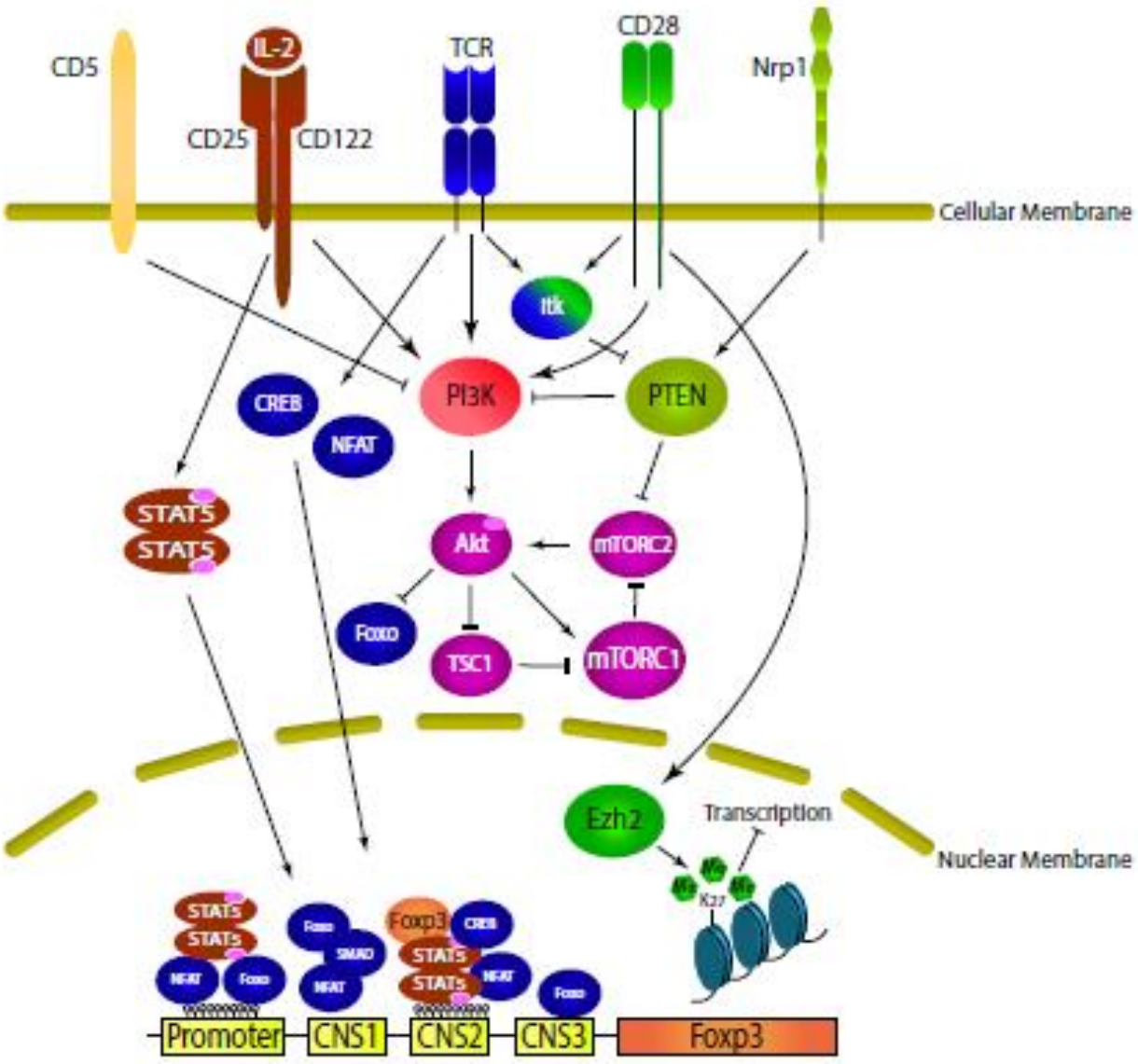


Figure 1: Coordinated signaling from extracellular inputs and their downstream targets in Treg cells.

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