Divergent Effects of OX40L on Regulatory T Cell Phenotype: Implications for Type 1 Diabetes Therapy

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THESIS
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This thesis work is dedicated to my mother, father and brothers who have shown me unwavering support and unconditional love at every stage in my life and have made many sacrifices to better my life. This could not have been accomplished without their encouragement and inspiration. I would also like to dedicate this work to my boyfriend who has always motivated and encouraged me. I could not have made it without his support.

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AD	Autoimmune Disease
APC	Antigen Presenting Cells
APC (stain)	Allo-Phycocyanin
GM-BMDCs	GM-CSF Bone Marrow-Derived Dendritic Cells
CFSE	Carboxyfluorescein Succinimidyl Ester
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
DC	Dendritic Cell
DP	Double Positive
EAE	Experimental Autoimmune Encephalitis
EAT	Experimental Autoimmune Thyroditis
FACS	Fluorescence-Activated Cell Sorting
FITC	Fluoroscein Isothiocyanate
Flt-3	FMS-like Tyrosine Kinase 3 Ligand
Foxp3	Forkhead-box-P3
GAD	Glutamate Decarboxylase
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
IL-	Interleukin
$InsB_{9-23}$	Insulin B-chain
IP	Intra-peritoneal
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked
iTreg	Inducible Regulatory Cell
MHC	Major Histocompatibility Complex
mTORC	Mechanistic Target of Rapamycin Complex
MG	Myasthenia Gravis
NFkB	Nuclear Factor Kappa B
NOD	Non Obese Diabetic

nTreg	Natural Regulatory T cell
PI3K	Phosphatidylinositide 3-Kinase
PLN	Pancreatic Lymph Nodes
SP	Single Positive
SpDC	Splenic Dendritic Cells
T1D	Type 1 Diabetes
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor-Beta
Th	T Helper
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor

1. INTRODUCTION

1.1 T-CELL MEDIATED IMMUNE RESPONSES

Innate and adaptive immune responses are needed to prevent the colonization of body organs by unwanted foreign organisms. Adaptive Immune responses require the activation of naïve T cells by a specific antigen, presented in association with the MHC molecule on the surface of antigen presenting cells (APC) (1). The activation of naïve T cells and their subsequent differentiation into effector cells require two essential signals: Signal one (recognition) requires the engagement of the T cell receptor (TCR) with the peptide:MHC complex on the surface of APC. 2) Signal 2 (verification) requires the interaction of co-stimulatory molecules on the surface of T cells (i.e. CD28) with co-stimulatory molecules on the surface of APC (i.e. CD80 and CD86) (1, 2). Signal 2 ensures that the T cell is responding to a foreign antigen (3). Additionally, signaling by cytokines controls the differentiation of naïve T cells into different subset of $CD4^+$ T cells with distinct functions (1). Currently, 4 main subsets of $CD4^+$ T cells have been recognized, which are mainly defined based on the cytokines they secrete: Th1, Th2, Th17 and regulatory T cells.

Th1 response is trigged by IL-12 and targeted against intracellular pathogens and protozoa. Th1 helper cells are characterized by the production of IL-2 and IFN-γ and the expression of the key Th1 transcription factors, STAT4 and T-bet (4). Th1 helper cells activate macrophages, cytotoxic T lymphocytes as well as $CD4^+$ T cells(5, 6). Excessive activation of Th1 responses has been implicated in the pathogenesis of delayed type hypersensitivity reactions as well as autoimmune diseases, such as Hashimoto's thyroiditis and type 1 diabetes (T1D) (1). In contrast, Th2 response is triggered by IL-2 and is directed against parasitic pathogens (6).

Th2 response produces IL-4, IL-5 and IL-13 and is characterized by the expression of transcription factors, STAT6 and GATAs (4). Th2 helper cells activate naïve B cells and mediate IgE and eosinophilic responses. Excessive activation of Th2 responses is known to produce allergic and hypersensitivity reactions, such atopic dermatitis and asthma (7). Th17, a more recently described subset of $CD4^+$ T cells, mediate responses against extracellular bacteria and fungi. They are triggered by IL-6 and transforming growth factor-β (TGF-β) and characterized by the production of IL-17 (8, 9). Th1, Th2 and Th17 cells are all involved in activating immune responses against foreign pathogens. On the contrary, the fourth main subset of CD4 T cells namely, regulatory T cells (Tregs), have an opposite function; Tregs function to suppress immune responses. TGF-β is required for the differentiation of Tregs from naïve CD4+ T cells, and Tregs produce immunoregulatory T cytokines, such as IL-10 and TGF-β (10-12).

The immune system has developed potent mechanisms to prevent infections and eliminate foreign antigens. However, immune responses need to be tightly regulated (13). Failure to properly regulate immune responses (i.e. Th1/Th2 or Teff/Treg imbalance) could lead to unwanted responses that cause tissue damage and lead to autoimmunity.

1.2 IMMUNOLOGICAL TOLERANCE

Immunological tolerance or self-tolerance, describes a state in which the body is capable of differentiating “self“ from “non-self”. In other words, tolerance

allows the immune system to ignore “self” antigens while generating a response against “non-self” antigens (1). Tolerance can be divided into two subcategories: central and peripheral tolerance. Central tolerance is the main mechanism of tolerance by which developing T and B-lymphocytes learn to discriminate self from non-self in the thymus or bone marrow, respectively (14, 15). Mostly, non-self antigen-specific lymphocytes develop in mature lymphocytes that enter the periphery. Peripheral tolerance offers a key “check-point” to control self-reactive lymphocytes that have escaped central tolerance and entered the periphery (1). Immunological tolerance is crucial for normal physiology. Deficit in central or peripheral tolerance can result in autoimmune disease (16). Different theories have been proposed to describe how immunological tolerance develops, however, the exact mechanism is still not fully understood.

A) Clonal deletion theory: When T cells enter the thymus, they express neither CD4 nor CD8, thus are called double negative (DN) T cells. DN T cells then give rise to double positive T cells (DP) that express both CD4 and CD8. DP cells then undergo positive selection, where they commit either to the $CD8^+$ lineage or $CD4^+$ lineage based on whether they interact with MHC class I or class II, respectively (17). Only cells that have low affinity interaction with MHC molecules get positively selected (18). T cells that react too strongly to the peptide:MHC complex displayed on stromal cells or dendritic cells in the thymus undergo negative selection or “clonal deletion” through the induction of apoptosis (19).

B) Clonal anergy: Anergy was initially described in B cells by Nossal (20). Nossal et al. described that antigen specific B cells were unresponsiveness to

subsequent activation by antigen. In T cells, anergy describes the state when T cell recognizes self-peptide on tissue cells in the absence of co-stimulatory signals (21). If T cells receive signal 1, in the absence of signal 2, they enter a state of anergy, Anergy provides a mechanism to maintain tolerance in the periphery, even if autoreactive T cells escape thymic negative selection (22).

C) Idiotype network theory: This was initially proposed by Jerne in 1973 (23) who was awarded Nobel Prize in Physiology or Medicine in 1984, partly for his proposal of the immune network concept. Jerne suggested that the immune system is compromised of a network of antibodies and lymphocytes that not only recognize foreign antigens, but also recognize and interact with each other (23, 24).

D) Regulatory T cell theory: indicates the presence of a suppressor population (regulatory T cells or Tregs) that functions mainly to suppress or modulate auto-reactive immune responses to maintain self-tolerance. Regulatory T cells are discussed in depth in the following section.

1.3 REGULATORY T CELLS

Regulatory T cells are a recently discovered subset of $CD4^+$ T cells that play a critical role in suppressing immune responses and maintaining self-tolerance (25-27). Tregs were initially identified as $CD4^+$ T cells that express that IL-2 receptor α-chain (CD25) molecule (25). The regulatory or suppressive function of Tregs was initially demonstrated by adoptive transfer studies (11). It was shown that adoptive transfer of T cells depleted of $CD25^+$ into athymic nude mice caused autoimmune

disease. However, co-transfer of $CD4^+CD25^+$ T cells was capable of preventing the development of autoimmunity (11). The suppressive function of Tregs was also later demonstrated in *ex-vivo* suppression experiments where co-culturing Tregs with effector T cells caused a suppression of effector T cell proliferation and cytokine production (26).

Later, it was discovered that Tregs constitutively express the transcription factor, forkhead-box-P3 (Foxp3), which has been described as the master regulator of Treg function(10, 12). The critical role of Foxp3 in Treg development and function is demonstrated by the fact that scurfy mice, which harbor a mutation in the FOXP3 gene, show lymphoproliferative disease with multi-organ autoimmunity(28). Additionally, a mutation in the FOXP3 gene in human patients leads to the development of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), which is characterized by multiple autoimmune diseases, such autoimmune endocrinopathies, eczamateous dermatitis, and type 1 diabetes (T1D) (29).

The critical role of Foxp3 in Treg function is implicated in the function of FOXP3 as both an activator and a repressor of transcription. FOXP3 activates genes that are up-regulated in Tregs (CD25, CTLA-4), and suppresses the production of Th1 and Th2 cytokines in Tregs (26). Additionally, FOXP3 has been shown to suppress the production of IL-2 in Tregs via physical interactions with the IL-2 promoter. FOXP3 has been shown to interfere with the interaction between the nuclear factor activator of T cells (NFAT) and the activator protein 1 (AP1), thus repressing the induction of IL-2 transcription (1, 30). Despite the suppression of IL-2

production in Tregs, IL-2 is well known to play a critical role in Tregs function, survival and homeostasis (31, 32).

1.3.1 REGULATORY T CELL SUBSETS: NATURAL VS. ADAPTIVE

There are two main subsets of Tregs: Natural regulatory T cells (nTreg) and adaptive or inducible regulatory T cells (iTregs). nTregs develop in the thymus through high-affinity binding to MHCII:self-peptide complex (33). nTregs are polyclonal as they are capable of recognizing diverse self-antigens (34, 35). Once generated in the thymus, nTregs migrate to the periphery where they function to suppress self-reactive effector T cells (35). nTregs are marked by the expression of high levels of CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and the glucocorticoid-induced TNF receptor family-related protein (GITR) (35, 36). The function of nTregs has been shown to be dependent on activation via TCR/CD3 complex (11). However, one key feature of nTregs is hyporesponsiveness (low proliferation and low cytokine production) upon TCR-stimulation(11, 26). Interestingly, Tregs hyporesonsiveness can be overcome with the addition of high levels of IL-2 (26). Although TCR activation has been shown to be critical for the suppressive function of nTreg, once activated, nTreg can mount antigen non-specific responses (26, 37). As discussed above, nTregs also produce only minimal amounts of IL-2, due to the suppression of IL-2 production by FOXP3.

iTregs on the other hand develop in the periphery from classical T cell subsets under certain conditions of antigenic stimulation (35). Unlike nTregs, iTregs have variable expression of CD25 (35). Evidence for the induction of Tregs from conventional T cells came from adoptive transfer studies of $CD4^{+}CD25^{-}$ T cells into

mice devoid of nTregs. The transferred $CD4^+CD25^-$ T cells were capable of converting into $CD4^+CD25^+$T cells upon homeostatic proliferation. The converted $CD4^+CD25^+$T cells were shown to have suppressive function, equivalent to nTregs (38). Another study showed that induction of $CD4^+CD25^+Foxp3^+$ Tregs occurred upon adoptive transfer of naive antigen-specific CD4(+) T cells into OVA-expressing $Rag^{-/-}$ mice, which develop severe autoimmune conditions resembling gaft-versus-host disease (39). Generation of $CD4^+CD25^+Foxp3^+$ Tregs occurred even in the absence of the thymus, was dependent on IL-2 and was associated with recovery from disease (39). Additionally, Chen et al. have shown that TCR-stimulation of naïve T cell in the presence of TGF-β converted them into $Foxp3^+$ Tregs, capable of suppressing inflammatory responses(40). In line with these data, TGF-β has been shown to control the development and function of Tregs, as well as protect against autoimmune diseases (41, 42). TCR stimulation with an α-CD3 antobody in the presence of TGF-β is widely used for the generation of $Foxp3^+$ Tregs from $Foxp3^-$ T cells *in-vitro* (40, 43).

1.3.2 REGULATORY T CELL MARKERS

Apart from Foxp3, CD25 and CTLA-4 (discussed below), other markers have been suggested to be associated with Treg phenotype and function. Two of these markers are the ectonucleoside triphosphate diphosphorhydrolase-1 (CD39) and ecto-5′-nucleotidase (CD73) (44). Expression of both molecules, particularly CD39, has been shown to correlate with Foxp3 expression (45) and has been suggested to identify a highly suppressive subset of Tregs (46).

Another marker that has been recently suggested to describe a highly suppressive subset of Tregs is CD44 (47, 48). CD44 is a cell surface H-CAM (homing-associated cell-adhesion molecule) that plays a role in cell adhesion, migration and interaction (26, 49). CD44 expression has been shown to be correlated with the expression of Foxp3 and has been suggested to mark a highly suppressive subset of $CD4^{+}Foxp3^{+}$ T cells (48, 50). Additionally, Tregs obtained from $CD44^{-/-}$ mice were shown to have impaired suppressive function in *ex-vivo* assays (49). Thus, CD44 has been suggested to be an important marker for purifying Tregs with the highest suppressive capacity(50)

1.3.3 MECHANISM OF REGULATORY T CELL SUPPRESSIVE FUNCTION

Although much has been discovered about Treg function, the exact mechanism by which Treg suppress immune responses is still not fully understood. Some studies have demonstrated, through transwell experiments, that Tregs suppressive function is contact-dependent (51). In these experiments, separation of Tregs and effector T cells (Teff) resulted in an abrogation of suppression by Tregs. Additionally, transfer of Treg supernatant into Teff cultures did not lead to suppression of Teff proliferation (51). Other studies, however, have shown a critical role for immune-regulatory cytokines, such as IL-10 (52) and TGF- β(41, 53, 54), as well cytokine depletion (55), in mediating Treg function. Some of the key proposed mechanism of Treg function are discussed below:

A) Contact-dependent mechanisms of Treg function: Contact-dependent mechanisms of suppression by Tregs require the involvement of markers on the surface of Tregs. One of the most well studied regulatory surface-marker is Cytotoxic

T-Lymphocyte Antigen 4 (CTLA-4), which has been shown to play a critical role in maintaining peripheral tolerance (56). CTLA-4 shares homology with the co-stimulatory receptor CD28, however it binds the B7 ligands (CD80/CD86) with a much higher affinity than CD28 (57). CTLA-4 is constitutively expressed on Tregs and has been demonstrated to play a role in the regulatory/suppressive function of Tregs (58). Blocking of CTLA-4 with an antagonstic monoclonal antibody has been shown to exacerbate autoimmune diabetes (59) and experimental allergic encephalomyelitis (60). Additionally, the loss of CTLA-4 expression on nTregs in conditional knockout mice leads to autoimmune pathologies through the loss of Treg function. CTLA-4 has been suggested to regulate immune responses at 2 levels: 1) by altering Teff function 2) by altering DC function (26). In terms of effector T cells, CTLA-4 can directly suppress effector T cell proliferation/function through ligation to CD80/CD86 on activated T cells (26). The ligation of CTLA-4 on Tregs to CD80/CD86 on activated T cells has been shown to prevent autoimmune diseases (61). Additionally, T cells from B7-deficient mice have been shown to be resistant to suppression by Tregs i*n-vitro* (61). A second mechanism by which CTLA-4 can indirectly inhibit T cell responses is through altering DC function (26). CTLA-4 has been shown to suppress T cell immune responses by inhibiting DC maturation(62). Additionally, Tregs have been shown to form aggregates around DCs and down-regulate CD80/CD86 expression on DC in a CTLA-4-dependent manner (58, 63). Inhibition of DC maturation and down-regulation of CD80/CD86 limit the capacity of DC to stimulate naïve T cells, thus leading to suppression of immune responses (27). Finally, it has been suggested that CTLA-4-Ig promotes the activity of Indoleamine

2,3-dioxygenase (IDO) in DC (26, 64). IDO expression is known to modulate immune responses and inhibit T cell proliferation and function through the depletion of tryptophan in the local tissue microenvironment (64-67).

Another surface molecule that has been implicated in the contact-dependent suppressive function of Tregs is lymphocyte activation gene-3 (LAG-3). LAG-3 is a MHC class II binding CD4 homolog with a negative regulatory function (68-70). Expression of LAG-3 on Tregs has been shown to modulate their suppressive function both *in-vivo* and *in-vitro* (71). Knockout of LAG-3 results in impaired suppressive function of Tregs, while ectopic expression of LAG-3 has been shown to induce regulatory function (71). Further studies have demonstrated that engagement of LAG-3 on Tregs to MHC-II on DCs inhibits DCs maturation and immunostimulatory capacity (72).

B) Cytokine secretion by Tregs: Apart from the contact-dependent mechanisms of Tregs suppressive function, there is some evidence suggesting a critical role for soluble molecules, such as IL-10 and TGF-β in mediating Treg function. TGF-β is well characterized for its role in the induction of inducible Tregs (iTregs) in the periphery (40, 41, 43), however evidence regarding its role in mediating the suppressive function of Treg remains controversial and inconclusive. Three different isoforms of TGF-β have been described (TGF-β1, TGF-β-2 and TGF-β-3). In the immune system, TGF-β-1 is the predominate isoform (26, 73). The critical role of TGF-β in regulating immune responses is evident in TGF-β-I knock-out mice, which develop a lethal lymphoproliferative autoimmune disorder, similar to that of $Foxp3^{-/-}$ mice (74). Alternatively, TGF-β-RI, TGF-β-RII knockout mice or mice

expressing a dominant negative form of the TGF-β-II receptor show multi-organ inflammation and T-cell infiltration, similar to the TGF-β-I KO mice (53, 73, 75). Nevertheless, studies using a TGF-β blocking antibody have failed to demonstrate the role of TGF-β in mediating Treg suppressive function in humans (76, 77). Some groups have proposed that TGF-β is in fact presented on the cell surface of Tregs, and that suppression by Tregs requires the interaction of TGF-β on the surface of Tregs with TGF-βR on the surface of T effector cells (26, 78). Despite the inconclusive evidence of the role of TGF-β in Treg-mediated suppression, it is widely accepted that TGF-β can induce regulatory T cells in the periphery, as discussed above (40, 43, 79, 80). The role of TGF-β in inducing Tregs is of strong clinical implications as it offers a method to generate large numbers of functional Tregs *ex-vivo*, which in turn could be used therapeutically in autoimmune diseases.

Another cytokine that has been well studied and strongly implicated in Treg function is IL-10. IL-10 is an immunoregulatory cytokine with potent anti-inflammatory functions (26). The anti-inflammatory properties of IL-10 are apparent in IL-10 knockout mice, which develop chronic enterocolitis (81). Additionally, mutations in the IL-10 receptor were found in patients with early-onset enterocolitis (82). Nevertheless, similar to TGF-β, the role of IL-10 in Treg-mediated suppression is controversial and not fully understood. Some studies indicate that IL-10 plays an important role in the suppressive function of Tregs (52). However, other studies suggest that $CD4^{+}CD25^{+}$ Tregs do not secrete IL-10 upon stimulation, and that the regulatory function of Tregs is independent of IL-10 (83)Although the role of IL-10 in Treg function is still not well established, IL-10 has a well-established anti-

inflammatory function and has been shown to inhibit the secretion of Th1 cytokines (84). IL-10 has also been shown to down-regulate MHC-II and B7-2 expression on DCs (84, 85). Pre-incubation of DCs with recombinant IL-10 prevents the induction of T cell responses by those DCs (84). Thus, it has been suggested that IL-10 may have a cell- or tissue-specific role in Treg function, but is probably not required for the general function of Tregs (26).

C) Cytokine deprivation: Tregs constitutively express high levels of CD25, a-chain of IL2 receptor. On the other hand, effector T cells, which require IL-2 for their proliferation, express CD25 only upon their activation. Tregs have been showed to suppress the activation of effector T cells by directly inhibiting IL-2 production (30, 86). Another proposed mechanism of Treg-mediated suppression is through the consumption of IL-2. A study by Pandiyan et al. showed that Treg inhibited proliferation and decreased cytokine accumulation in Teff through cytokine-deprivation. Treg induced apoptosis in Teff through the enhanced activation of proapoptotic proteins and suppression of Akt signaling. This Treg induced apoptosis was reversed by the addition of cytokines, notably IL-2 and IL-7. Thus, it was concluded that cytokine-deprivation induced apoptosis is an important mechanism of Treg mediated-suppression (55).

1.4 AUTOIMMUNITY

Autoimmunity is the failure of an organism to recognize its parts as “self” thus leading to an immune response against self-antigens. The concept of autoimmunity

was first presented at the beginning of the 20th century by Paul Ehrlich (1). Ehrlich received a Nobel Prize in Physiology or Medicine in 1908 for his contributions to immunology. Ehrlich described autoimmunity as “horror autotoxicus” where, in normal physiology, the body does not mount a response against its own tissues (87). Now we know that autoimmune responses, kept low and under control, are vital to the development and functioning of the immune system (88). In fact, self-reactive T cells and autoantibodies are detectable in healthy humans (89-91). The presence of immunological tolerance in healthy individuals prevents the progression of this low-level self-reactivity into pathological autoimmunity. There are about 80 different autoimmune diseases (AD) known, with rheumatoid arthritis, multiple sclerosis and type 1 diabetes being some of the most common ones (92). Collectively, AD are among some of the most common diseases affecting about 5-8% (or 14 to over 22 million people) of the US population (93). NIH estimates annual direct health care costs for AD to be in the range of $100 billion (93). AD are more common in women than men, and they are considered to be among the leading causes of death in young and middle aged women (94). Thus, a further understanding of autoimmunity and finding therapeutic targets is of highly clinical relevance.

1.5 TYPE 1 DIABETES

Insulin-dependent diabetes mellitus (IDDM) or type 1 diabetes (T1D) is a T cell-mediated autoimmune disease resulting in islet β-cell destruction, hypoinsulinemia, and severely altered glucose homeostasis.

Hyperglycemia occurring in diabetes is responsible for chronic complications, such as microvascular complications (retinopathy, nephropathy and neuropathy), as well as macrovascular complications (coronary artery disease, stroke and peripheral arterial disease) (95). Diabetic nephropathy, peripheral arterial disease, coronary heart disease, and ischemic stroke are the main causes of morbidity/mortality in diabetic patients representing a major clinical and economic challenge (96). Thus, current treatment in T1D is aimed at preventing the development and/or progression of these complications. Even with intensive management, T1D patients are still at a 10-fold increased risk for developing cardiovascular complications compared to non-diabetic patients (97). Currently, the only treatment option for T1D patients is insulin therapy, which helps maintain normal blood glucose but does not cure the underlying autoimmune responses.

It is widely accepted that T1D develops from the autoimmune destruction of insulin-producing β-cells in the pancreatic islets. The histopathology of T1D is defined by two hallmark findings: 1) the decrease or absence of β-cell mass and 2) insulinitis; increased inflammatory/lymphocytic infiltration of the islets (98, 99), which is believed to be the cause of destruction of β-cells. Although both genetic and environmental factors have been implicated in the pathogenesis of T1D (100), it is still not fully understood what exactly triggers the lymphocytic infiltration and destruction of β-cell. T1D was initially described as a T cell-mediated disease. Both $CD4^+$ and $CD8^+$ T cells have been shown to be involved in the development of T1D (98, 99). Nevertheless, there is strong evidence in the literature suggesting the involvement of

other cell types, such as dendritic cells (101-103), B-cells (104-106) and NK-cells (107-109) in disease pathogenesis.

1.6 IMMUNOMODULATORY THERAPIES IN TYPE 1 DIABETES

Several lines of evidence suggest that a loss of balance between pathogenic immune responses and regulatory responses plays an important role in the pathogenesis of many autoimmune diseases, including T1D. The functional loss of the immunoregulatory mechanisms has been shown to play a role in the development of T1D in the non-obese diabetic (NOD) mouse, a well-established mouse model for T1D (110-113). Regulatory T cells are key players in maintaining self-tolerance. As discussed above, there is strong evidence indicating that the suppressive function of Tregs is crucial for the prevention of autoimmune diseases. Thus, given the importance of Treg function in regulating unwanted autoreactive immune responses, many studies have focused on understanding the role of Tregs in the pathogenesis and treatment for autoimmune diseases, including type 1 diabetes.

The importance of Tregs in T1D is implicated in IPEX patients, who have a mutation in the FOXP3 gene, and develop several autoimmune diseases, including T1D. Furthermore, some studies in T1D have reported a decrease in the number of Tregs (114) and the function of Tregs (115), although other reports indicate normal Treg number and function in T1D patients (116). Nevertheless, studies in NOD mice have demonstrated the importance of Tregs in the prevention of disease. In adoptive transfer studies, the co-transfer of $CD4^+CD25^+$ with CD25-depleted splenocytes into

pre-diabetic NOD mice protected against the development of T1D (110). Additionally, adoptive transfer of *ex-vivo* generated antigen-specific Tregs has been shown to prevent the onset of disease in pre-diabetic mice, as well as reverse diabetes in hyperglycemic mice (113).

In line with these data, many studies have focused on finding therapeutic targets that can modulate Treg number and/or function to prevent T1D. Some of the therapeutic targets included administration of oral insulin, using anti-CD3 monoclonal antibodies, GAD65 and IL-2 therapy.

Induction of oral tolerance was one of the very first concepts proposed to expand Tregs in T1D patients. Pabst describes oral tolerance as "*the state of local and systemic immune unresponsiveness that is induced by oral administration of innocuous antigen such as food proteins*" (117) (abstract section, para 1). The gut-associated-lymphoid-tissue (GALT) is a key player in inducing oral tolerance. GALT allows the recognition and destruction of harmful antigens while inducing anergy towards food proteins and gut flora (118). Thus, it had been proposed earlier that various autoimmune diseases could be suppressed by administering oral antigen (118-120). Further studies in prediabetic NOD mice showed that oral administration of insulin prevented T1D onset (121) and even restored a euglycemic state in diseased animals (122) via the induction of antigen-specific Tregs (123, 124). This led to the initiation of a clinical trial in the relatives of T1D patients (125). Unfortunately, results obtained from human subjects conflicted with the results obtained from mouse studies. Overall, it was concluded that oral insulin did not delay or prevent T1D (125).

Later proposals aimed at using an auto-antigen based vaccine, specifically a glutamic acid decaboxylase 65 (GAD65)-based vaccine. Pre-clinical studies in the NOD mouse model showed that the initiation of insulitis, β -cell destruction and T1D are associated with a pathogenic T-cell response to GAD65 (126). The same study by Kaufman and other studies showed that tolerization to GAD65 induce regulatory T cells (127) and delay and/or prevent T1D onset (126, 128, 129). Based on these preclinical data, a clinical trial with GAD-alum (recombinant human GAD + adjuvant alum) in latent autoimmune diabetes of adults (LADA) was initiated. Initial results were very promising; patients receiving GAD-alum showed controlled C-peptide levels and had higher numbers of $CD4^+CD25^+$ Tregs (130). Although CD25 is a well-recognized marker for Tregs, CD25 is also expressed on activated effector T cells (131); therefore, an increase in $CD4^+CD25^+$ T cells does not necessarily correlate with an increase in Tregs (118). Nevertheless, data from a subsequent larger study concluded that administration of GAD65 did not reduce insulin requirements and did not improve C-peptide levels. Treated patients were also shown to have increased GAD65 autoantibodies (118, 132).

Subsequent trials proposed using an anti-CD3 monoclonal antibody to induce tolerance in T1D. Preclinical studies in the NOD mouse model and other diabetes model showed that treatment with anti-CD3 mAb induced tolerance, reversed hyperglycemia, and even protected from adoptive transfer of diabetes(133-135). Later studies showed that the therapeutic effect of anti-CD3 mAb was also mediated through the induction of TGF-β production by Tregs (111). These preclinical findings led to the initiation of clinical trials with two anti-CD3 antibodies: teplizumab (136,

137) and otelixizumab (138, 139). Both studies showed initial promising results of higher C-peptide levels, lower HbA1c, and lower insulin requirements (136-139). However, these positive outcomes diminished over time (after ~15-18 months) (118). Both studies concluded that the effects are only short term and treatment is more effective in younger patients (136, 139) in patients with higher levels of C-peptide, and in patients who have been recently (<6 wks) diagnosed with T1D (118, 136).

The most recent clinical trial employed a co-treatment with Rapamycin and IL-2 to induce Tregs (140). Rapamycin is known to block activation of the mammalian target of rapamycin complex 1 (mTORC1) (141). Th1 and Th17 cells, but not Tregs, require mTORC1 for survival and growth, therefore, rapamycin has been suggested to specifically target effector T cell populations, mainly Th1 and Th17, without altering Tregs proliferation (140, 142, 143). IL-2 on the hand acts on activated effector T cells and Tregs, both of which express the IL-2 receptor α-chain (144). Impaired IL-2 production and IL-2R signaling have been suggested to play a role in the pathogenesis of T1D (31). IL-2 treatment in NOD mice has been shown to expand $Foxp3^+$ Tregs and prevent diabetes onset (145, 146). Additionally, preclinical studies in NOD mice showed that co-treatment with rapamycin and IL-2 prevented the onset of T1D in NOD mice (147). Based on these findings, a phase I clinical trial was conducted in T1D patients. Surprisingly, rapamycin/IL-2 combination therapy resulted in transient β-cell dysfunction, despite the concomitant induction of Tregs.

Vitamin D has also been suggested to have Treg inducing properties. In animal models, vitamin D has been shown to prevent insulitis (148) reduce disease onset (149) and suppress pro-inflammatory cytokines (150). Based on the pre-clinical

data, a clinical trial was conducted to determine the effects of Vitamin D on β-cell function (151). Unfortunately, the trial was conducted in healthy non-diabetic individuals, thus the results are difficult to interpret. Nevertheless, the study showed that administration of Vitamin D caused a significant increase in the percentage of Tregs (151). Further studies in diabetic patients are needed to confirm the potential beneficial effects of vitamin D on Treg induction and treatment of T1D (118).

1.7 EXPANSION OF REGULATORY T CELLS BY GM-CSF-BONE MARROW DERIVED DENDRITIC CELLS (GM-BMDCs)

Studies in our laboratory have shown that treatment with low-dose GM-CSF was sufficient to prevent the development of T1D in NOD mice (152), Experimental Autoimmune Thyroditis in CBA mice (153, 154) and Experimental Autoimmune Mysthenia Gravis in C57BL mice (155). This GM-CSF induced suppression was primarily mediated through the mobilization of $CD11c^{+}CD8a^{-}$DCs, which in turn caused the expansion of Tregs (154). Interestingly, adoptive transfer of DCs from NOD.*scid* mice treated with GM-CSF into NOD mice caused a significant increase in Tregs and delayed the onset of T1D, compared to adoptive transfer of DCs from untreated mice (152). Surprisingly, when SpDCs were cultured with GM-CSF *ex-vivo* and then adoptively transferred into NOD mice, they failed to expand Tregs or protect against disease (152). These findings suggested that GM-CSF might be acting on DC precursors present in the bone marrow.

To directly address this question, we cultured bone marrow from CBA mice with GM-CSF (GM-BMDCs) *ex-vivo*. After 7 days of GM-CSF culture, GM-BMDCs

were co-cultured with total $CD4^+$ T cells to determine their capacity to expand or induce $Foxp3^+$ Tregs. GM-BMDCs caused significant proliferation of $Foxp3^+$ Tregs compared to SpDCs controls (156). Interestingly, this Treg proliferation occurred in the absence of TCR stimulation or any exogenous antigen. This was further confirmed by using GM-BMDCs from MHC-II-/-, which were capable of expanding Tregs in the absence of canonical TCR stimulation, but required exogenous IL-2. These data suggested that the GM-BMDC-induced Treg proliferation was TCR-independent, however MHC-II:TCR interaction between GM-BMDCs and $CD4^+CD25^-$ effector T cells was required for IL-2 production (156). Subsequently, in transwell experiments, we showed that GM-BMDC-induced Treg expansion required contact between GM-BMDCs and Tregs. Therefore, GM-BMDC-induced Treg expansion was 1) TCR-independent, 2) required IL-2 (supplied exogenously or produced by Teff), 3) contact-dependent.

To identify surface molecules that were involved in GM-BMDC-induced Treg expansion, we characterized the expression of different co-stimulatory molecules on GM-BMDCs, in comparison to SpDCs, which are incapable of expanding Tregs. Interestingly, we noted that OX40L was highly expressed on GM-BMDCs, but not on SpDCs (157). OX40/OX40L (discussed below) has been shown to promote effector T cell survival and expansion (158, 159). Alternatively, the role of OX40L/OX40 signaling in Treg proliferation and function is controversial and poorly understood. Some studies have suggested that blocking OX40L-OX40 pathway can diminish different autoimmune diseases (160-162), while others suggest that OX40 plays a critical role in Treg homeostasis and suppression of EAE and colitis (163, 164). We

further investigated the role of OX40L/OX40 signaling in Treg expansion and found that the addition of OX40L blocking antibody in the GM-BMDC-CD4+ co-cultured abrogated Foxp3+ Treg proliferation in a dose-dependent manner. Similarly, we sorted GM-BMDCs into OX40L+ and OX40L- subsets and noted that only the OX40L+ GM-BMDCs were capable of expanding Tregs.

Surprisingly, the addition of an OX40 agonist alone in the presence of SpDCs could not expand Tregs. This suggested that other molecules were required for Treg proliferation by GM-BMDCs. Notch signaling has been shown to be involved peripheral T-cell differentiation and proliferation (165, 166). APCs over-expressing Notch family ligand Jag-1, and hematopoietic progenitors expressing Jag-2 can activate Tregs (166, 167). We found higher expression of Jagged-1 on the surface of GM-BMDCs compared to SpDCs (157). Further studies using sorted GM-BMDCs showed that only $OX40L^{+}Jag\text{-}1^{-}$ GM-BMDCs were capable of expanding Tregs, compared to $OX40L^{+}Jag\text{-}1^{-}$ GM-BMDCs or SpDCs. Furthermore, addition of a blocking antibody to either Jag-1 or OX40L abrogated Treg expansion. Blockade of both ligands simultaneously abrogated Treg to a greater extent (157). Therefore, these findings indicated that OX40L/Jag-1 co-signaling is required for TCR-independent Treg proliferation.

1.8 OX40L/OX40 SIGNALING IN T-CELL FUNCTION AND IMMUNITY

As described above, T cell activation requires signaling through TCR (signal 1) and co-stimulatory molecules (signal 2) (1). The interaction of CD28 with the B7

molecules (CD80/CD86) is one of the most studied and well-defined co-stimulatory pathways. However, other co-stimulatory signals can also lead to the activation of T cells. OX40 is a member of the tumor necrosis factor (TNF) receptor superfamily. OX40 was initially described in 1987 as an activation marker on T cells (168), however it was later shown to be an important co-stimulatory molecule (159). OX40 is expressed on activated T cells upon TCR ligation, and as opposed to other members of TNFR superfamily, it is absent on naïve or memory T cells (158). The ligand for OX40 is OX40L, a type II glycoprotein with a 23 amino acid cytoplasmic tail and a 133 amino acid extracellular domain (158). OX40L was initially identified as glycoprotein (gp34) expressed on human T-cell leukemia virus type I (HTLV-I)-transformed T cells (169). Gp34 was later found to bind OX40 and thus was identified as OX40L (158, 170). OX40L was originally thought to be primarily expressed on APCs (171, 172), however, more recent studies have shown that OX40L is expressed on vascular endothelial cells (173), activated natural killer cells (174), and mast cells (175). Current evidence suggests that OX40L can bind no receptor other than OX40. Similarly, OX40 can only be ligated by OX40L (176).

1.8.1 ROLE IN EFFECTOR T CELL FUNCTION

In terms of function, OX40/OX40L interaction has been strongly implicated in the proliferation and survival of T cells (158). OX40 is expressed on T cells following TCR ligation, and usually reaches a peak around 48 hrs, although there are mixed results regarding the timing of expression (158, 177). OX40L on the other hand is expressed on APCs following LPS or CD40 stimulation and reaches a maximum level at 48-72 following stimulation (171, 172, 177). Thus, OX40/OX40L interaction seems

to occur around 2-3 days following antigen presentation (158, 177). The critical role of OX40/OX40L signaling in T cell proliferation is evident in $OX40^{-/-}$ mice, in which T cells show decreased proliferation 5-6 days following antigen encounter, and a significant decrease in survival by 12-13 days, despite a normal initial proliferation during the first 48 hrs (177).

OX40, similar to other TNFR family molecules, is known to signal through the TNF Receptor Associated Factors (TRAF) adaptor proteins (178). TRAF family members consist of 7 different adaptor proteins (TRAF1–TRAF7) (179). OX40 signaling has been linked to transduction through TRAF 2, TRAF3, TRAF5 and TRAF6 (158, 180). OX40 signaling through TRAF proteins has been shown to lead to the activation of both the canonical and non-canonical NF-κB pathways (158, 159). It has been suggested that in addition to signaling as a co-stimulatory molecule, OX40 can signal alone independent of TCR stimulation (158, 181). In the context of antigen-presentation, TCR signaling leads to the activation of PI3K and Akt. Subsequently, OX40L/OX40 ligation leads to the recruitment of TRAF2, TRAF3 and TRAF5 (158, 178). Recruitment of TRAF molecules leads to a signalosome formation with PI3K, Akt. This signalosome then leads to the activation of the NF-κB canonical pathway and activation of p50 and RelA (Fig. 1) (158). Thus, the TCR-driven Akt activation, along with the OX40-driven NF-κB activation, provides a strong signal for cell proliferation, survival and cytokine production (158). However, in the absence of TCR signaling, OX40 can form a signalosome with CARMA1, PKC-θ and TRAF2 leading to NF-κB activation and enhanced cell survival, even in the absence of PI3K and Akt activation (158, 181).

Based on several lines of evidence indicating a role for OX40 in augmenting $CD4^+$ effector T cell function (162, 177), OX40 has gained strong interest as a therapeutic target for autoimmune diseases as well as cancer. Recent reports have shown that blocking OX40L-OX40 signaling can diminish Experimental Autoimmune Encephalomyelitis (EAE) (160), inflammatory bowel disease (IBD) (161), and T1D (162). OX40 signaling has also been implicated in tumor immunology as OX40 expressing T lymphocytes have been detected in the tumor-microenvironment in melanoma patients (182). Therefore OX40 has been proposed as a potential target in cancer therapy. Treatment with an OX40 agonistic antibody has been shown to enhance antitumor responses in several tumor models, such as melanoma, breast, lung and colon cancer (182-185). Based on the promising preclinical findings, OX40 agonist is currently being tested in clinical trials as a treatment for cancer patients (172, 186).

1.8.2 ROLE IN REGULATORY T CELL

In addition to its co-stimulatory function in effector T cells, OX40 has also been shown to be an important regulator of Tregs proliferation and suppressive function (172, 187-189). As opposed to effector T cells, which express OX40 upon TCR ligation, Tregs have constitutive expression of OX40 (190). Studies with OX40-KO mice show a reduction in the number of $Foxp3^+$ Tregs in younger mice (<8 wks) compared to WT mice (187). Additionally, studies using transgenic mice overexpressing OX40L show increased Treg numbers in both the spleen and the thymus (187, 191), although others have reported that Tregs from OX40L-tg mice have reduced Foxp3 expression but show normal proliferative capacity and survival

(189). Furthermore, ligation of OX40 on Tregs has been shown to block their suppressive function in Graft-versus-host-disease models (190, 191) and in tumor mouse models (192). In this context, several studies have demonstrated an enhanced OX40 expression in sites of inflammation, such as a tumor-microenvironment (191, 193, 194). Thus numerous reports indicate that OX40 ligation on Tregs could enhance immune responses against tumors (192, 195) and may even block the conversion of effector T cells into iTregs (196, 197). A study by Piconese et al. demonstrated that intratumoral injection of an OX40 agonistic antibody could block the generation of iTregs (192). Thus, targeting OX40L-OX40 pathway holds promising results as an effective therapy for cancer patients.

On the contrary to the above evidence suggesting a role for OX40 as a negative regulator of Treg function, accumulating data suggest that OX40 signaling may in fact enhance Treg proliferation and suppressive function (157, 172). OX40L-expressing artificial APCs, preloaded with anti-CD3 and anti-CD28, were shown to drive Treg proliferation from human umbilical cord blood without altering their suppressive function (198). Furthermore, studies have shown that OX40L-OX40 interaction can contribute to Treg survival, proliferation and suppressive function depending on the cytokine milieu in mouse models of colitis (164) and EAE (163). A recent report by Bresson et al. also demonstrated that OX40 ligation can prevent T1D by enhancing antigen-specific immune responses (199). Finally, work from our lab has shown that GM-BMDCs could expand Tregs *ex-vivo* through an OX40L/OX40-dependent, TCR-independent mechanism. Adoptive transfer of $OX40L^{+}$ GM-BMDCs caused Treg expansion *in-vivo* and suppression of EAT (157). Thus, these conflicting

data suggest that OX40L/OX40 signaling may play a complex and dual role in Treg homeostasis and function, and the outcome of this signaling is perhaps dependent on the microenvironment and the presence of other co-stimulatory signals.

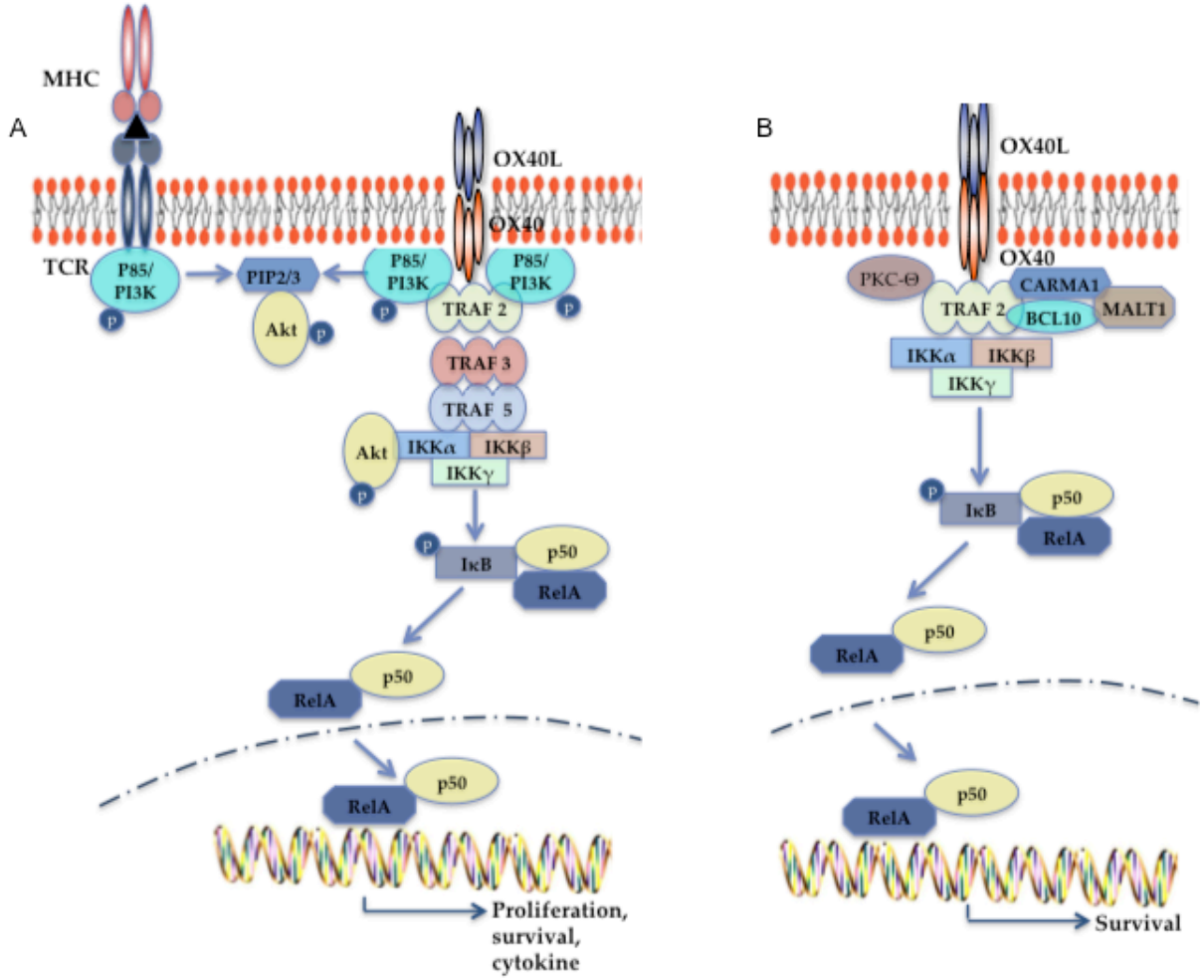

Fig. 1: TCR-dependent and TCR-independent OX40 signaling in T cells: In the presence of TCR signaling, OX40 functions as a co-stimulatory molecule, leading to NF-κB activation, which synergizes with TCR-driven Akt activation to enhance cell proliferation/survival. In the absence of TCR, OX40 can form a signalosome with CARMA1, PKC-θ and TRAF-2, leading to NF-κB activation and cell survival. (source: adopted from Croft, 2010)

1.9 OBJECTIVE OF THIS STUDY

Due to the critical suppressive function of regulatory T cells, recent work in the field of immunology has focused on trying to modulate Treg function as a therapy for autoimmune diseases, including type 1 diabetes as well as transplantation. Although preclinical and clinical studies have proposed various methods to enhance Treg function and/or number, the feasibility of these approaches is still very poor, mainly due to the complex physiology of Tregs.

Tregs have been shown to constitutively express the TNFRS molecule, OX40. OX40L/OX40 signaling has been well described as a co-stimulatory molecule involved in effector T cell proliferation and function, however, its role in Treg function is still not very well understood. Some data suggest that OX40 signaling can inhibit Treg function and suppress autoimmune disease (160-162), while other data show that OX40 can in fact enhance Treg survival and proliferation (157, 163, 164, 199). Previous work from our lab has demonstrated a critical role for OX40L/OX40 signaling for *ex-vivo* expansion of regulatory T cells by GM-CSF-differentiated bone marrow-derived dendritic cells (GM-BMDCs). Thus, the aim of the work presented here is to further investigate the role of OX40L/OX40 signaling in *ex-vivo* and *in-vivo* Treg expansion in the non-obese diabetic (NOD) mouse model of type 1 diabetes. More specifically, the presented work intends to determine, although not conclusively, the following: 1) Whether GM-BMDCs can expand Tregs *ex-vivo* in NOD mice, 2) Whether OX40L/OX40 signaling is critical for GM-BMDC-induced Treg expansion? 3) the effects of *in-vivo* administration of soluble OX40L on Treg phenotype and function as well as disease onset in the NOD mice. In the first part of this study, we establish

the capacity of GM-BMDCs to expand functional Tregs *ex-vivo.* I additionally show that this expansion is dependent on OX40L/OX40 signaling. In the second part of this study, I investigate the effects of soluble OX40L administration on disease onset and Treg phenotype. I show that OX40L has divergent effects based on the age of the animal. Finally, in chapter 5, I explore the potential mechanism responsible for driving the divergent effects of OX40L.

2. MATERIALS AND METHODS

2.1 ANIMALS

Six to eight week old NOD mice and twelve week Balb/c were purchased from the Jackson laboratory. Thymectomized NOD mice were also purchased from the Jackson Laboratory. Thymectomy was performed on 5 or 8 week old NOD mice by the vendor (Surgical Services, Jackson Lab). Foxp3.GFP (C57B6/j background) mice were kindly provided by Dr. Chenthamarakshan Vasu (Department of Surgery, Medical University of South Carolina).

All Mice were housed in the Biological Resources laboratory facility at the University of Illinois (Chicago, IL) and provided food and water *ad libitum*. All animal experiments were approved by the University of Illinois at Chicago animal care and use committee.

6 or 12 week old non diabetic NOD mice were treated i.p. with OX40L once a week over a period of 3 weeks (200ug/mouse/treatment). For experiments with IL-2, 12 wk old mice received 25,000 IU of IL-2(145), following the same treatment regimen described above. Blood glucose was measured using tail vein bleed.

2.2 VARIOUS REAGENTS

Recombinant mouse GM-CSF (PMC2013) and Cell Trace Violet (C34557) were purchased from Invitrogen (Carlsbad, USA). Mouse IL-2 recombinant protein (34-8021) was purchased from eBiosciences (San Diego, CA). Recombinant mouse TGF-β (7666-MB-005) was purchased from R&D Systems (Minneapolis, MN). Soluble Fc-muOX40L was a kindly provided by Dr. Alan Epstein (Department of Pathology, Keck School of Medicine, University of Southern California) (185).

Carboxy Fluorescein Succinimidyl Ester (CFSE) (65-0850); PE-conjugated anti-OX40L (12-5905), anti-OX40 (12-1341), anti-CD25 (12-0251); APC-conjugated anti-OX40L (17-5905), anti-Foxp3 (17-5773), anti-CD8α (17-0081), anti-CD11c (17-0114); eFlour-450 conjugated anti-CD11c (48-0114); FITC conjugated anti-CD8α (11-0081), anti-CD4 (11-0041); anti-mouse CD3 (16-0032) were purchased from eBioscience (San Diego, CA). Pacific Blue-conjugated anti-CD4 (100428) was purchased from Biolegend (San Diego, CA).

2.3 ISOLATION OF DC AND T-CELL POPULATIONS

Bone marrow cells were cultured in complete RPMI medium containing 10% heat-inactivated FBS in the presence of 20ng/ml GM-CSF. Fresh media containing 20ng/mL GM-CSF was added on days 4 and 6. On the eighth day, non-adherent $CD11c^+$ DCs (total GM-BMDCs) or specific sub populations of GM-BMDCs (i.e. $OX40L^+$ or $OX40L^-$) was sorted using a MoFlo flow cytometer (Beckman/Coulter) following staining with appropriate antibodies (CD11c, OX40L). $CD4^+$ cells were purified from the spleens by either using the Mouse CD4+ T Cell Isolation Kit II (130-095-248) from Miltenyi Biotec (San Diego, CA), or sorted using a MoFlo flow cytometer (Beckman/Coulter) following staining with anti-CD4 antibodies.

2.4 *IN-VITRO* CO-CULTURES OF DCs AND T-CELLS:

GM-BMDCs (5×10^4) and $CD11c^+$ SpDCs were cultured with $CD4^+$ T-cells at a ratio of 1:2 for 5 days. For proliferation assays, total $CD4^+$ T-cell were labeled with CFSE at 10uM according to manufacturer's instruction (eBioscience, San Diego, CA)

or CellTrace Violet prior to co-culturing them with DCs. After 5 days of co-culture, cells were fixed/permeabilized, stained with CD4 and Foxp3 and analyzed using a Cyan Flow Cytometer. Each experiment was set up in triplicates for each sample, and was repeated at least 3 times (3 independent experiments).

2.5 INDUCTION OF REGULATORY T CELLS *EX-VIVO*

Splenocytes from NOD mice were stained with CD4 and CD25 antibodies, and then sorted using a MoFlow flow cytometer (Beckman.Coulter). $CD4^+CD25^-$ ($5x10^5$ cells) were cultured in the presence of anti-CD3 (2ug/mL) and TGF-β (3 ng/mL) or anti-CD3 alone (control) for 48 hrs. After that, cells were washed thoroughly to remove any anti-CD3/TGF-β, labeled with CFSE and then co-cultured for 5 days with GM-BMDCs or SpDCs at a ratio of 2:1 ($1 X 10^5$ T cells: $5 X 10^4$ DCs). After 5 days, cells were then stained for CD4, Foxp3 and analyzed for Treg proliferation using Cyan flow cytometer (Beckman/Coulter). Experiment was set up in triplicates for each sample and was repeated at least 2 times.

2.6 ISOLATION OF *IN-VITRO* EXPANDED REGULATORY T CELLS

Total CD4+ T cells were co-cultured with BMDCs for 5 days, as described above. After 5 days, cells from GM-BMDC-CD4+ T cells co-cultures were stained with CD4 and CD25 antibodies. $CD4^+CD25^+$ T cells were sorted using a MoFlo Cell sorter. Purified Tregs were then co-cultured with CFSE-$CD4^+CD25^-$T cells in the suppression assay, described below.

2.7 SUPPRESSION ASSAY

$CD4^+CD25^-$ T cells were isolated from the spleens of NOD mice using a MoFlo sorter (Beckman/Coulter) following staining with anti-CD4 and anti-CD25 antibodies. Cells were then labeled with CFSE and plated in a U-bottom 96-well plate (5 X 10^4 cells/well) in the presence of anti-CD3 (2ug/mL) and splenic APCs. Freshly isolated or BMDC-expanded Tregs were sorted and co-cultured with CFSE-$CD4^+CD25^-$ T effectors at different ratios. After 3 days, cells were fixed, stained with CD4 and Foxp3 and analyzed using a Cyan Flow Cytometer (Beckman/Coulter). Each experiment was set-up in triplicates for each sample, and repeated at least two times.

2.8 *IN-VITRO Treg* CONVERSION ASSAY

$CD25^+CD4^+$ Tregs were isolated from the spleens and PLN of 6 and 12 wk old NOD mice using a MoFlow flow cytometer (Beckman/Coulter) following staining with anti-CD4 and anti-CD25 antibodies. Purified Tregs were then labeled with CFSE. Labeled cells from each age group mice were mixed back with splenocytes or PLN. Cells were cultured in the presence or absence of Cell-Stimulation Cocktail (PMA/Ionomycin) (eBiosciences) and/or IL-2 (10ng/mL) for 24 hrs. After 24 hrs, cells were stained with CD4 and Foxp3, according to the staining protocol described below. Cells were analyzed for Foxp3 expression in the $CFSE^+$ population using a Cyan flow cytometer (Beckman/Coulter). Each experiment was set-up in duplicates for each sample, and repeated two times.

2.9 INTRACELLULAR STAINING

Briefly, at the end of co-culture experiments, T-cells were first stained with Pacific blue labelled anti-mouse CD4 antibody. Then, for intracellular staining, surface stained cells were fixed and permeabilized using a Foxp3/Transcription Factor Staining Buffer Set (Ebioscience, San Diego, CA) and incubated with specified antibodies.

2.10 TETRAMER STAINING

Mouse InsB9-23 MHCII tetramer (peptide sequence:HLVERLYLVAGEEG) was obtained from the NIH Tetramer Core Facility. Single-cell suspensions were prepared from the spleens or pancreatic lymph nodes of 6 or 12 weeks old NOD mice either treated with OX40L alone or untreated. Cells were incubated with the MHCII tetramer in .5% BSA at room temperature for 1 hr. Tetramer labeled cells were then stained with a anti-CD4 antibody. Cells were then analyzed using a Cyan flow cytometer (Beckman/Coulter).

2.11 FLUORSCENCE-ASSOCIATED CELL SORTING (FACS)

Freshly isolated and *ex vivo* cultured cells were washed with PBS-.5% BSA-EDTA. For surface staining, the cells were labeled with specified FITC, PE, APC conjugated antibodies for 30 min. For cell proliferation assays, the cells were labeled with CFSE, fixed, permeabilized and incubated with fluorescent-coupled antibodies for intracellular staining. Stained cells were washed three times and analyzed by Cyan flow cytometer (Beckman/Coulter).

2.12 RNA ISOLATION, DNA SYNTHESIS AND RT^2-PCR

Splenocytes and PLN isolated from either 12 or 6 wk treated or untreated mice were either stimulated with cell stimulation cocktail (eBiosciences) for 6 hrs or left unstimulated. Total RNA from different samples was isolated using RNeasy Mini Kit (Qiagen). An aliquot of each RNA sample was run on a denaturing agarose gel to verify the presence of the 18S and 28S ribosomal RNAs. Total RNA was reverse transcribed using the RT^2 First Strand Kit (Qiagen). RT-PCR was performed using the mouse inflammatory cytokines and receptor RT^2 Profiler PCR Array and RT^2 SYBR Green Mastermix (SABiosciences, CA). PCR was performed on ABI Vii 7 (384-well block) instrument (Applied Biosystems). Data were analyzed using the web-based software provided by the manufacturer (SABioscience, CA). The relative mRNA expressions for each cytokine in stimulated samples were normalized to the mRNA levels in unstimulated samples.

2.14 ADOPTIVE TRANSFER

Three groups of 3 mice each were adoptively transferred, via i.v. injection, with either i) PBS, ii) 1 x 10^6 purified $CD11c^+$ DCs from untreated NOD mice or iii) 1 x 10^6 $CD11c^+$ BMDC purified and sorted from BM cultures. Seven days after the adoptive transfer, mice were sacrificed, and the spleens and lymph nodes were isolated and analyzed for percentage of $Foxp3^+$ Tregs.

2.15 STATISTICAL ANALYSIS

Mean, standard deviation, and statistical significance were calculated using the MS-Excel application software. Statistical significance was determined using the one tailed Students t-test. A *p*-value of ≤ 0.05 was considered significant.

3. EXPANSION OF REGULATORY T CELLS BY BMDCs *EX-VIVO* AND *IN-VIVO* IN NOD MICE: CRITICAL ROLE OF OX40L/OX40 SIGNALING

3.1 INTRODUCTION

Recently, we reported that bone marrow (BM) precursor cells differentiated in the presence of GM-CSF (GM-BMDCs) were able to selectively expand Tregs in co-cultures while spleen derived DCs (SpDCs) could not (156). Further, using GM-BMDCs from MHC-II KO mice, we have shown that Treg expansion occurred in the absence of canonical TCR engagement, but required GM-BMDC-T cell contact and exogenous IL-2 (156). Investigation into expression of cell surface molecules on GM-BMDCs that could provide signals to Tregs revealed significantly higher levels of expression of OX40L as compared to SpDCs. OX40 is constitutively expressed on Tregs, whereas its expression is up-regulated on effector T cells upon TCR ligation. Addition of an OX40L blocking antibody to the GM-BMDC-$CD4^+$ T cell co-culture abrogated expansion of Tregs. This abrogation was reversed upon addition of an OX40 agonist. Adoptive transfer of an OX40L+ subset, but not $OX40L^-$, GM-BMDCs into mice with experimental autoimmune thyroiditis (EAT) caused *in-vivo* expansion of IL-10 secreting Tregs with a concomitant suppression of EAT (157). These results suggested that OX40/OX40L interaction is critical for GM-BMDC induced Treg expansion and *in-vivo* IL-10 production. Earlier reports showing reduced numbers of Tregs in the spleens of $OX40^{-/-}$ mice and increase in Tregs in OX40L over-expressing mice support this notion (187, 191).

Thus, in this part of the study, we confirm the critical role of OX40L/OX40 signaling in GM-BMDC-induced Treg expansion *ex-vivo* in NOD mice, a well-established model for T1D. We additionally show that adoptive transfer of GM-BMDC into NOD mice expands Tregs *in-vivo*.

3.2 EXPERIMENTAL RESULTS:

3.2.1 GM-BMDCs FROM NOD MICE EXPRESS OX40L AND ARE CAPABLE OF EXPANDING Tregs *EX-VIVO*

In our earlier studies, we have shown that GM-BMDCs-mediated Treg expansion is dependent on OX40/OX40L signaling in CBA mice (157). To determine if this phenomenon is consistent in the NOD mice, we cultured bone marrow from NOD mice with GM-CSF to determine the expression of OX40L on GM-CSF-differentiated BMDCs. Consistent with our previous reports, about 30 % of the $CD11c^+$ BMDCs were $OX40L^+$ after 7 days of GM-CSF cultures (Fig. 2).

To test whether GM-BMDCs can expand Tregs *ex-vivo* in NOD mice, we co-cultured GM-CSF differentiated BMDCs with naïve $CD4^+$ T cells from NOD mice. Briefly, $CD4^+$ T cells were purified from the spleens of NOD mice, labeled with CFSE, and then co-cultured with 7-day old GM-BMDCs at a 2:1 ratio, in the absence of any exogenous antigen. After 5 days, cells were analyzed for proliferation of $Foxp3^+$ Tregs (Fig. 3). Consistent with our previous findings, only GM-BMDCs, and not SpDCs (negative control), were capable of inducing the proliferation of $Foxp3^+$ Tregs (11.3% ± 1 vs. 1.3% ± 0.4; p-value = 1.5E-06).

Additionally, we further tested the full capacity of GM-BMDCs to induce Treg expansion by co-culturing GM-BMDCs with *ex-vivo* generated Tregs (iTregs). iTregs were generated by sorting $CD4^+CD25^-$ from the spleen of NOD mice, then culturing them in the presence of α-CD3 and TGF-β to induce $Foxp3^+$ Tregs, as previously reported (40, 43). After 48 hrs, a fraction of the cells were stained with CD4 and

Foxp3 to confirm the presence of a $Foxp3^+$ T cell population. About 50% of the cells were converted and expressed Foxp3 (Fig. 4 upper panel). The cells were then labeled with CFSE and co-cultured with GM-BMDCs or SpDCs for 5 days. After 5 days, iTregs showed robust proliferation only when co-cultured with GM-BMDCs (35 ± 3 %), but not when co-cultured with SpDCs (7.13 ± 0.7%; p-value = 3.5E-05) (Fig. 4 lower panel). These data confirm that GM-BMDCs are capable of expanding nTregs, isolated from the spleens of NOD mice, as well as iTregs generated *ex-vivo* with TGF-β.

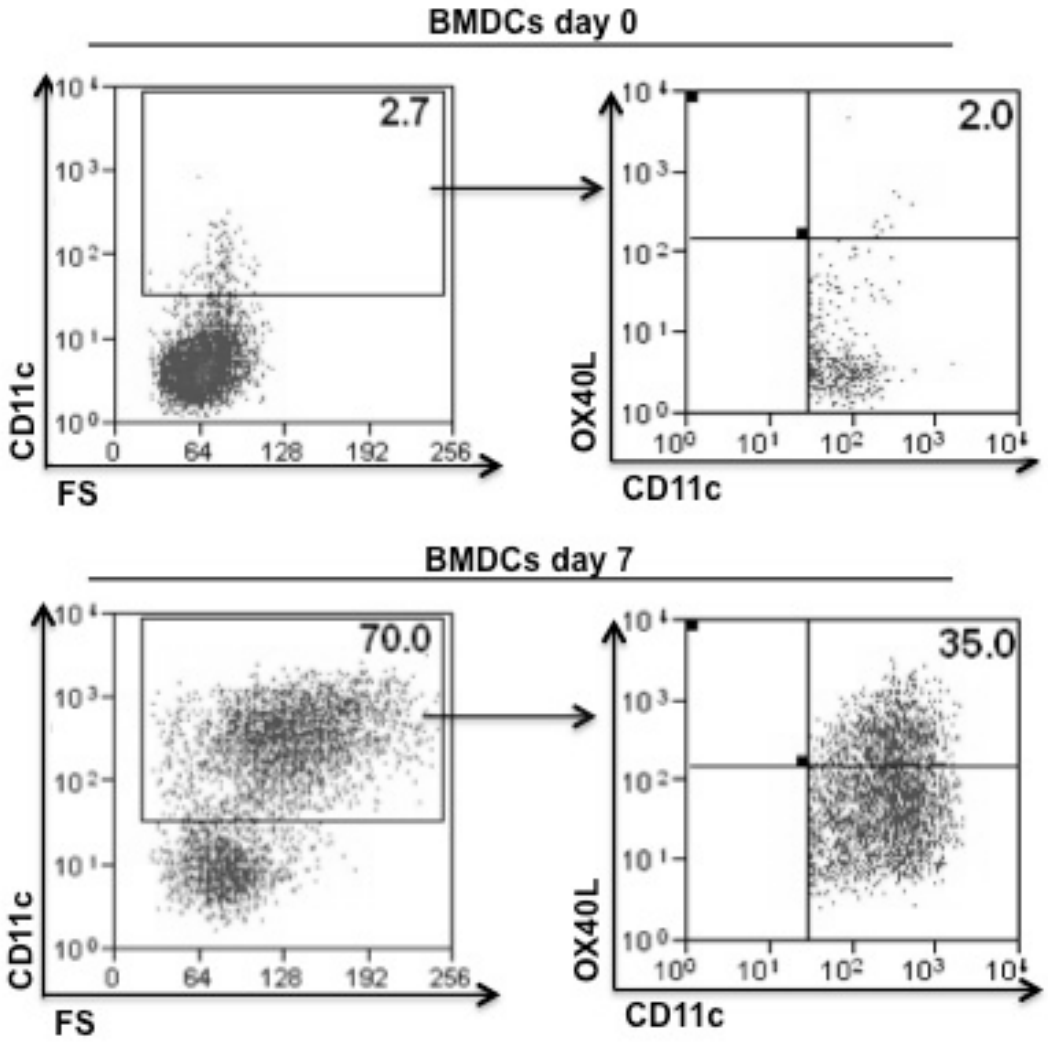

Fig 2. GM-CSF-Differentiated BMDCs Derived from NOD mice express OX40L: BMDCs were isolated from the femur of NOD mice and cultured with GM-CSF (20ng/mL) for 7 days. GM-BMDCs were analyzed for CD11c and OX40L expression at day 0 (upper panel) and day 7 (lower panel) of GM-CSF culture. After 7 days of GM-CSF culture, about 35% of CD11c+ BMDCs expressed OX40L (lower right).

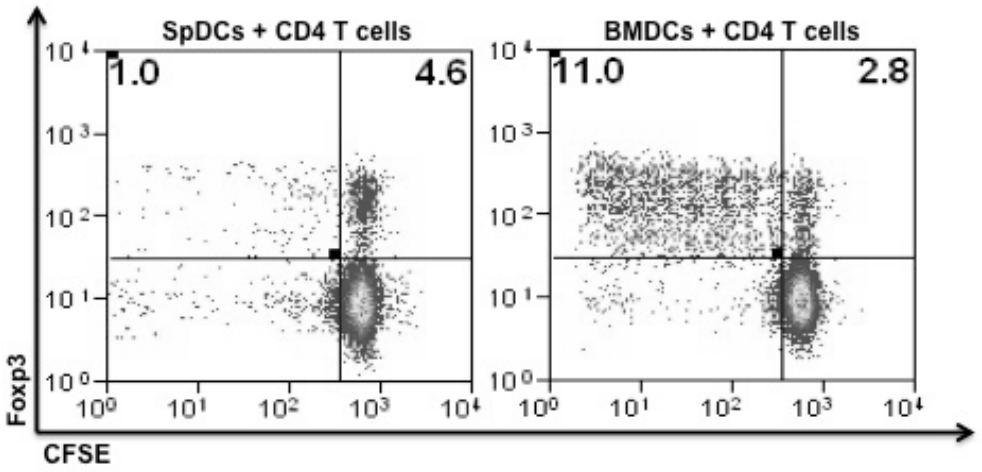

Fig. 3. NOD GM-BMDCs induce nTreg proliferation in *ex-vivo* co-cultures: CFSE labeled CD4+ T-cells were co-cultured, in the absence of any exogenous antigen, with SpDCs (Control) or GM-BMDCs from NOD mice at 2:1 ratio. After 5 days of co-culture, cells were analyzed by FACS for Treg proliferation.

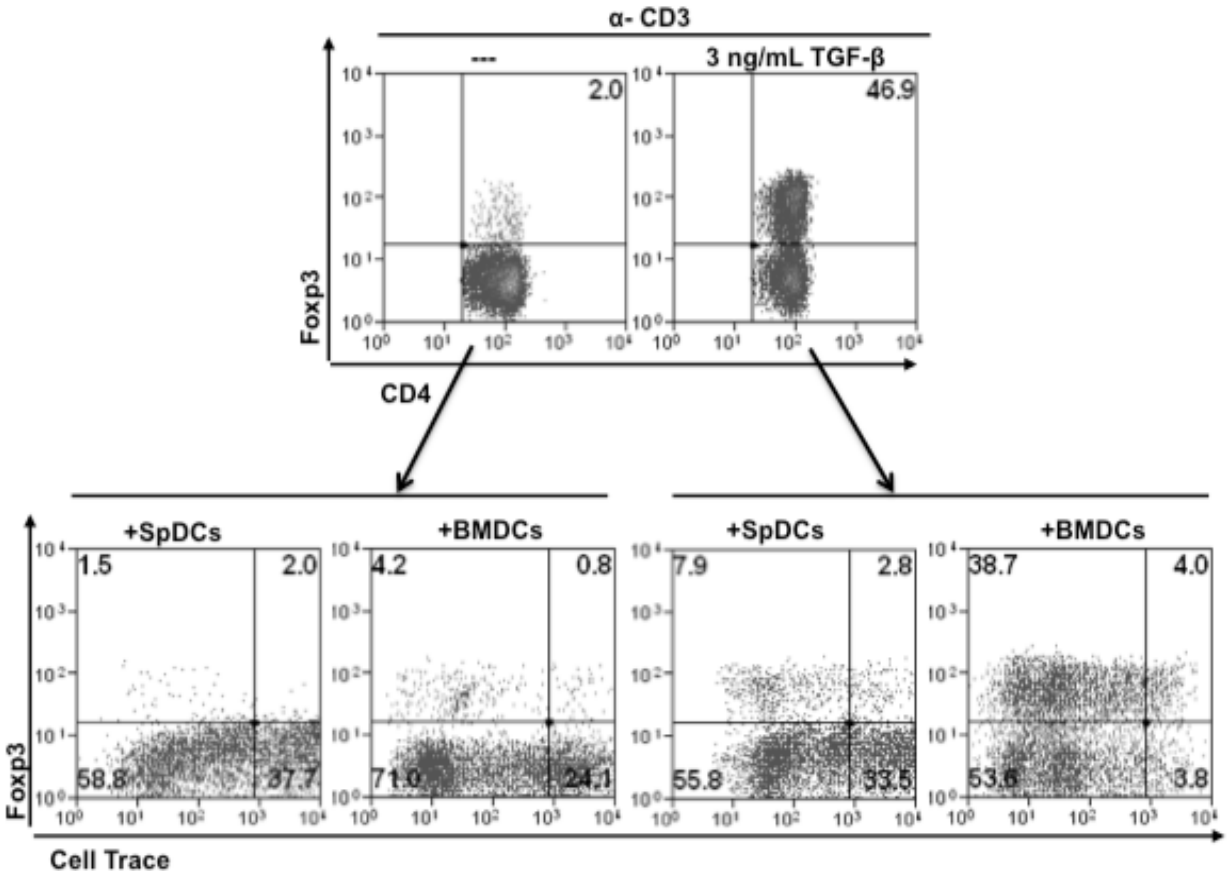

Fig. 4. GM-BMDCs are capable of expanding *ex-vivo* generated iTregs: $CD4^{+}CD25^{-}$ T cells were purified from the spleens of NOD mice and cultured for 48hrs with α-CD3 alone (2ug/mL) (control) or α-CD3+TGF-β (3ng/mL) to induce Tregs. About 50% of $Foxp3^{-}$ cells converted into $Foxp3^{+}$ Tregs in the presence of TGF-β (top panel). Cells were then washed, labeled with Cell-Trace Violet, and co-cultured with either SpDCs (control) or GM-BMDCs. Only GM-BMDC-CD4 co-cultured show sustained expression and proliferation of $Foxp3^{+}$ Tregs, compared to SpDCs controls (bottom panel).

3.2.3 *EX-VIVO* EXPANDED Tregs ARE FUNCTIONALLY SUPPRESSIVE

Previous studies have demonstrated that *in-vitro* expansion of Tregs leads to a loss of Foxp3 expression and suppressive function of Tregs (200, 201). Therefore, to rule out this possibility, we set-up an *in-vitro* suppression assay to determine whether Tregs expanded *ex-vivo* using GM-BMDCs retain their capacity to suppress effector T cell proliferation. $CD4^+CD25^-$ T effectors (Teff) were isolated from the spleens of NOD mice. Sorted cells were labeled with CFSE and stimulated with α-CD3 (2ug/mL) and splenic APCs. *Ex-vivo* expanded $CD4^+CD25^+$ (GM-BMDC-Tregs) sorted from 5-day old GM-BMDC-$CD4^+$ T cell culture were co-cultured with the CFSE labeled-$CD4^+CD25^-$ Teff at different ratios (Fig. 5). Tregs sorted from freshly obtained splenocytes, cultured at 1:1 ratio with Teff cells, were used as a positive control. After 3 days, cells were analyzed for effector T cell proliferation. Effector T cells proliferated robustly when stimulated in the absence of Tregs (85.0 ± 7.0%). In contrast, proliferation was markedly reduced in the presence of GM-BMDC-Tregs (33 ± 4.0 %; p-value = .006). As expected, the suppression by GM-BMDC-Tregs was dependent on the ratio of Tregs in the co-culture. Furthermore, the suppressive function of GM-BMDC-Tregs was comparable to that of freshly isolated splenic Tregs (30 ± 1.4 %). These data suggest that Tregs expanded *ex-vivo* with GM-BMDCs retain their suppressive function. Therefore, this system is a potential therapeutic tool for large-scale production of highly suppressive Tregs, which could in turn be used as a therapy in patients with T1D, and other autoimmune diseases.

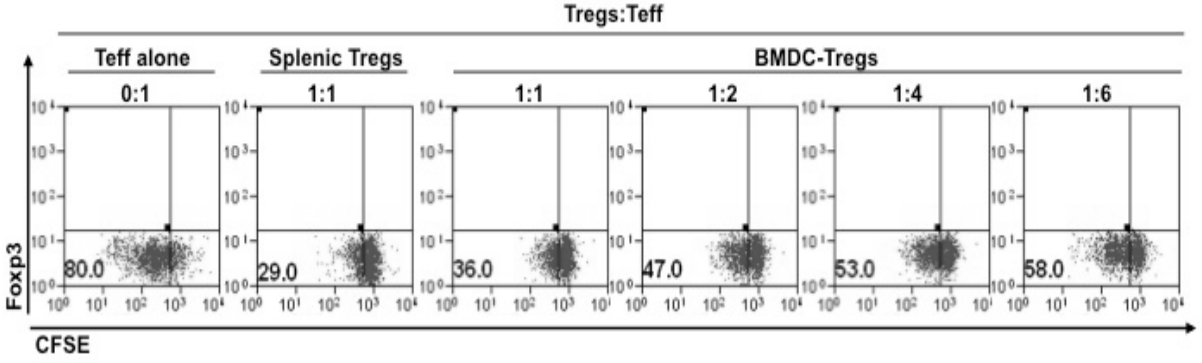

Fig. 5. GM-BMDC-expanded Tregs retain their suppressive function: $CD4^+CD25^-$ T effectors were isolated from the spleens of NOD mice, CFSE labeled and cultured in the presence of APCs + α-CD3 (2ug/mL) to induce proliferations. $CD4^+CD25^+$ Tregs were sorted from 5 day GM-BMDC-$CD4^+$ T cells co-cultures. Tregs sorted directly from freshly obtained spleens (splenic Tregs) were used as a positive control. GM-BMDC-Tregs were co-cultured with CFSE-labeled Teff at different ratios. After 3 days in culture, cells were analyzed for T effector proliferation using FACS. Teff cells show reduced proliferation in the presence of GM-BMDC-Tregs.

3.2.3 GM-BMDC-INDUCED Tregs EXPANSION IS DEPDENT ON OX40L

To further confirm the role of OX40L in the expansion of Tregs *ex-vivo* in NOD mice, we sorted GM-BMDCs into $OX40L^+$ and $OX40L^-$ subsets and co-cultured each subset with CFSE-labeled naïve $CD4^+$ T cells for 5 days, in the absence of any exogenous antigen. As indicated by the extent of CFSE dilution, $Foxp3^+$ Tregs proliferated only when co-cultured with $OX40L^+$ GM-BMDCs but not $OX40L^-$ GM-BMDCs (17.3 % ± .8 vs. 1.5 ± .5; p-value = 4.4e-6) (Fig. 6). Expectedly, $OX40L^+$ GM-BMDCs induced a more robust proliferation of Tregs than total BMDCs (17.8 % ± .8 vs. 11.3 % ± .98; p-value = 5.96E-05), which consist of only about 35 % $OX40L^+$ GM-BMDCs (Fig. 2). Collectively, these data highly suggest that OX40L is critical for GM-BMDC-induced Treg expansion *ex-vivo*.

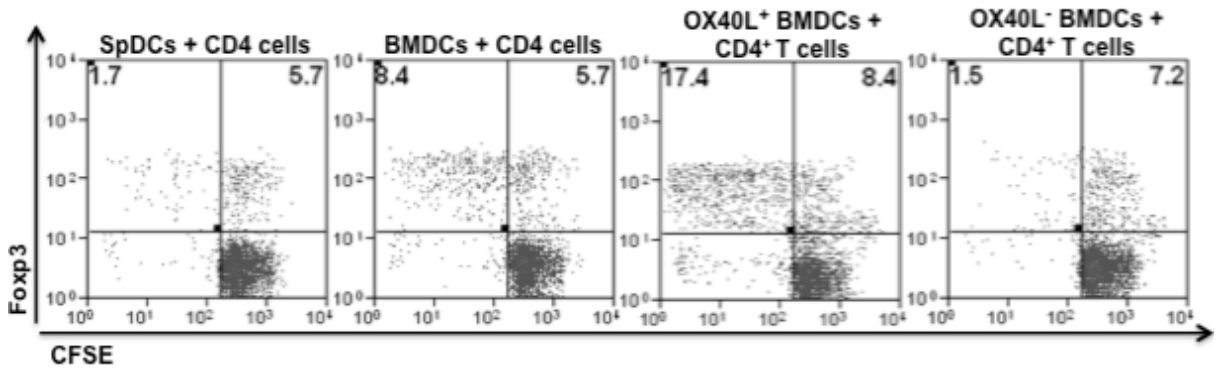

Fig. 6. Expansion of Tregs by GM-BMDCs is dependent on OX40L: CFSE labeled $CD4^+$ T-cells were co-cultured with either splenic dendritic cells (SpDCs), or total, OX40L+ or OX40L- enriched GM-BMDCS for 5 days without exogenous antigen and analyzed by FACS for Treg proliferation. Only $OX40L^+$, and not $OX40L^-$, BMDCs induce robust proliferation of $Foxp3^+$ Tregs.

3.2.4 OX40L$^+$ GM-BMDCs INDUCE PROLIFERATION OF NATURALLY EXISTING REGULATORY T CELLS

We noted that expansion of Foxp3$^+$ Tregs by GM-BMDCs could be the result of 1) proliferation of existing Tregs in the total CD4$^+$ population, and/or 2) conversion of Foxp3$^-$ T cells into Foxp3$^+$ followed by their proliferation. Therefore, to specifically address the role of OX40L$^+$ GM-BMDCs on Foxp3$^+$ Tregs, we made use of Foxp3-GFP transgenic mice. We set up co-cultures of sorted OX40L$^+$ and OX40L$^-$ GM-BMDCs with sorted and Cell-Trace Violet–labeled CD4$^+$GFP$^+$ or CD4$^+$GFP$^-$ (Fig. 7B) T cells isolated from Foxp3-GFP mice (Fig. 7A), in the presence or absence of IL-2. The extent of Cell-Trace Violet dilution revealed that, in the absence of IL-2, a very small fraction of GFP$^+$ T cells proliferated after 5 d of co-culture with either total, OX40L$^+$, or OX40L$^-$ GM-BMDCs. However, in the presence of IL-2, Foxp3$^+$ T cells proliferated efficiently only when co-cultured with either total (25.0 ± 1.7%) or OX40L$^+$ (34 ± 3.2%), and not with OX40L$^-$, GM-BMDCs (7.4 ± 1.0%) (Fig. 7B). In contrast, GFP$^-$ T cells (Foxp3$^-$) showed relatively either modest or robust proliferation based on absence or presence of IL-2 irrespective of whether they were co-cultured in the presence of total, OX40L$^+$, or OX40L$^-$ BMDCs. Most notably, we failed to see any adaptive conversion of effector T cells into Tregs in any cultures involving GFP$^-$ cells. It is important to note that none of these co-cultures was stimulated with anti-CD3 or any exogenous Ag. Thus, our data strongly suggest that only OX40L$^+$ GM-BMDCs, a subset of the CD11c$^+$CD11b$^+$CD8a$^-$ GM-BMDCs can cause efficient proliferation of existing Foxp3+ Tregs. (Originally published in The Journal of Immunology. Gopisetty, A., Bhattacharya, P., Haddad, C., Bruno, JC Jr., Vasu, C., Miele, L., Prabhakar, BS. OX40L/Jagged1 Cosignaling by GM-CSF-Induced Bone Marrow-

Derived Dendritic Cells Is Required for the Expansion of Functional Regulatory T Cells. J Immunol. 2013;190(11):5516-25. Copyright © [2013] The American Association of Immunologists, Inc.)

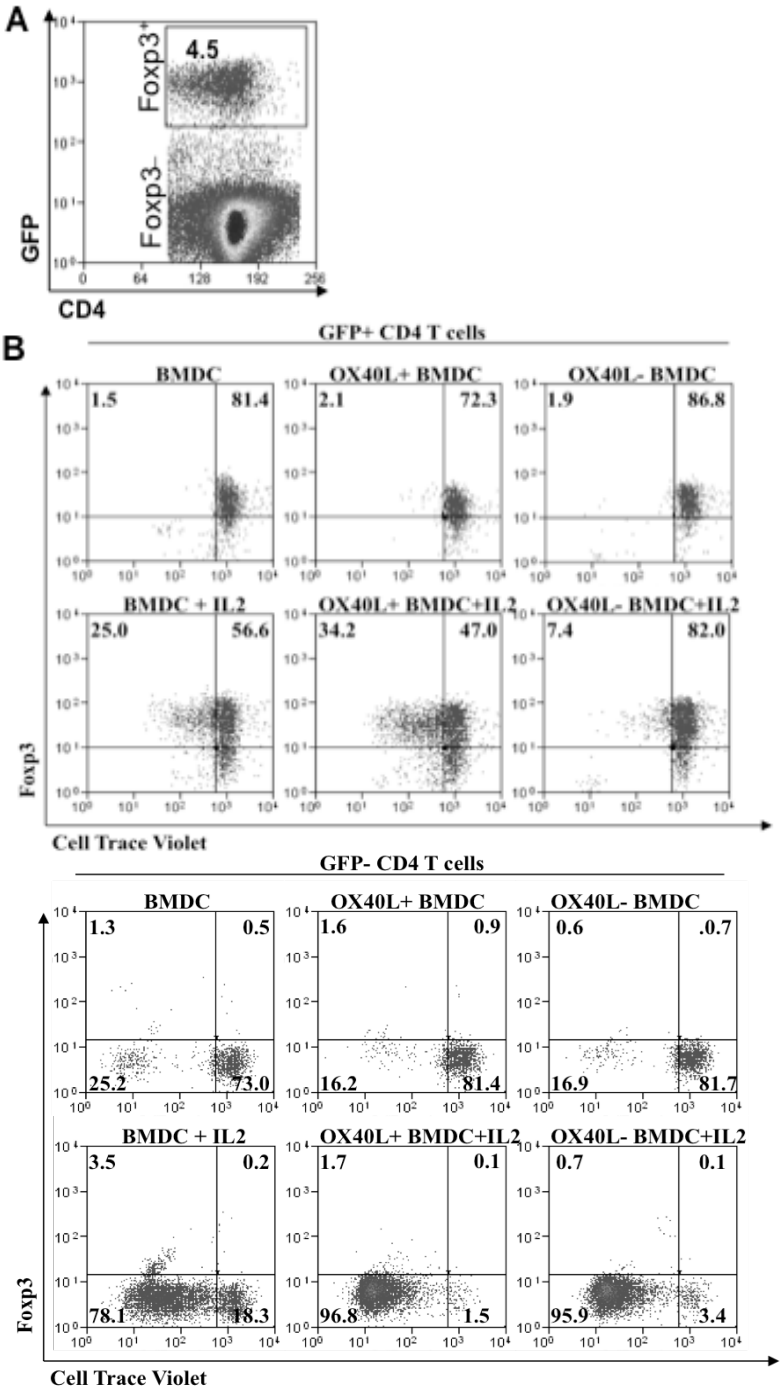

Fig. 7. OX40L+ GM-BMDCs induce proliferation of existing Foxp3$^+$ Tregs, rather than convert effector T cells: A) sorting of GFP$^+$ and GFP$^-$ Tregs from the spleen of GFP.Foxp3 mice. B) Total, OX40L$^+$ or OX40L$^-$ were co-cultured with cell-trace labeled GFP$^+$ (top panel) and GFP$^-$ (lower panel) T-cells, in the presence or absence of IL-2, for 5-days, without exogenous antigen and analyzed by FACs. (Originally published in The Journal of Immunology. Gopisetty, A., Bhattacharya, P., Haddad, C., Bruno, JC Jr., Vasu, C., Miele, L., Prabhakar, BS. OX40L/Jagged1 Cosignaling by GM-CSF-Induced Bone Marrow-Derived Dendritic Cells Is Required for the Expansion of Functional Regulatory T Cells. J Immunol. 2013;190(11):5516-25. Copyright © [2013] The American Association of Immunologists, Inc.)

3.2.5 ADOPTIVE TRANSFER OF GM-BMDCs INTO NOD MICE EXPANDS Tregs *IN-VIVO*

To test the capacity of GM-BMDCs to expand Tregs *in-vivo* in NOD mice, we adoptively transferred total GM-BMDCs or SpDCs (as a control) (1x10^6 cells/mouse) to 10-week-old mice. One week following the adoptive transfer, mice were sacrificed and analyzed for any increase in the percentage of $Foxp3^+$ Tregs in the spleen and pancreatic lymph nodes (PLN) (Fig. 8). Mice adoptively transferred with total GM-BMDCs showed increased percentage of $Foxp3^+$ Tregs in both the spleens (18 ± 2.5 % vs. 13.8 ± .5 %; p-value= .02) and PLN (15.3 ± .35 % vs. 11.5 ± .4 %; p-value= .005) compared to the PBS control. In contrast, adoptive transfer of SpDCs did not increase Tregs in either the spleen (13.5 ± .7 %) or the PLN (11.6 ± .38%). Taken together, these results strongly suggest that GM-BMDCs can induce the proliferation of Tregs *ex-vivo* and *in-vivo*.

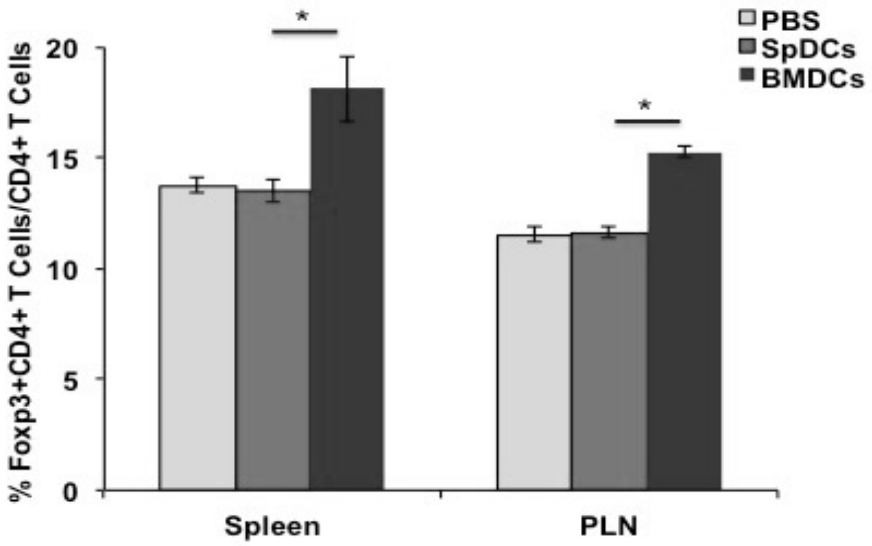

Fig. 8. Adoptive transfer of GM-BMDCs into NOD mice expands Tregs *in-vivo*: $1x10^6$ GM-BMDCs or SpDCs were adoptively transferred via i.v. tail injection into 10-wk-old NOD mice. 1 week post-transfer, spleens and PLNs were analyzed for $Foxp3^+$ Treg expansion. Only mice adoptively transferred with BMDCs show an increase in the frequency of $Foxp3^+$ Tregs in the spleen and PLNs. n= 3 mice/group. (*) indicates *p*-value < .05.

3.3 DISCUSSION

The immune system has developed effective mechanisms to eliminate foreign antigens and prevent infections. However, immune responses need to be tightly regulated in order to prevent unwanted immune responses against self-antigens (13). The failure to regulate immune responses and/or the failure to distinguish "self" from "non-self" lead to autoimmunity (1). Autoimmune diseases are caused by many factors, such as environmental factors, genetic susceptibility and immunoregulatory dysfunction (202). However, it is now strongly accepted that an imbalance between effector responses and regulatory responses is an important basis of many autoimmune diseases, including type 1 diabetes (110, 115, 203). Regulatory T cells, a subset of $CD4^+$ T cells, play a crucial role in suppressing and modulating immune responses (11, 25, 113). Since their discovery, considerable efforts have been focused on trying to manipulate Tregs as a therapy for autoimmune diseases, organ transplantation and even cancer therapy. Although the knowledge of Tregs, in terms of markers and function, has been expanding, manipulating Tregs as a therapeutic target for autoimmune diseases has remained a challenge (118). First, many studies have demonstrated the poor proliferative capacities of Tregs (11, 26). Additionally, studies have demonstrated that Tregs show a loss or a decline in their suppressive function following *in-vitro* expansion (200, 204). Finally, as Tregs are a subset of $CD4^+$ T cells, targeting Tregs without expansion of harmful effector T cells is very critical to prevent unwanted immune responses (118). Even purifying Tregs from human subjects for *in-vitro* expansion remains a challenge as the markers used for purification (i.e. CD25) are also expressed on activated T cells (131). Therefore,

finding approaches/targets that can lead to the expansion of functionally suppressive Tregs, without contamination with effector T cells, is crucial for advances in the field.

Our laboratory has previously used GM-CSF in the treatment of different autoimmune diseases, such as EAT (153, 154), MG (155) and T1D (152). We have shown that GM-CSF treatment induced a significant increase in the $Foxp3^+$ Tregs population in the spleen. Furthermore, we later showed that the Treg expansion was mediated through an increase in $CD8\alpha^-$ DC population caused by GM-CSF treatment. In an effort to further understand how GM-CSF was mediating its effects, we found that GM-CSF was acting primarily on bone marrow precursors and that culturing bone marrow cells in the presence of GM-CSF differentiated a special subset of DCs that was $CD11c^+CD11b^+CD8a^-$, expressed low levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), but expressed higher levels of TGF-ß (i.e. tolerogenic phenotype). These tolerogenic GM-BMDCs were capable of expanding $Foxp3^+$ Tregs *ex-vivo*, in the absence of TCR-stimulation (156). Later work showed that this GM-BMDC-induced expansion of Tregs was 1) TCR-independent, 2) required IL-2, normally supplied by effector T cells present in the co-cultures and 3) dependent on signaling through OX40L (156, 157).

In the first part of this study, we wanted to explore the full potential of using BMDCs to expand Tregs in a mouse model of T1D. Our data show that GM-BMDCs derived from NOD mice express OX40L after 7 days of GM-CSF culture (Fig. 2). Moreover, in GM-BMDC-$CD4^+$ co-cultures, GM-BMDCs are capable of expanding nTregs (Fig. 3) (purified from the spleen) as well as *ex-vivo* generated Tregs (Fig. 4). We also show that Tregs expanded *ex-vivo* using GM-BMDCs are fully functional as

they are capable of suppressing effector T cells proliferation in *ex-vivo* suppression experiments (Fig. 5).

Further, our data indicate that OX40L is critical for the induction of Treg expansion by GM-BMDCs, as only $OX40L^+$, but not $OX40L^-$, GM-BMDCs were capable of expanding Tregs (Fig. 6). Using $Fop3^+$ Tregs from GFP mice, we also show that the increase in $Foxp3^+$ Tregs in $OX40L^+$ GM-BMDC-$CD4^+$ T cells is primarily due to proliferation of existing Tregs, rather than adaptive conversion of $Foxp3^-$ into $Foxp3^+$ T cells (Fig. 7).

Although Tregs have been successfully expanded *in-vitro* by others, most methods relied on stimulation through TCR and the co-stimulatory molecule, CD28 (116). It is well known that T-cell activation and proliferation requires signaling through TCR and signaling through co-stimulatory molecules (1). Therefore, methods that target TCR and co-stimulatory signals to expand Tregs have a higher risk of also expanding harmful effector T cells and thus contaminating Tregs that could be used for adoptive transfer into patients. In the absence of either signal, T cells fail to become activated or proliferate (21, 22). In this context, expansion of Tregs using $OX40L^+$ GM-BMDCs system has unique and strong implications as it occurs in the absence of TCR signaling, therefore eliminating the risk of effector T cell proliferation. Additionally, since OX40 is constitutively expressed on Tregs, but only expressed on effector T cells following TCR stimulation (158), this further ensures that Tregs can be expanded without effector T cell contamination. Accordingly, our results have strong clinical implications as: 1) total or $OX40L^+$ GM-BMDCs could be used directly to expand Tregs *in-vivo*, 2) total or $OX40L^+$ BMDCs could be used as a system to

generate large numbers of Tregs (nTregs or iTregs) *ex-vivo*, which in turn could be adoptively transferred into murine models, and ultimately humans, as a therapy in many autoimmune diseases, and 3) soluble OX40L may be sufficient to expand Tregs *in-vivo*, and thus could be used as a potential therapy for autoimmunity. Although our data indicate that GM-BMDCs can expand Treg efficiently both *ex-vivo* and *in-vivo*, cell based therapy is still an expansive and complicated procedure. Thus, in the next chapter, we will focus on understanding the effects of using soluble OX40L to expand Tregs *in-vivo* in NOD mice, and determining how this therapy can be modulated to cause the most efficient Treg expansion and thus disease protection.

4. DIVERGENT EFFECTS OF OX40L ON REGULATORY T CELL PHENOTYPE AND FUNCTION IN NOD MICE

4.1 INTRODUCTION

In the first part of this study, we show that OX40L is critical for GM-BMDC induced Treg expansion. OX40L, expressed on APCs, bind to OX40 on T cells. Both OX40 and OX40L are members of the tumor necrosis factor superfamily with co-stimulatory function. Signaling through OX40 has been shown to play an important role in effector T cell activation, proliferation and survival (158). Although OX40 has been well studied in effector T cell function, its role in Treg is still controversial. As opposed to effector T cells, which express OX40 upon activation, Tregs constitutively express OX40, implying a critical role for OX40L/OX40 signaling in Treg homeostasis and function. Some studies have suggested that blocking OX40-OX40L can diminish experimental autoimmune diseases including EAE (183), inflammatory bowel disease (161), and T1D (162), while others have demonstrated a critical role for OX40 in intestinal Treg homeostasis and the suppression of colitis (164) as well as protection from T1D (199). Interestingly, Ruby et al has shown, in a model of EAE, that OX40 signaling can have different outcomes depending on the time of administration and local cytokine milieu (163). These contradictory data suggest that OX40 signaling plays a complex dual role in Treg biology and homeostasis.

In light of these data and given our findings that OX40L is critical for GM-BMDC-induced Treg expansion, we wanted to further understand the role of OX40L in Treg expansion and homeostasis *in-vivo* in NOD mice. We hypothesized that treatment of NOD mice with soluble OX40L can induce *in-vivo* Treg expansion and prevent/delay disease onset.

4.2 EXPERIMENTAL RESULTS

4.2.1 OX40L INDUCES RAPID ONSET OF T1D IN 12-WEEK BUT NOT 6-WEEK-OLD NOD MICE

In order to test whether soluble OX40L is sufficient to expand Tregs *in-vivo*, we treated NOD mice with soluble OX40L. NOD mice begin to develop diabetes around 12 weeks of age, and by 20 weeks of age about 80% of NOD female mice become diabetic. Therefore, we initially treated diabetes-free 12-week-old (n=9/group) NOD mice with OX40L (200ug/mouse x 3 treatments, 1 treatment/week) right before disease onset to determine whether the treatment can expand Tregs and prevent hyperglycemia. To our surprise, OX40L treatment induced a rapid onset of hyperglycemia in the 12-week-old NOD mice, with 100% of mice becoming diabetic within one week of treatment (Fig. 9). Recent immunomodulatory agents used in clinical trials of T1D were shown to exacerbate disease due to β-cell damage (140). Therefore, to rule out any β-cell toxicity induced by OX40L treatment, we treated 6-week-old NOD mice (n=6/group) with OX40L (200ug/mouse x 3 treatments, 1 treatment/week). Interestingly, treatment of 6-week-old NOD mice did not induce rapid onset of T1D, and mice remained diabetes-free following three treatments with OX40L. Our data are consistent with a previous report showing that OX40L treatment, in a model of EAE, can have different outcomes on disease phenotype depending on the time of administration (163). OX40L seems to exacerbate disease at the age when NOD mice begin to spontaneously develop T1D. However, if administered prior to that, OX40L most likely has a protective role.

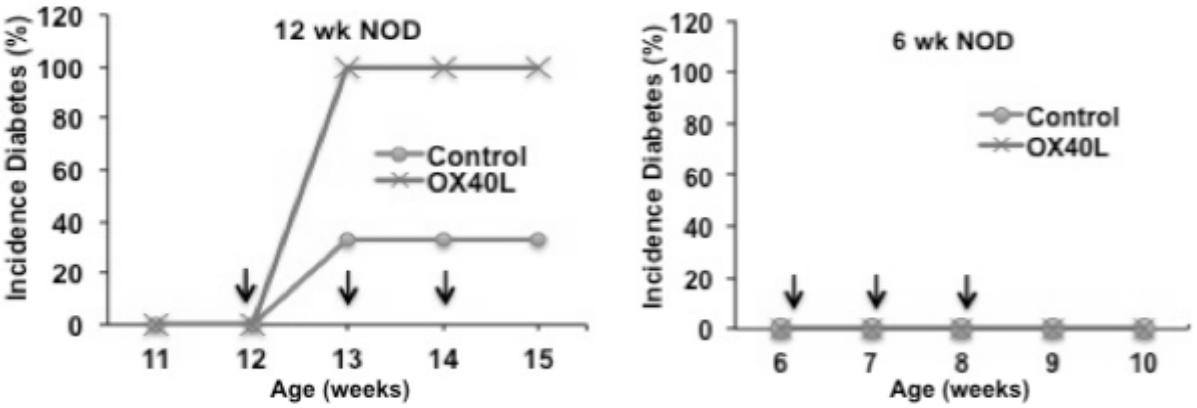

Fig. 9. Soluble OX40L treatment induced rapid onset of T1D in 12-week but not 6-week old NOD mice: Blood glucose measurements from 12- (left) or 6 wk old NOD mice treated with OX40L (200 ug/mouse x 3 treatments) or PBS (control). OX40L treatment induced rapid onset of hyperglycemia in 12- but not 6-wk-old NOD mice. 12-week control n= 9, 12-week OX40L n= 9, 6-week control n= 6, 6-week OX40L n= 6.

4.2.2 OX40L EXPANDS Tregs IN THE PERIPHERY OF 6-WEEK BUT NOT 12-WEEK OLD NOD MICE

To further understand why OX40L induced rapid onset of disease in 12-week-old but not 6-week-old NOD mice and to further elucidate the effects of OX40L on Treg phenotype and function, spleens and PLNs from 12 and 6-week-old treated mice were analyzed for Foxp3 expression (Fig. 10). The percentage of $Foxp3^+$ Tregs in 12-week-old mice treated with OX40L was comparable to that of 12-week-old controls in the spleen (16.3 ± 1.5 % vs. 14.3 ± 1.3 %; *p*-value = 0.07) and PLN (9.7 ± 1.3 % vs. 12.5 ± .5%; *p*-value = 0.053). Interestingly, 6-week-old NOD mice treated with OX40L showed a marked increase in the frequency of $Foxp3^+$ Tregs in both the spleen and PLNs compared to 6-week-old controls (41.0 ± 1 % vs. 12.8 ± 1.4%; *p*-value = 0.0003 and 19.2 ± 3.4 % vs. 9.7 ± .6 %; *p*-value = 0.01, respectively).

To test if OX40L had any effect on other T lymphocytes subsets (i.e. $CD4^+$ and $CD8^+$ T cells), spleens and PLNs from the different groups were additionally analyzed for the percentages of total $CD4^+$ and $CD8^+$ T cells (Fig. 11). Interestingly, total $CD4^+$ T cells in the spleen of 12-week-old NOD mice were increased upon OX40L compared to 12-week controls (34.0 ± 1.5 % vs. 23.0 ± 1.7%; *p*-value = 0.007). This increase in $CD4^+$ T cells was not noted in the PLN (47.0 ± 2.0 % vs. 45.0 ± 4.0%; *p*-value = 0.31). In contrast, the percentage of $CD4^+$ in 6-week-old NOD mice was comparable to that of the 6-week-old controls in the spleen (23.0 ± 2.1 % vs. 28.0 ± 3.6 %; *p*-value = 0.06) and PLN (42.0 ± 3.0 % vs. 47.5 ± 1.0% ; *p*-value = 0.06).

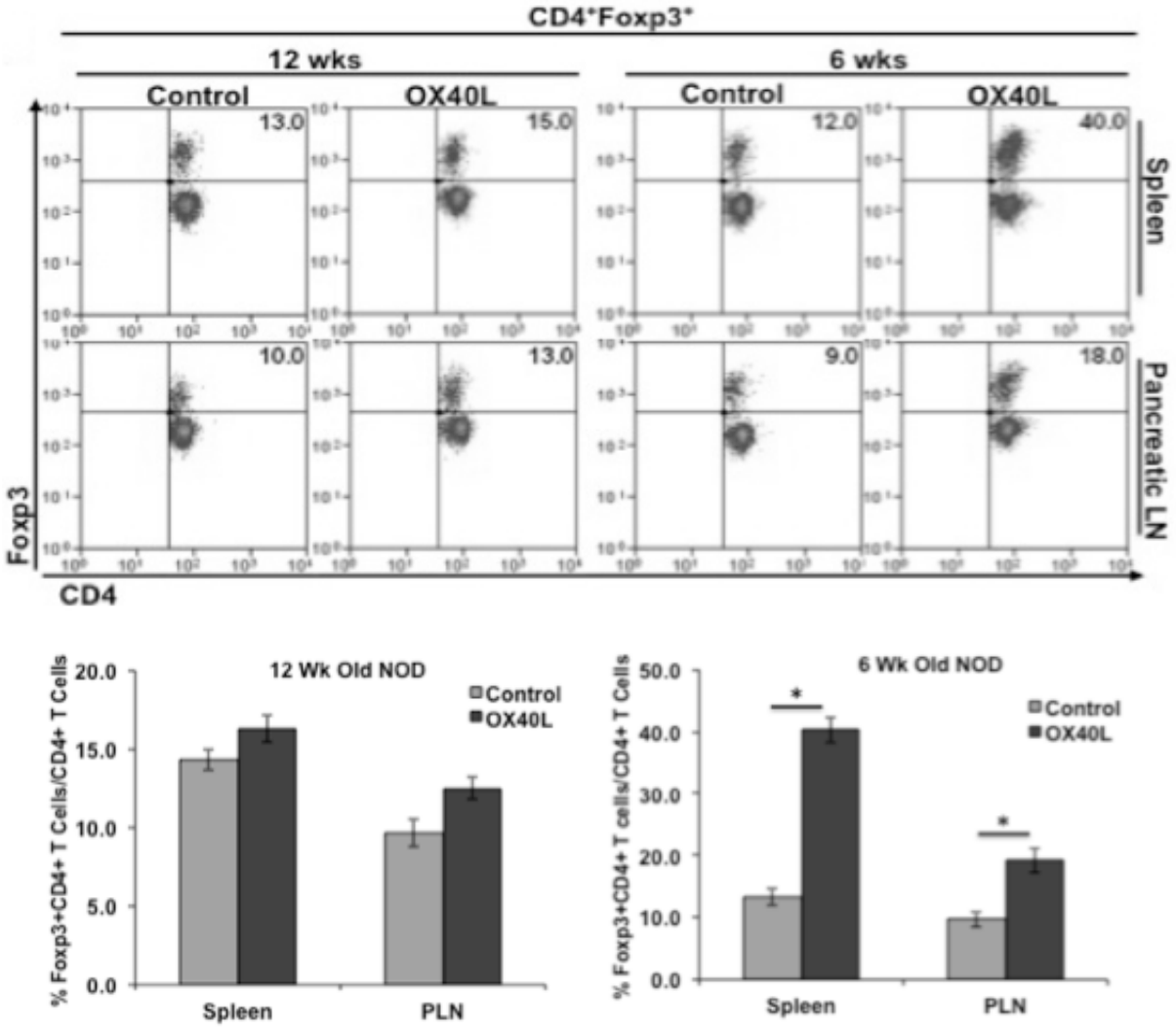

Fig. 10. OX40L treatment expands Tregs *in-vivo* in 6-week-old but not 12-week-old NOD mice: Spleens (top) and PLN (bottom) were isolated from 12- or 6-wk-old NOD mice, after three treatments of OX40L or PBS (Control). Cells were analyzed for Foxp3 expression by FACS. Bar graphs are statistical representation of Treg percentages in different treatment groups. 6-wk-old NOD mice treated with OX40L showed increased frequency of $Foxp3^{+}$ Tregs. (*) indicate *p*-value < 0.05. n = 5 mice/group.

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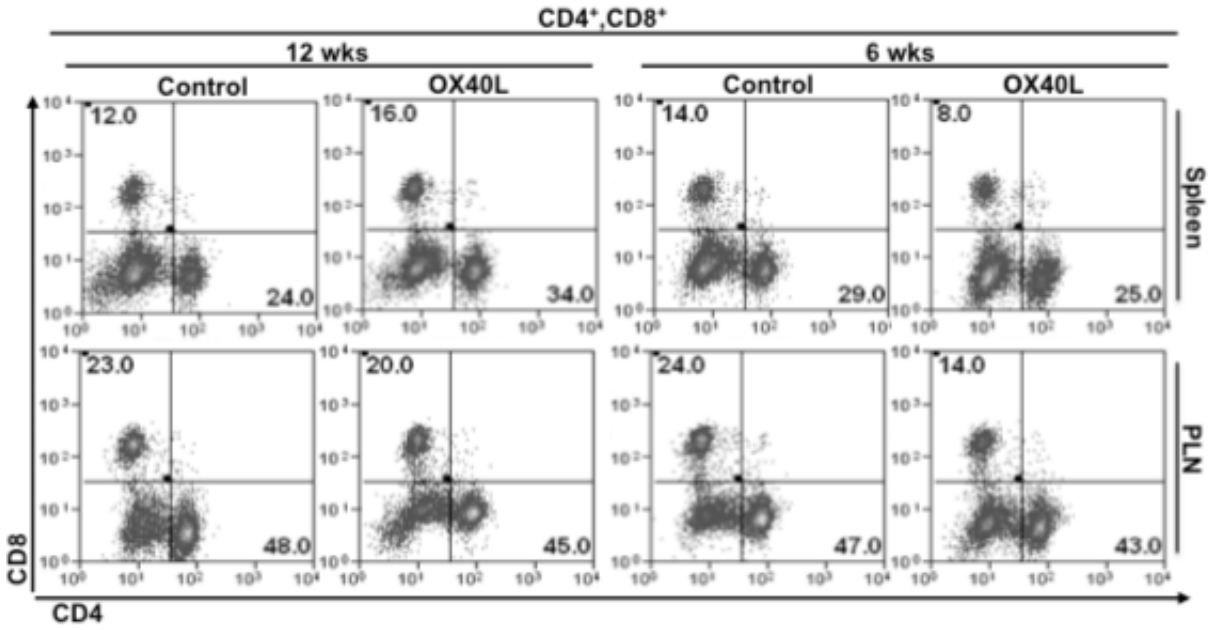

Fig. 11. OX40L increases total CD4$^+$ T cells in the spleens of 12-week-old NOD mice: Spleens (top) and PLN (bottom) were isolated from 12-wk or 6-wk-old NOD mice, following three treatments of OX40L. Cells were stained with CD4 and CD8 antibodies and analyzed by FACS. Numbers indicate percentages of CD4 or CD8 cells in splenocytes or PLN cells.

4.2.3 OX40L INDUCES Treg EXPANSION IN THE THYMUS OF 12-WEEK AND 6-WEEK-OLD NOD MICE

To further understand the dichotomy in the effects of OX40L on Treg expansion between 12 and 6-week-old NOD mice, we determined the frequency of $Foxp3^+$ Tregs in the thymus of 12 and 6-week old mice upon OX40L treatment (Fig. 12). Interestingly, in contrast to the periphery, 12-week-old NOD mice showed an increase in the percentage of thymic $Foxp3^+$ Tregs upon OX40L treatment compared to the 12-wk controls (5.3 ± .6% vs. 1.5 ± .5%; *p*-value = 0.004). The increase in thymic $Foxp3^+$ Tregs was also noted 6-week-old NOD mice treated with OX40L compared to the 6-week-old controls (5.2 ± 1.0% vs. 1.3 ± .3%; *p*-value = 0.006).

Thymocytes from different groups were also analyzed for $CD4^+$ and $CD8^+$ T cells percentages (Fig. 13). Typically, $CD4^+CD8^+$ DP T cells make up the majority of the thymus (~ 60-70 %). Interestingly, we noted that 12-week-old mice treated with OX40L showed a significant decrease in the frequency of $CD4^+CD8^+$ DP T cells (23 ± 10%) with a concomitant increase in the frequency of $CD4^+$ single positive (SP) T cells (48 ± 10%) compared to the 12-week-old controls (57 ± 5%; *p*-value = 0.007 and 13 ± 2.5%; *p*-value = 0.009). Surprisingly, this thymic phenotype was absent in 6-week old mice treated with OX40L, which showed normal distribution of DP and SP T cells compared to the age-matched controls (61 ± 3% vs. 57 ± 3%; *p*-value = 0.15; and 26 ± 1.4% vs. 27 ± 3%; *p*-value = 0.34). The increase in the percentage of CD4 T cells in the thymus of 12-week-old NOD mice is consistent with the increase in CD4 cells in the spleens.

The increase in Tregs in thymus but not in the periphery of 12 wk old NOD mice raised the following questions:

1) Is there an age-dependent decrease in Treg migration from the thymus to the periphery?
2) Is OX40L treatment inducing proliferation of effector T cells due to increased expression of OX40 on effector T cells in 12-week-old NOD mice?
3) Is there an increase in auto-reactive T cells in OX40L-treated 12-week old NOD mice that is perhaps responsible for the rapid onset of hyperglycemia in these mice?
4) Does OX40L act primarily on the thymus or the periphery?
5) Is there conversion of Tregs into effector T cells in the periphery of 12 wk old NOD mice? If so, is the conversion due to:
    a. The presence of a pro-inflammatory cytokine milieu in the periphery
    b. An inherent defect in Tregs making them more susceptible to reprogramming.
    c. Cytokine depletion, particularly IL-2 which is important for the survival of Tregs

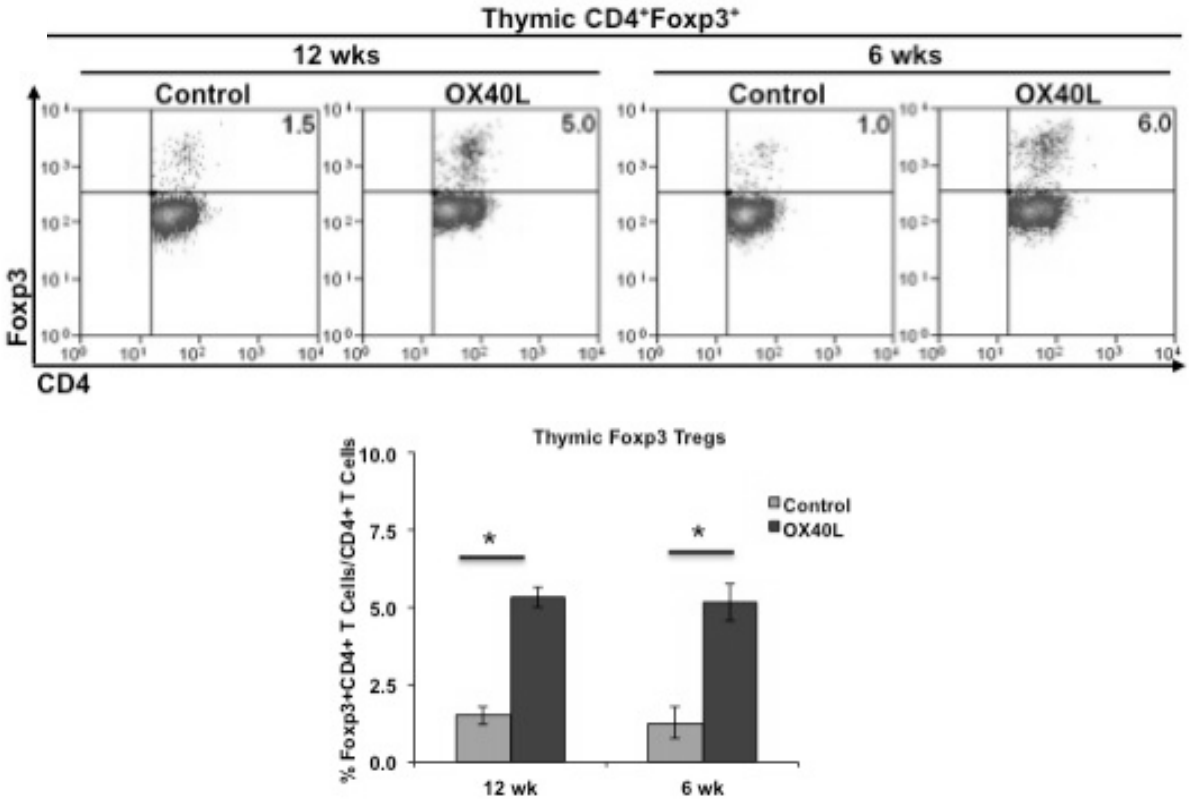

Fig. 12. OX40L treatment expands thymic Tregs *in-vivo* in 12-week and 6-week-old mice: Thymic tissues were isolated from 12- or 6-wk-old NOD mice, after three treatments of OX40L or PBS (Control). Cells were analyzed for Foxp3 expression by FACs. Bar graphs are statistical representation of percentages of Tregs upon OX40L in 12- and 6-wk-old NOD mice. OX40L increases the frequency of $Foxp3^+$ Tregs in both 12- and 6-wk-old mice. (*) indicate p-value < 0.05. n = 5 mice/group

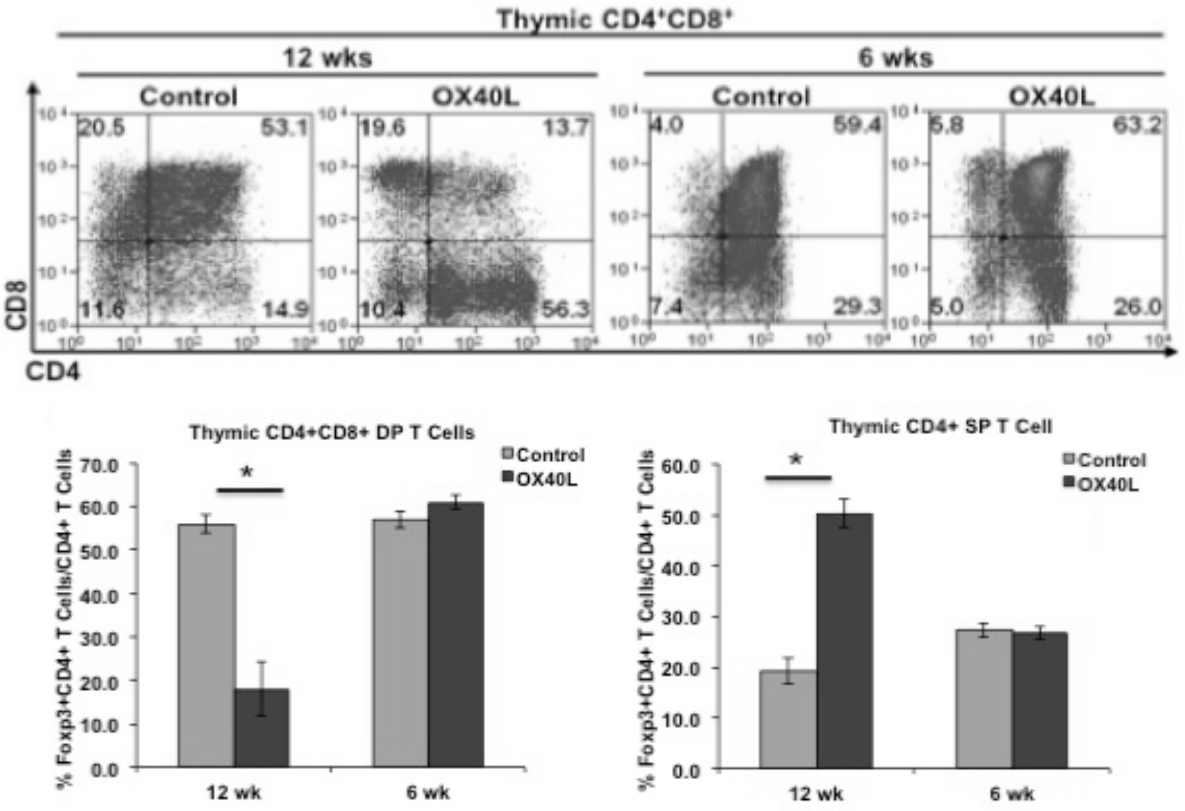

Fig. 13. OX40L treatment enhances differentiation of DP T cells into SP CD4 T cells in the thymus of 12-week-old NOD mice: Thymic tissues were isolated from 12 or 6 wk old NOD mice, after three treatments with OX40L or PBS (Control). Cells were analyzed for CD4 and CD8 expression by FACs. Bar graphs are statistical representation of percentages of DP (left) and SP (right) upon OX40L in 12- and 6 wk-old NOD mice. OX40L-treated 12-wk-old NOD mice show enhanced CD4 SP cells. (*) indicate *p*-value < 0.05. n = 4 mice/group

4.2.8 PHENOTYPIC CHARATERIZATION OF REGULATORY T CELLS

In addition to Foxp3, different surface molecules and transcription factors have been implicated as important for the function of Tregs. CD39 is the cell surface-located prototypic member of the ecto-nucleoside triphosphate diphosphohyrolase (E-NTPDase) family (205). E-NTPDases are involved in the hydrolysis of extracellular nucleotides into nucleosides, which in turn can activate the adenosine receptors (205). CD39 is expressed on B cells, NK cells, monocytes, DCs, some activated T cells and Tregs (205). CD39 has been suggested to play a crucial role in the suppressive machinery of Tregs (45, 206). Additionally it has been shown that $CD39^{+}Foxp3^{+}$ are capable of suppressing pathogenic Th17 responses (46). Therefore, we tested for the expression of CD39 on Tregs of 12 and 6-week-old, OX40L treated and control mice, to determine if there is any difference in the suppressive phenotype of Tregs (Fig. 14). Interestingly, 6-week-old NOD mice showed a significant increase in $CD39^{+}Foxp3^{+}$ Tregs upon OX40L treatment in their spleen compared to 6-week-old control mice (39 ± 10.0 % vs. 9.7 ± 3.1 %; *p*-value = .004) and PLN (26.5 ± 7.8 % vs. 3.8 ± 1.8 %; *p*-value = 0.03). On the contrary, the percentages of $CD39^{+}Foxp3^{+}$ Tregs in the spleens and PLNs of OX40L-treated 12-week-old were similar to that of the age-matched PBS-controls (9.5 ± 2.0 % vs. 12.0 ± 1.0 %; *p*-value = 0.08) and (5.3 ± 1.1 % vs. 6.5 ± 0.7 %; *p*-value = 0.15), respectively. Thus, the expanded Tregs in 6-week-old mice have a highly suppressive phenotype.

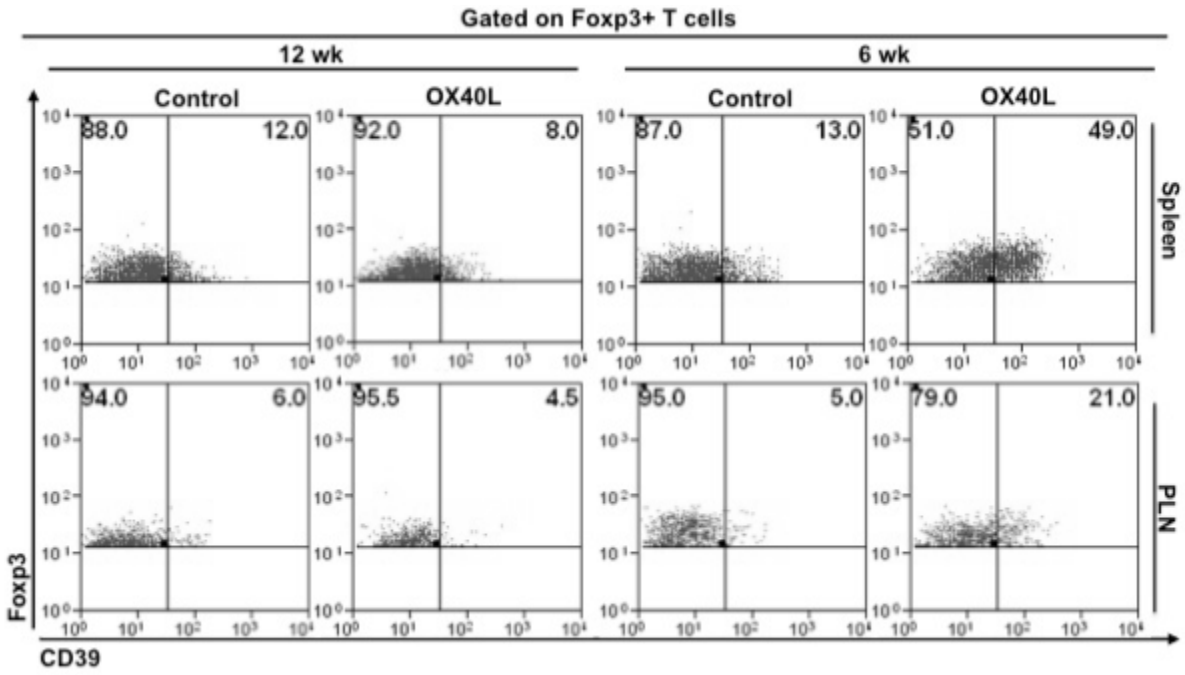

Fig. 14. Foxp3^{+}CD39^{+} Tregs are increased in 6-week-old NOD mice upon OX40L treatment: Spleens (top) and PLN (bottom) were isolated from mice following three treatments of OX40L (200ug/mouse) or PBS. Cells were analyzed for Foxp3 and CD39 expression by FACS. OX40L treated 6-wk-old NOD mice show enhanced expression of CD39 on Tregs, suggesting an enhanced suppressive function upon OX40L treatment. n = 3 mice/group.

Another marker that has been suggested to discriminate the highly suppressive subset of Tregs is CD44. CD44 is a cell surface-glycoprotein that is expressed on the surface of many cell types (47). CD44 participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis (207). It has been shown that CD44 expression is up-regulated in the most actively suppressive subset of $CD4^+CD25^+$ regulatory T cells (50). Therefore, we tested for the expression of CD44 on Tregs in 12- and 6-week-old, treated and untreated, mice (Fig. 15). Similar to CD39, CD44 expression in the $Foxp3^+$Treg population was up-regulated in the 6-week-old group upon OX40L treatment compared to 6-week controls in both the spleen (43.0 ± 5.7 % vs. 24.0 ± 6.1 %; *p*-value = 0.009) and PLN (21 ± 1.4 % vs. 13.0 ± 1.4%; *p*-value = 0.015). However, OX40L treatment of 12-week NOD mice failed to up-regulate CD44 expression, as the frequency of $CD44^+Foxp3^+$Tregs was comparable to that of 12-week control in the spleen (20.0 ± 1.4% vs. 22.7 ± 2.1 %; *p*-value = 0.11) and PLN (9.3 ± 1.0 % vs. 11.0 ± 2.8 %; *p*-value = 0.25). Collectively, these findings suggest that early administration of OX40L leads to an increase in a highly suppressive subset of Foxp3+ Tregs, which protects against disease onset, as shown by others (199). However, if administered closer to the onset of the clinical disease, OX40L not only fails to expand Tregs in the periphery but can induce rapid onset of hyperglycemia in 12-week-old NOD mice (Fig. 9, 100% hyperglycemia within 1 week of OX40L administration).

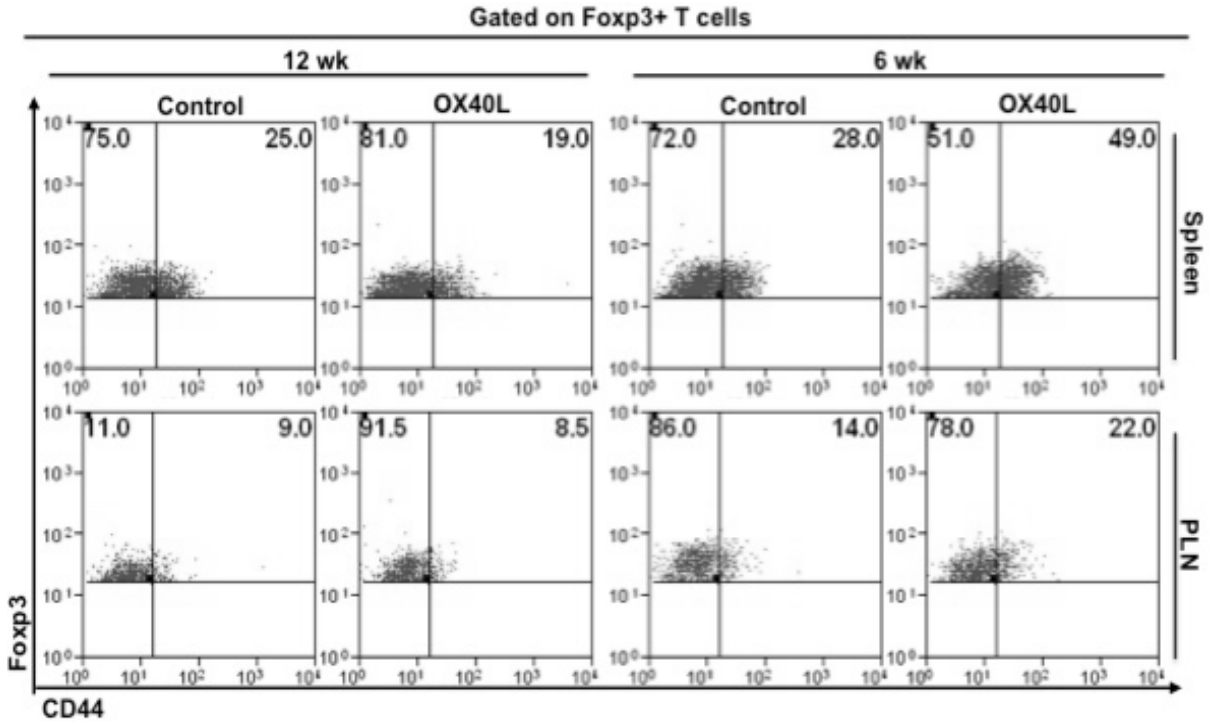

Fig. 15. Foxp3^{+}CD44^{+} Tregs are increased in 6-week-old NOD mice upon OX40L treatment: Cells were isolated from the spleen (top) and PLN (bottom), following three treatments of OX40L (200ug/mouse) or PBS. Cells were analyzed for Foxp3 and CD44 expression by FACS. 6-wk-old mice treated with OX40L shown enhanced expression of CD44.

4.2.4 AGE-DEPENDENT DIVERGENT EFFECTS OF OX40L ARE SPECIFIC TO NOD MICE

OX40L induced Tregs expansion in the thymus of 12-week-old NOD mice, but not the spleens or PLNs. On the contrary, 6-week-old NOD mice treated with OX40L showed increased Tregs in the thymus, spleen and PLNs. To determine if the discrepancy between 6 and 12-week-old NOD mice was due to a general phenomenon of aging (i.e. decreased migration of Tregs from the thymus to the periphery in aged mice) or due to the microenvironment or a genetic defect in NOD mice, we treated 12-week-old Balb/c mice with OX40L (Fig. 16) Interestingly, upon OX40L treatment, 12-week-old Balb/c mice showed a significant increase in Foxp3+ Tregs in the thymus (5 ± 0.3% vs. 1 ± .4%; *p*-value = 0.001), spleen (22 ± 1.6% vs. 14 ± 0.4%; *p*-value = 0.009) and PLNs (17 ± 0.9% vs. 10 ± 2.8%; *p*-value = 0.02) compared to the control group. Therefore, OX40L is capable of inducing Tregs in the thymus and periphery of 12-week-old Balb/c mice. These data highly suggest that OX40L treatment leads to vastly different biological outcomes in NOD mice most likely due to an inherent defect in these mice to sustain higher numbers of Tregs. This defect becomes apparent around 12 weeks of age and is most likely responsible for the more acute onset of the disease. More specifically, the failure to increase Tregs in the periphery of 12-week-old NOD upon OX40L treatment mice may be due to either a change in the peripheral microenvironment or a change in Treg phenotype, which prevents sustained Tregs survival or expansion in the periphery.

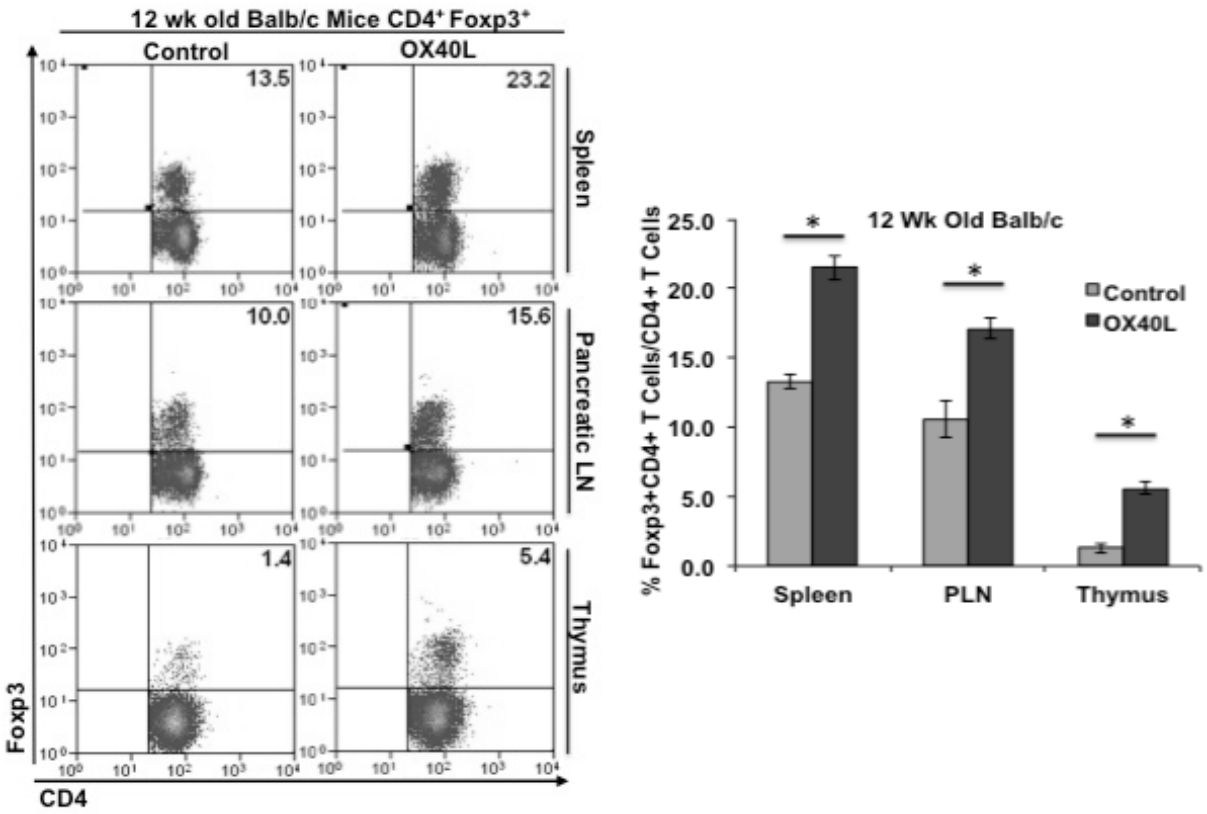

Fig. 16. OX40L expands Tregs in the thymus and periphery of 12-week old Balb/c mice: Spleens (top), PLN (middle) and thymus (bottom) were isolated from 12-wk-old Balb/c mice, following three treatments of OX40L or PBS. Cells were analyzed for Foxp3 expression by FACS. OX40L induces expansion of Tregs in central and peripheral lymphoid organs in Balb/c mice. Bar graphs are statistical representation of Treg percentages in different treatment groups; (*) indicate *p*-value < 0.05. n = 3 mice/group

4.2.6 OX40L ACTS PRIMARILY ON CELLS IN THE THYMUS

To further understand the dichotomy in sustaining Treg numbers between the thymus and periphery of 12-week-old NOD mice upon OX40L treatment, we used thymectomized mice to determine whether the OX40L acts primarily on the thymus or peripheral lymphoid organs. As 6-week-old NOD mice showed Treg expansion in both the thymus and periphery, 6-week-old thymectomized mice with OX40L to test if Treg expansion occurs in the periphery in the absence of the thymus. Although thymectomized mice showed a higher frequency of splenic Tregs, which was likely compensatory due to the absence of the thymus, OX40L treatment failed to induce further increase in Treg numbers in thymectomized mice, as the frequency of Tregs in the treatment group was similar to that of the thymectomized PBS controls in the spleen (19.3 ± 1.1 vs. 20.5 ± 1.4; *p*-value = 0.15) and PLNs (14.5 ± 2.1 vs. 11.0 ± 1.0; *p*-value = 0.68). As expected, OX40L treatment of WT 6-week-old NOD mice induced Treg expansion in the spleen (34.3 ± 3.1 vs. 11.4 ± 1.5; *p*-value = 0.15E-3) and PLNs (20.0 ± 2.6 vs. 11.0 ± 1.0; *p*-value = 0.006). These data suggest that OX40L acts primarily on the cells in the thymus and induces expansion of Tregs, which then migrate to the periphery and account for their increase. Thus, the absence of an increase in Tregs in the periphery of 12-week-old NOD mice may be due to either a failure of the peripheral microenvironment to sustain Tregs or a conversion in Treg phenotype.

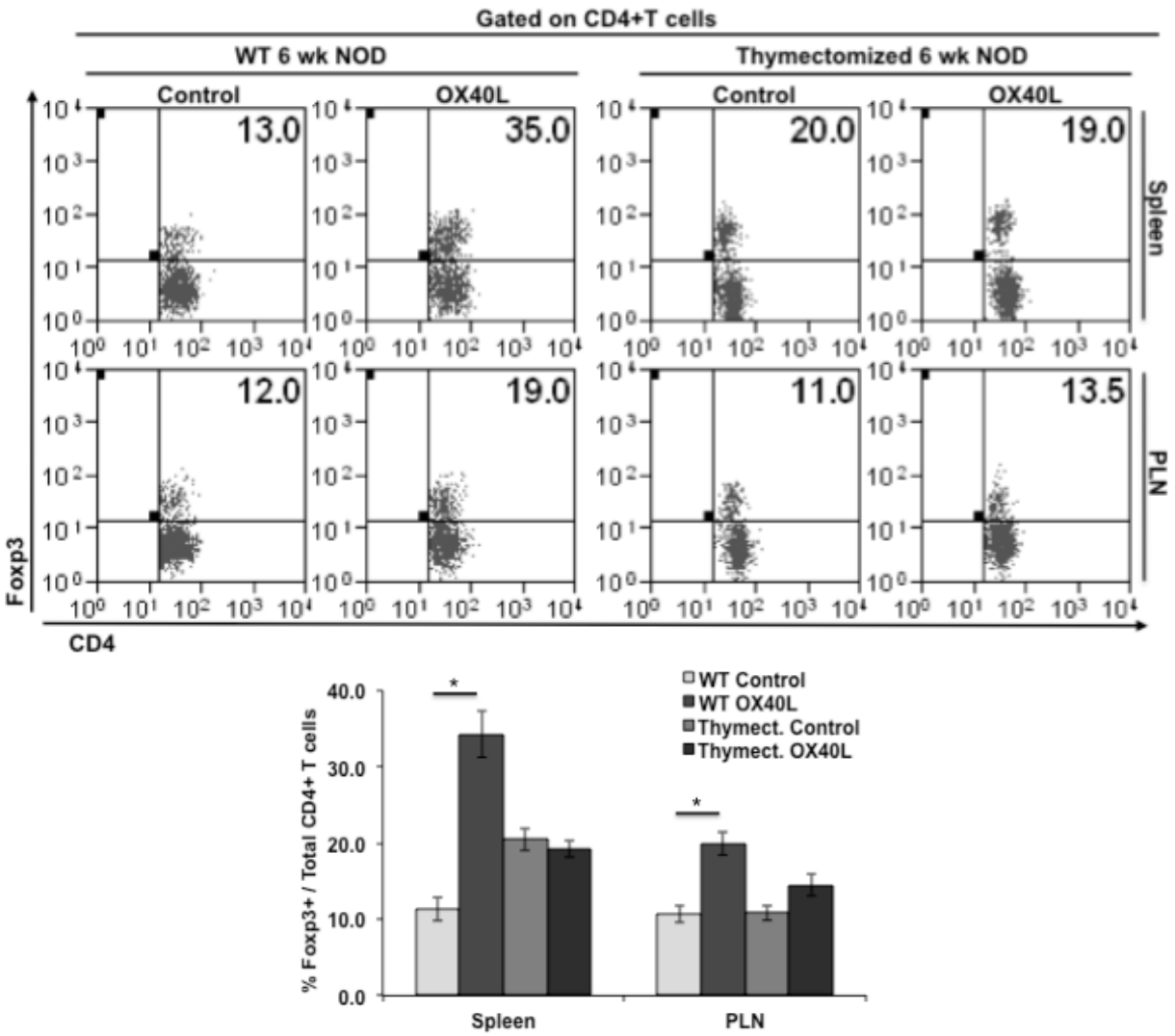

Fig. 17. OX40L treatment does not expand Tregs in the periphery of 6-week-old thymectomized NOD mice: Spleens (top) and PLN (bottom) were isolated from WT or thymectomized 6-week-old NOD mice, after three treatments of OX40L or PBS (Control). Cells were analyzed for Foxp3 expression by FACS. Bar graphs are statistical representation of Treg percentages in different treatment groups. OX40L treatment fails to expand Tregs in peripheral lymphoid organs in the absence of the thymus. (*) indicates *p*-vaule < .05. n = 4 mice/group.

4.2.5 BOTH 12-WEEK AND 6-WEEK-OLD NOD MICE SHOW SIMILAR EXPRESSION OF OX40 ON $CD4^+$ T CELLS

Effector T cells express OX40 transiently upon TCR activation (158). Thus, we tested whether $CD4^+$ T cells from 12-week old NOD mice had higher levels of expression of OX40 to which OX40L was binding and leading to robust proliferation of harmful effector T cells. Thus, the thymus, spleens and PLN from 12 and 6-week-old NOD mice were analyzed for the expression of OX40 on total $CD4^+$ (Fig. 18) and $Foxp3^+$ T cells. Interestingly, 12 and 6-week-old NOD mice showed similar levels of expression of $OX40^+CD4^+$ T cells in the thymus (3.6 ± 0.6 vs. 3.7 ± 0.3; *p*-value = 0.4), spleen (4.0 ± 0.5 vs. 3.7 ± 0.6; *p*-value = 0.4) and PLN (2.2 ± 0.8 vs. 2.8 ± 0.35; *p*-value = 0.24). Additionally, OX40L treatment did not increase OX40 expression in 12-week-old NOD in either the thymus (4.6 ± 0.6 vs. 3.6 ± 0.6; *p*-value = 0.12), spleen (3.5 ± 0.7 vs. 4.0 ± 0.5; *p*-value = 0.2), or PLN (2.3 ± 0.4 vs. 2.2 ± 0.8; *p*-value = 0.5). 6-week-old NOD mice showed a slight increase in OX40 expression in the spleen (5.5 ± 0.7 vs. 3.7 ± 0.6; *p*-value = 0.03) and PLN (7.5 ± 0.7 vs. 2.8 ± 0.4; *p*-value = 0.006) compared to 6-week controls, most likely due to the significant increase in $Foxp3^+$ Tregs seen in these mice. Finally, OX40 was mainly expressed on $Foxp3^+$ Tregs (about 80% of $OX40^+$ T cells were also $Foxp3^+$), which was consistent among the 4 groups (data not shown). Therefore, it is unlikely that higher levels of expression of OX40 was responsible for the rapid proliferation of effector T cells in 12-week old NOD mice when compared to 6-week-old NOD mice.

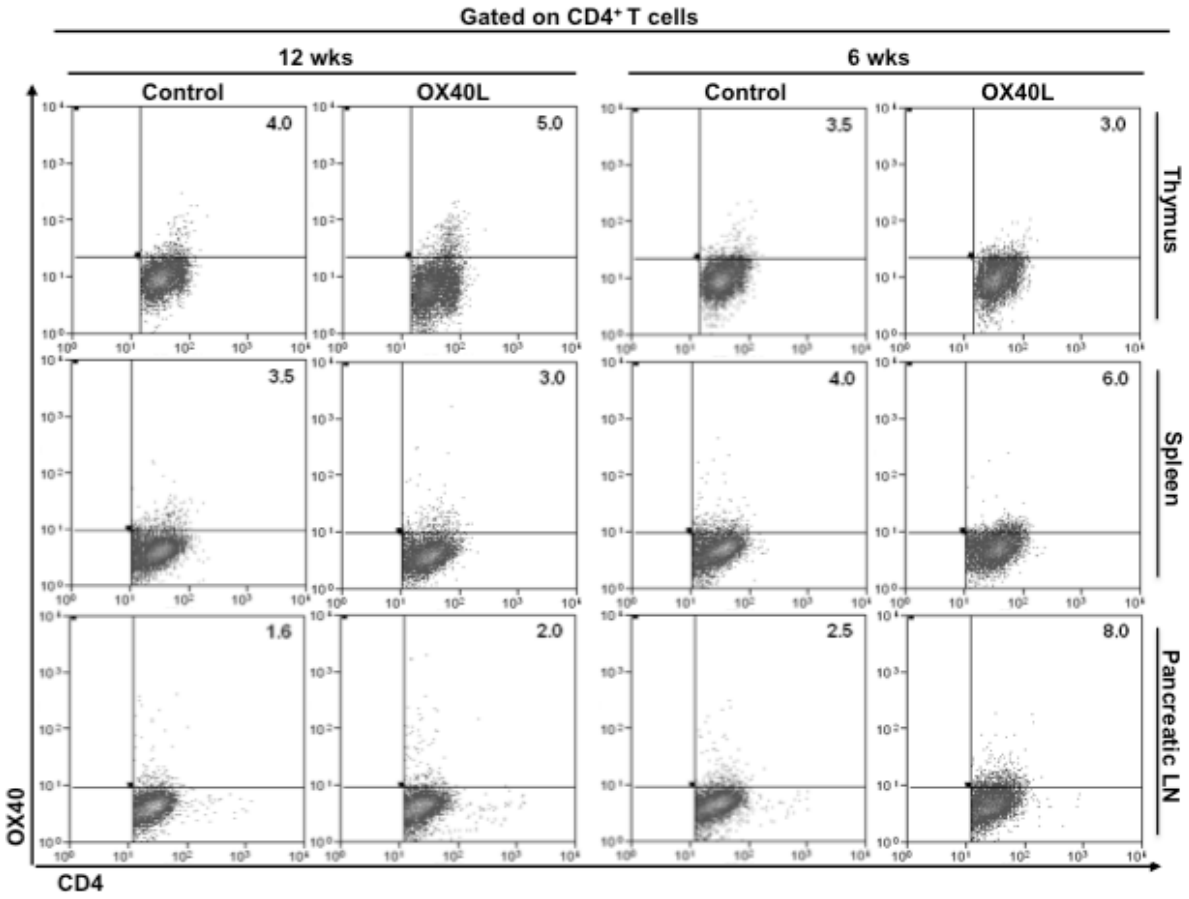

Fig. 18. OX40 expression in the thymus and periphery of 12-week and 6-week-old NOD mice: Thymus (top), spleen (middle) and PLNs (bottom) from 12- and 6-week-old NOD mice were stained with OX40 and CD4 to determine surface expression of OX40 on $CD4^+$ T cells. 12 and 6-wk-old NOD mice show similar expression of OX40 on total $CD4^+$ T cells.

4.2.7 AUTOREACTIVE T-CELLS ARE INCREASED IN 12-WEEK-OLD MICE AND DECREASED IN 6-WEEK-OLD MICE UPON OX40L TREATMENT

As discussed earlier, during T cell development in the thymus, T cells that react too strongly to the peptide:MHC complex displayed on stromal cells or dendritic cells in the thymus undergo negative selection (19). This process allows the elimination of auto-reactive T cells. It has been suggested that autoimmunity occurs due to auto-reactive T cells escaping thymic negative selection (15, 19). The increase in CD4 SP T cells in the thymus of 12-week-old mice, along with the rapid onset of disease, suggested that there might be an increase in auto-reactive T cells upon OX40L treatment. MHC class II tetramers have emerged as an important tool to recognize antigen-specific $CD4^+$ T cells (208). HLA class II tetramers are widely used in studying vaccine development, antitumor responses, allergy monitoring and autoimmunity (208). In the context of autoimmunity, MHC-II tetramers allow the recognition of self-Ag-specific $CD4^+$ T cells, which are usually present at very low frequencies.

Autoreactivity towards the insulin peptide has been strongly implicated in the pathogenesis of T1D (209, 210). Autoreactive T cells directed against the Ins1/2$B_{9–23}$ peptide have been detected and linked to diabetogenesis in NOD mice (210-212). Therefore, in order to determine if OX40L treatment in 12-week-old mice caused an increase in auto-reactive $CD4^+$ T cells, we obtained InsB_{9-23} pMHC-II tetramers (NIH Tetramer Facility). Interestingly, 6-week-old mice treated with OX40L showed a marked reduction in the frequency of InsB_{9-23}-specific $CD4^+$ T cells in both the spleen (0.5 ± 0.05% vs. 0.9 ± 0.2%; *p*-value = 0.04) and PLNs (1 ± 0.14% vs. 2.9 ± 0.3%; *p*-value = 0.007) compared to 6-week PBS controls (Fig. 19). In contrast, OX40L-

treated 12-week-old mice showed a significant increase in $InsB_{9-23}$-specific $CD4^+$ T cells in the PLN compared to 12-week controls (10.1 ± 1.2% vs. 3.7 ± 0.4%; *p*-value = 0.009). The frequency of $InsB_{9-23}$-specific $CD4^+$ T cells in spleens of OX40L-treated was similar to that of 12-week-old PBS controls (1 ± 0.17% vs. 1.2 ± 0.4%; *p*-value = 0.24).

Collectively, these data suggest that the significant increase in $Foxp3^+$ Tregs in 6-week-old NOD mice induces a robust suppression of auto-reactive responses, thus leading to a decrease in the frequency of $InsB_{9-23}$-specific $CD4^+$ T cells in the peripheral lymphoid organs. However, OX40L treatment fails to increase $Foxp3^+$ Treg percentage in the periphery of 12-week-old NOD mice; rather, these mice show a significant increase in $InsB_{9-23}$-specific $CD4^+$ T cells, which most likely contribute to the rapid onset of T1D in these mice. The increase in $InsB_{9-23}{}^+CD4^+$ T cells could be due to a direct effect of OX40L on effector T cells or an indirect effect on Tregs. However, given the data showing that 12-week and 6-week-old NOD mice have comparable levels of OX40, which was mainly expressed on Tregs, it is unlikely that OX40L is causing proliferation of effector T cells. Therefore, we hypothesize that OX40L acts primarily on cells in the thymus where it induces marked expansion of Tregs in both 12-week and 6-week-old NOD mice. However, upon emigration from the thymus, these Tregs are not sustained as well in the peripheral lymphoid organs of 12-week-old mice as they are in the 6-week old mice. Failure to sustain Tregs in the periphery could be due to 1) increased inflammatory cytokines in the periphery, 2) a defect in Tregs themselves 3) a decrease in the levels of IL-2, which is crucial for

survival of Tregs. It is important to note that a loss of Foxp3 expression upon proliferation has been reported in the literature before (200).

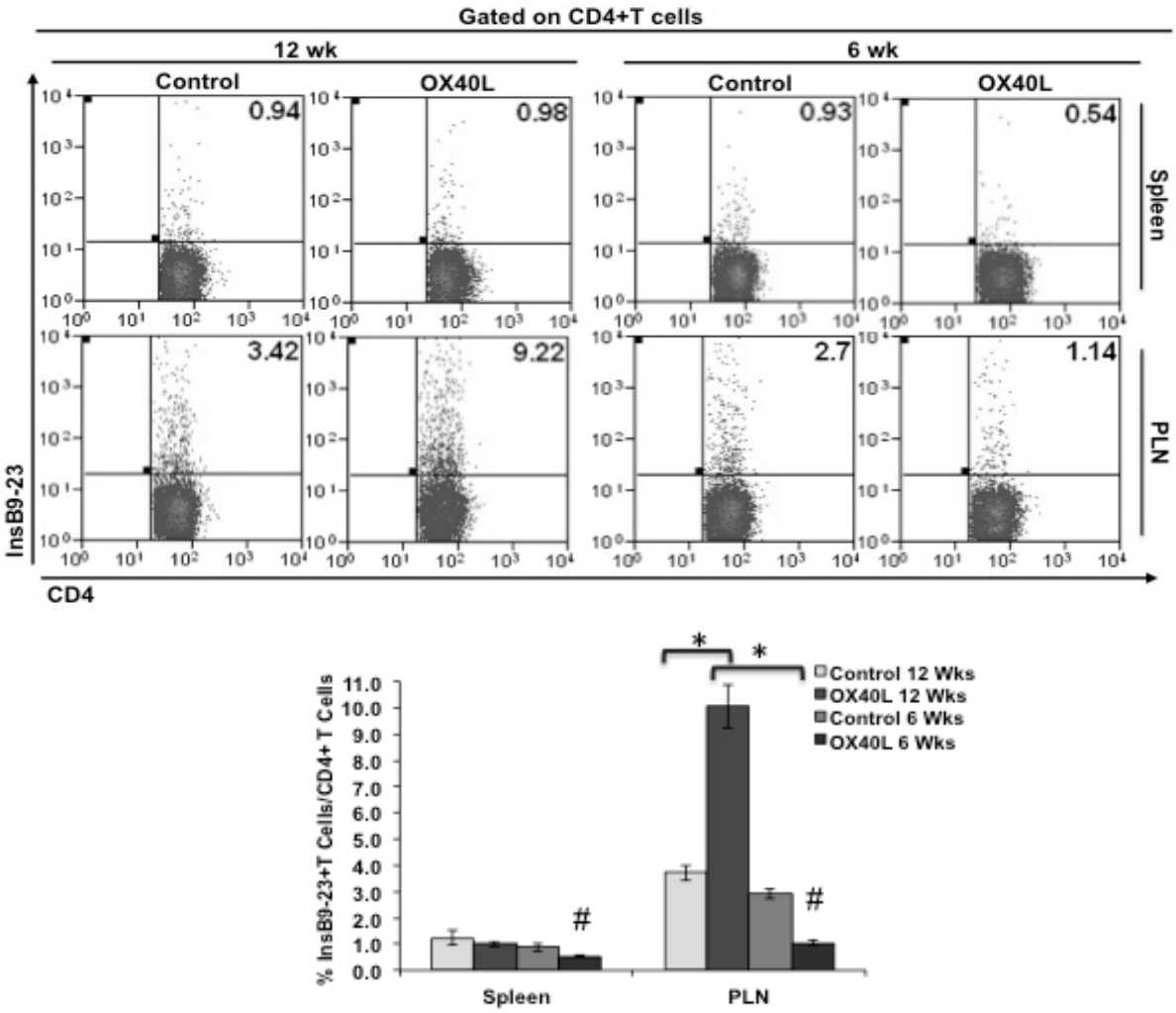

Fig. 19. OX40L treatment increases autoreactive T cells in the PLNs of 12-week-old NOD mice: Spleens (top) and PLN (bottom) were isolated from 12- or 6-wk-old NOD mice, following three treatments of OX40L. Presence of autoreactive T cells was determined using InsB9-23 MHCII tetramers. Bar graphs are statistical representation of percentages of InsB9-23^{+} CD4^{+} T cells; Autoreactive T cells are increased in 12-wk-old NOD mice treated with OX40L. (*) and (#) indicate *p*-values < 0.05 between 12-wks OX40L and control groups and 6-wks OX40L and control groups, respectively. n = 4 mice/group.

4.2.8 INCREASED LABILE TREGS IN 12-WEEK-OLD NOD MICE

A recent study by Sharma et al. has identified a new subset of "labile" $Foxp3^+$ Tregs that have the capacity to undergo reprogramming and attain helper-like function by promoting the differentiation of naïve $CD8^+$ T cells into effector cells under certain pro-inflammatory conditions (213). These labile Tregs are characterized as $Foxp3^+CD103^-CD38^+$ cells (Fig. 20). In contrast, stable Tregs, characterized as $Foxp3^+CD103^+CD38^-$, sustain their phenotypic and functional properties under pro-inflammatory conditions (213). It is suggested that the labile subset is present early in thymic development; therefore it is a part of the nTregs repertoire (213). Since OX40L treatment increased $Foxp3^+$Tregs in the thymus but not in the periphery of 12-week-old NOD mice, we tested whether 12-week-old NOD mice have altered Treg phenotype (i.e. increased frequency of labile T cells). We hypothesized that such a Tregs might contribute to the increase noted in the thymus upon OX40L treatment, but are lost in the periphery due to reprogramming in the peripheral microenvironment. Thus, the thymus, spleens and PLNs of 12 and 6-week-old mice were stained with CD38 and CD103 to determine differences in the distribution of stable vs. labile Tregs (Fig. 21). Interestingly, we noted that, in 6-week-old mice, OX40L induced Treg expansion mainly in the stable Treg subset "$Foxp3^+CD103^+CD38^-$" in the thymus (20.3 ± 1.5 % vs. 8.3 ± 0.6 %; *p*-value = 0.0001), spleen (16 ± 2.0 % vs. 8.2 ± 0.8 %; *p*-value = 0.002) and PLNs (19 ± 4.0 % vs. 8.0 ± 1.0 %; *p*-value = 0.007), compared to 6-week PBS controls. Moreover, these OX40L-treated 6-week-old mice had a concomitant decrease in the labile population in the thymus (2.7 ± 0.25 % vs. 4.7 ± 0.6 %; *p*-value = 0.003), spleen (7.2

± 1.0 % vs. 10.0 ± 1.0 %; *p*-value = 0.013), and PLNs (4.4 ± 0.7 % vs. 7.0 ± 1.0 %; *p*-value = 0.009). In contrast, the increase seen in thymic Tregs of 12-week-old NOD mice upon OX40L treatment occurred mainly due to an increase in the labile "$Foxp3^{+}CD103^{-}CD38^{+}$" compared to 12-week-old PBS controls (13.7 ± 3.8 % vs. 6.7 ± 0.6 %; *p*-value = 0.02). The frequency of labile Treg in OX40L-treated 12-week-old mice in the spleen (14.5 ± 2.0 % vs. 15 ± 0.7 %; *p*-value = 0.4) and PLN (14.3 ± 3.0 % vs. 12.0 ± 2.0 %; *p*-value = 0.2) was similar to that of 12-week PBS controls. Additionally, the frequency of stable Tregs was also consistent between treated and control 12-week-old mice in the spleen (11.3 ± 0.6 % vs. 10.5 ± 0.6 %; *p*-value = 0.07) and PLNs (7.3 ± 0.6 % vs. 8.0 ± 2.0 %; *p*-value = 0.3). Lack of an increase in labile Tregs in the periphery of treated 12-week-old NOD mice is consistent with lack of an increase in the total $Foxp3^{+}$ Treg population in these mice. Considering the expanded thymic Tregs in treated 12-week-old mice were more "labile" in nature, it is likely that these cells had already converted upon reaching the periphery, and thus had lost Foxp3 expression and cannot be detected in the labile Tregs, which were gated on $Foxp3^{+}$ Tregs. It is also important to note that 12-week-old mice showed an increase in the labile Treg subset "Foxp3+CD103-CD38+" compared to the 6-week-old mice in the thymus (6.7 ± 0.6 % vs. 4.7 ± 0.6 %; *p*-value = 0.007), spleen (15 ± 2.0 % vs. 10.0 ± 1.0 %; *p*-value = 0.008) and PLNs (12 ± 3.0 % vs. 7.0 ± 1.0 %; *p*-value = 0.025). This altered Treg homeostasis may be an important underlying defect involved in the pathogenesis in T1D, which normally begins to develop starting at 12-weeks of age in NOD mice.

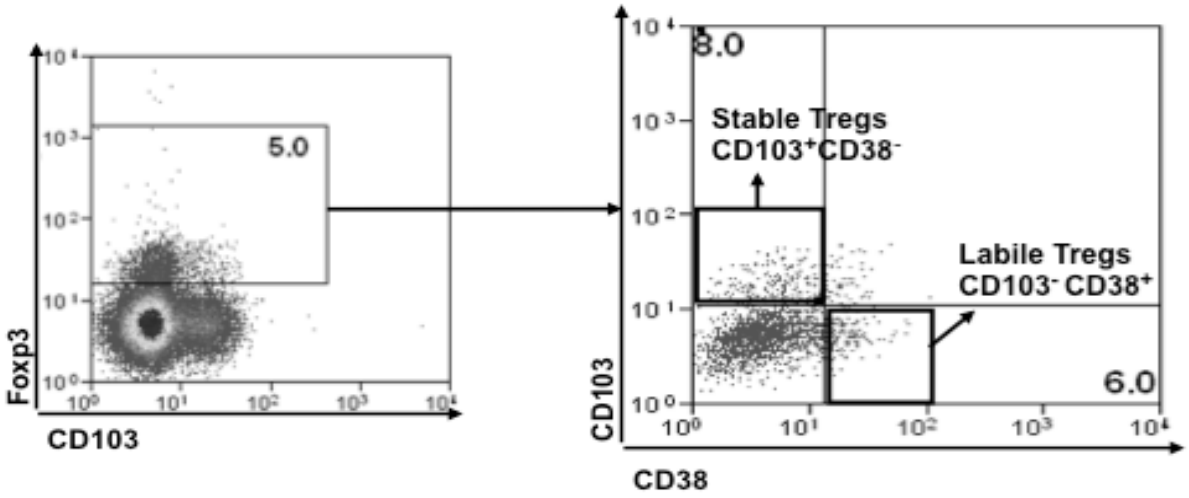

Fig. 20. Gating strategy for labile vs. stable Tregs: cells from different tissues are isolated and stained with Foxp3, CD103, and CD38. Cells are gated on the Foxp3$^+$ Treg population. Foxp3$^+$ Tregs are then divided into the stable vs. labile subsets based on CD103 and CD38 expression.

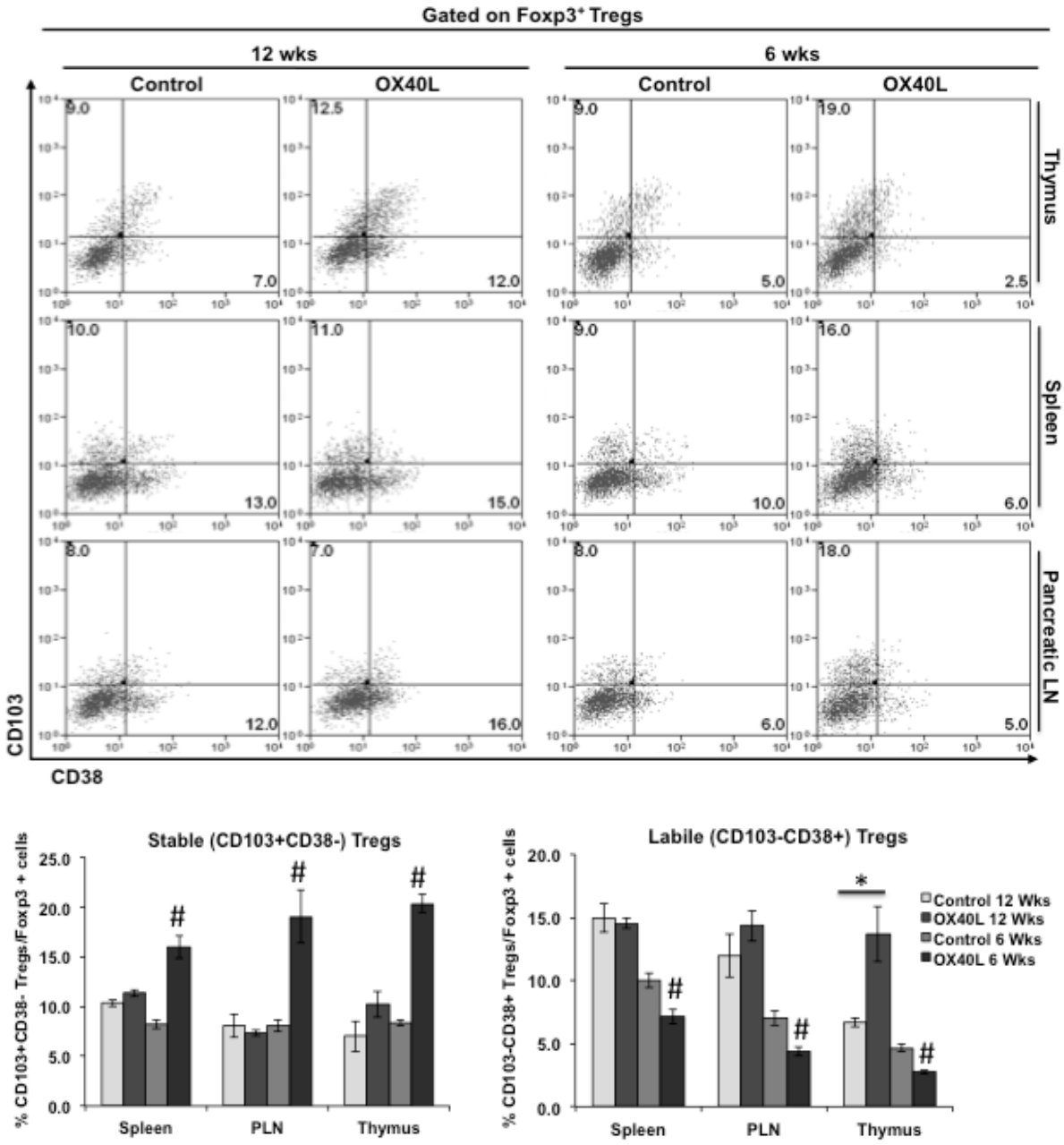

Fig. 21. Increased labile Tregs in the thymus of 12-week-old mice treated with OX40L: Thymus (top), spleen (middle), PLNs (bottom), from OX40L or control 12- and 6-week-old mice, were analyzed for stable ($Foxp3^+CD103^+CD38^-$) and labile ($Foxp3^+CD103^-CD38^+$) Tregs subset. Labile Tregs are increased in the thymus of 12-wk-old NOD mice upon OX40L treatment, whereas 6-wk-old mice show an increase in stable Tregs in the thymus, spleen and PLNs. (#) Indicates *p*-value < .05 between 6-week-old OX40L vs. 6-week-old controls. (*) Indicates *p*-value < .05 between 12-week-old OX40L group vs. 12-week-old controls. n = 3 mice/group

4.3 DISCUSSION

Activation of naïve T cells requires two essential signals: Signal one (recognition) requires the engagement of the T cell receptor (TCR) with the peptide:MHC complex on the surface of APC (1). 2) Signal 2 (verification) requires the interaction of co-stimulatory molecules on the surface of T cells (i.e. CD28) with co-stimulatory molecules on the surface of APC (i.e. CD80, CD86) (1). Although CD28-CD80/CD86 interaction is perhaps the most critical and well-studied co-stimulatory pathway, other molecules such as PD-1, CTLA-4, ICOS (Inducible COStimulator), OX40 and 4-1BB, have been described (214-216). OX40/OX40L has been shown to play a role in T cell activation, function and survival (158, 159). Blocking OX40L-OX40 signaling has been shown to inhibit unwanted immune responses in autoimmunity (160, 161), whereas stimulating OX40-OX40L has been shown to enhance anti-tumor responses in cancer (183, 217-219).

Although OX40L/OX40 signaling has been well studied in effector T cell function, its role in regulatory T cell biology and homeostasis is still poorly understood. While some studies have demonstrated that triggering OX40 on Tregs can block their suppressive function (189, 190), other studies have shown an important role for OX40L/OX40 signaling in Treg proliferation and survival (164, 199). Additionally, triggering OX40 has been shown to drive Treg proliferation given the "right" cytokine milieu (163). Our previous studies in a model of EAT have demonstrated a critical role for OX40L/OX40 signaling in BMDC-induced Treg expansion *ex-vivo* (157). In the first part of this study, we further confirmed the requirement for OX40L/OX40 in BMDC-induced *ex-vivo* in the NOD mouse model.

Given that the literature has demonstrated controversial roles of OX40L/OX40 signaling in Treg biology, the objective of this part of the study was to determine the effects of soluble OX40L on regulatory T cell phenotype and function *in-vivo* in NOD mice.

Interestingly, treatment of 12-week-old NOD mice resulted in rapid onset of T1D, whereas treatment of 6-week-old mice did not (Fig. 9). In addition, OX40L treatment failed to induce Treg expansion in the periphery of 12-week-old NOD mice, but resulted in a robust expansion of Tregs in 6-week-old mice (Fig. 10). This divergent effect of OX40L on disease phenotype has been reported in the literature before in a model of EAE (163). Thus, these findings prompted us to further investigate this dichotomy of OX40L function on Treg phenotype and disease induction in the NOD mice, and to determine how OX40L can be modulated to induce its protective effects in 12-week-old mice.

On the contrary to the effects on the periphery, OX40L treatment caused an increase in thymic $Foxp3^+$ Tregs in both 6- and 12-week-old NOD mice. These data suggested that there is perhaps a decrease in Treg migration from the thymus in 12-week-old mice. However, treatment of 12-week-old Balb/c mice with OX40L induced Treg increase in both the thymus and periphery. Additionally, data from thymectomized mice suggested that OX40L primarily targets cells in the thymus, where it induces robust Treg expansion. Thus, the dichotomy between the thymus and periphery in 12-week-old NOD mice is most likely due to an inherent defect in these mice, which may be either in the peripheral microenvironment or in the Tregs.

OX40L treatment of 6-week-old NOD mice resulted in increased expression of surface markers CD39 and CD44, both of which have been suggested to identify a highly suppressive subset of Tregs. Concomitantly, there was a decrease in InsB9-23^{+} autoreactive T cells in 6-week-old mice treated with OX40L. The increase in Tregs and decline in autoreactive T cells most likely confer disease protection in these mice. Although we did not follow 6-week-old NOD mice beyond 10-weeks of age, a recent study has shown that administration of an OX40 agonist to 6-week-old NOD mice prevents disease onset (199). On the contrary, an increase in surface molecules CD39 and CD44 was absent in 12-week-old NOD mice treated with OX40L. Concurrently, 12-week-old mice showed a significant increase in InsB9-23^{+} autoreactive T cells in the PLN upon OX40L treatment. This imbalance between regulatory and auto-reactive responses is consistent with the rapid onset of hyperglycemia in 12-week-old NOD mice upon OX40L.

OX40 expression on effector T cells was comparable between 12-week-old and 6-week-old NOD mice. Additionally, treatment with OX40L did not increase CD4^{+}OX40^{+} effector T cells in either 12-week-old or 6-week-old NOD mice. Thus, it is unlikely that OX40L is inducing proliferation of effector T cells.

A recent study identified a subset of Tregs that is capable of reprogramming into “helper-like” cells under inflammatory conditions. This population was characterized as Foxp3^{+}CD103^{-}CD38^{+}. The Foxp3^{+}CD103^{+}CD38^{-} population on the other hand was described as “stable Tregs” that do not have the capacity to undergo reprogramming. Therefore, we determined if labile Tregs are increased in thymus of 12-week-old mice. Interestingly, the increased thymic Tregs in treated 12-week-old

NOD mice were mainly of the labile phenotype. As total Tregs did not increase in the periphery of treated 12-week-old mice, labile Tregs were comparable between the treated and control groups. In contrast, 6-week-old mice, which showed Treg expansion in the thymus and periphery, had significantly higher percentages of stable Tregs Foxp3^{+}CD103^{+}CD38^{-}.

Based on these findings, we hypothesize that OX40L treatment increased Tregs in the thymus. However, these Tregs underwent rapid conversion in the periphery of 12-week-old mice. The conversion of Tregs, known to be self-antigen specific, into T effector cells may even contribute to disease onset resulting from increased Autoreactive T cells. Although, OX40L-treated 12-week-old NOD mice showed an increase in the labile population in the thymus, labile Tregs have been shown to sustain their function under normal physiological condition, but undergo conversion only under pro-inflammatory conditions. Additionally, defects in IL-2 signaling have been reported in the NOD mice (31, 145, 146). Thus, conversion of Treg is perhaps driven by either a decreased IL-2 and/or increased pro-inflammatory cytokines in the periphery of 12-week-old NOD mice. In the next chapter, we will study, although not to completion, the mechanism that is perhaps responsible for Treg conversion in the periphery of 12-week-old NOD mice.

5. PROPOSED MECHANSIM BY WHICH OX40L INDUCES DIVERGENT EFFECTS IN NOD MICE

5.1 INTRODUCTION

Many studies have focused on finding therapeutic targets to expand Tregs *in-vitro* and *in-vivo*. Unfortunately, the risk of effector T cell contamination and the tendency of Tregs to lose their suppressive function upon repeated proliferation remain as central challenges (118, 200). Work in our laboratory has demonstrated a critical role for OX40L/OX40 signaling in GM-BMDC-induced Treg expansion *ex-vivo*. Additionally, in this study, we showed that administration of OX40L to NOD mice prior to disease onset expands Tregs *in-vivo*, and others have shown that it can protect against disease onset (199). Interestingly, administration of OX40L at a later stage caused rapid onset of disease and failed to expand Tregs in the periphery, although Treg expansion occurred in the thymus. Thus, based on the data in chapter 4, we hypothesize that OX40L expands Tregs mainly in the thymus. However, upon migration into the periphery, the expanded Tregs undergo rapid conversion into Foxp3$^-$ Tregs in 12-week-old NOD mice. As Tregs are known to be "self-reactive", it is possible that converted Tregs in the periphery contribute to the increase in auto-reactive T cells seen in the periphery of 12-week-old NOD mice cause direct destruction of pancreatic β-cells.

IL-2 is well known to be critical for Treg survival and function (220). IL-2, IL-2R or CD25 knock-out mice have reduced Treg numbers and develop lymphoproliferative disease (144, 221-223). Additionally, deficiencies in IL-2 production and IL-2 signaling have been implicated in the pathogenesis of T1D in the NOD mice (31, 140, 145). Thus, IL-2 deficiencies in the periphery of 12-week-old NOD mice may contribute to the failure to sustain increased Tregs upon OX40L

treatment. On the other hand, the presence of pro-inflammatory cytokines, such as IL-6, has been shown to contribute to the conversion of labile Tregs (213). Thus, in the following chapter, we will further determine whether there is increased conversion of Treg from 12-week-old NOD mice. Additionally, we will determine, if not to completion, the mechanism underlying the conversion of Tregs in the periphery of 12-week-old NOD mice.

5.2 EXPERIMENTAL RESULTS

5.2.1 12-WEEK-OLD NOD MICE SHOW RAPID CONVERSION OF Tregs *EX-VIVO*

To determine if Tregs from 12-week-old NOD mice undergo conversion, we tested for loss of Foxp3 expression in Tregs in *ex-vivo* cultures. Briefly, we sorted out $CD4^+CD25^+$ Tregs (highest 2%) from the freshly isolated spleens and PLNs of 12- and 6-week-old NOD mice. Since CD25 is also expressed on activated effector T cells, we further confirmed the purity of the sort by staining each purified sample with Foxp3 (~ 80% Foxp3+ in the 4 different samples) (Fig. 22A). Tregs were then labeled with CFSE and co-cultured back with their respective total splenocytes/PLN. Splenocytes were stimulated with cell stimulation cocktail (eBioscience) to induce cytokine secretion and create a cytokine milieu similar to that present *in-vivo*. After 24-hrs, cells were stained with CD4/Foxp3 and analyzed for the percentages of $CFSE^+Foxp3^+$ vs. $CFSE^+Foxp3^-$ (Fig. 22B). Interestingly, in stimulated cultures, the majority of CFSE labeled cells remained $Foxp3^+$ in both the spleens (72 ± 3.1) and PLN (71 ± 1.4) of 6-week-old NOD mice. However, cells from 12-week-old NOD mice showed higher conversion of $Foxp3^+$ T cells in the spleen (57 ± 2.1; *p*-value = .001)

and PLN (53 ± 2.8; *p*-value = .008) as marked by increased percentages of $CFSE^{+}Foxp3^{-}$ T cells in these cultures. In the unstimulated cultures, cells from both 12- and 6-week old mice showed marked conversion in the spleen (39.3 ± 3.1 vs. 46.3 ± 1.5; *p*-value = .011) and PLN (40.5 ± 0.7 vs. 50.0 ± 1.4; *p*-value = 0.007) although 12-week cultures showed slightly higher loss of $Foxp3^{+}$ cells compared to the 6-week cultures.

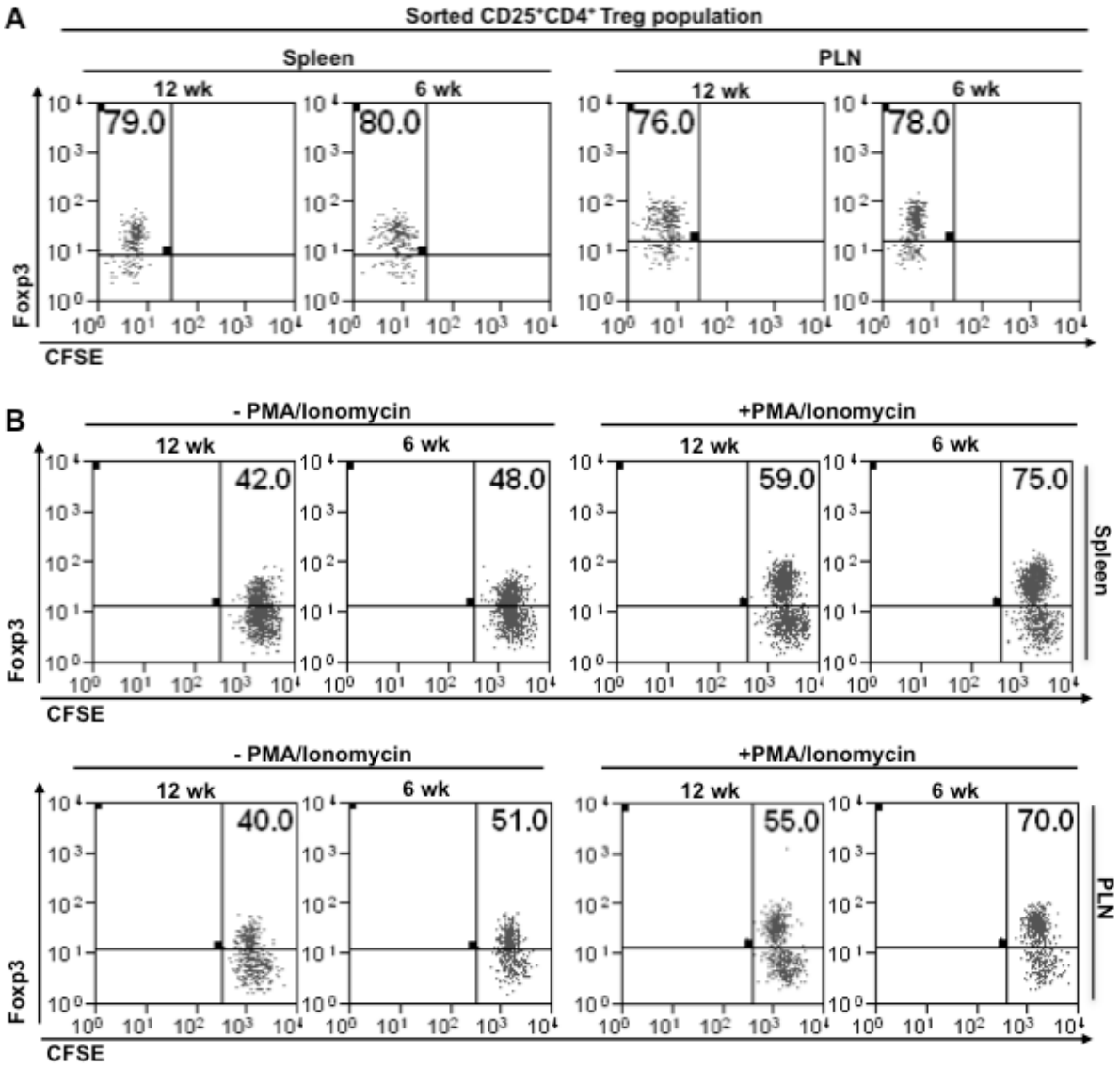

Fig. 22. Increased conversion of Foxp3$^+$ Tregs from 12-week-old NOD mice *ex-vivo*: A) sorted CD25$^+$ CD4$^+$ Tregs were stained with Foxp3 to determine the purity of the sorted population. B) Tregs isolated from spleens (top) or PLNs (bottom) of 12 or 6 wk old NOD mice were CFSE labeled and co-cultured with the respective total splenocytes, in the presence or absence of cell stimulation cocktail (PMA/ionomycin). After 24-hrs, cells were analyzed for Foxp3 Expression in the CFSE$^+$ population. 12-wk-old Tregs show increased loss of Foxp3 expression in the CFSE$^+$ population.

5.2.2 INCREASED *EX-VIVO* CONVERSION OF 12-WEEK Treg IS DEPENDENT ON THE LOCAL MICROENVIRONMENT

To elucidate if the local cytokine environment present in 12-week-old NOD mice drives their conversion *ex-vvio,* we co-cultured CFSE-labeled Tregs from 12-week-old mice with total splenocytes from 6-week-old, and Tregs from 6-week-old mice with splenocytes from 12-week-old mice. If the cytokine microenvironment was the main driver of Treg conversion, then Tregs from 12-week-old old mice should show sustained Foxp3 expression when co-cultured with 6-week-old splenoctyes, while 6-week-old Tregs should show enhanced loss of Foxp3 when co-cultured with 12-week-old splenoctyes. As described above, $CD4^+CD25^+$ Tregs (highest 2%) were sorted from freshly isolated spleens of 12 and 6-week-old NOD mice, labeled with CFSE and co-cultured back with 12 or 6-week splenocytes in the presence or absence of cell stimulation cocktail (PMA/ionomycin) (Fig. 23). Interestingly, co-culturing 12-week-Tregs with 6-week-splenocytes prevented the conversion of 12-weekTregs as the majority of $CFSE^+$ Tregs remained $Foxp3^+$ (71 ± 3.9 %) compared to 12-week Tregs + 12-week splenocytes cultures (57 ± 2.1 %). On the contrary, culturing 6-week Tregs with 12-week splenocytes resulted in decreased percentage of $CFSE^+Foxp3^+$ Tregs (61 ± 2.5 %) compared to the 6-week Tregs + 6-week splenocytes cultures (72 ± 3.1).

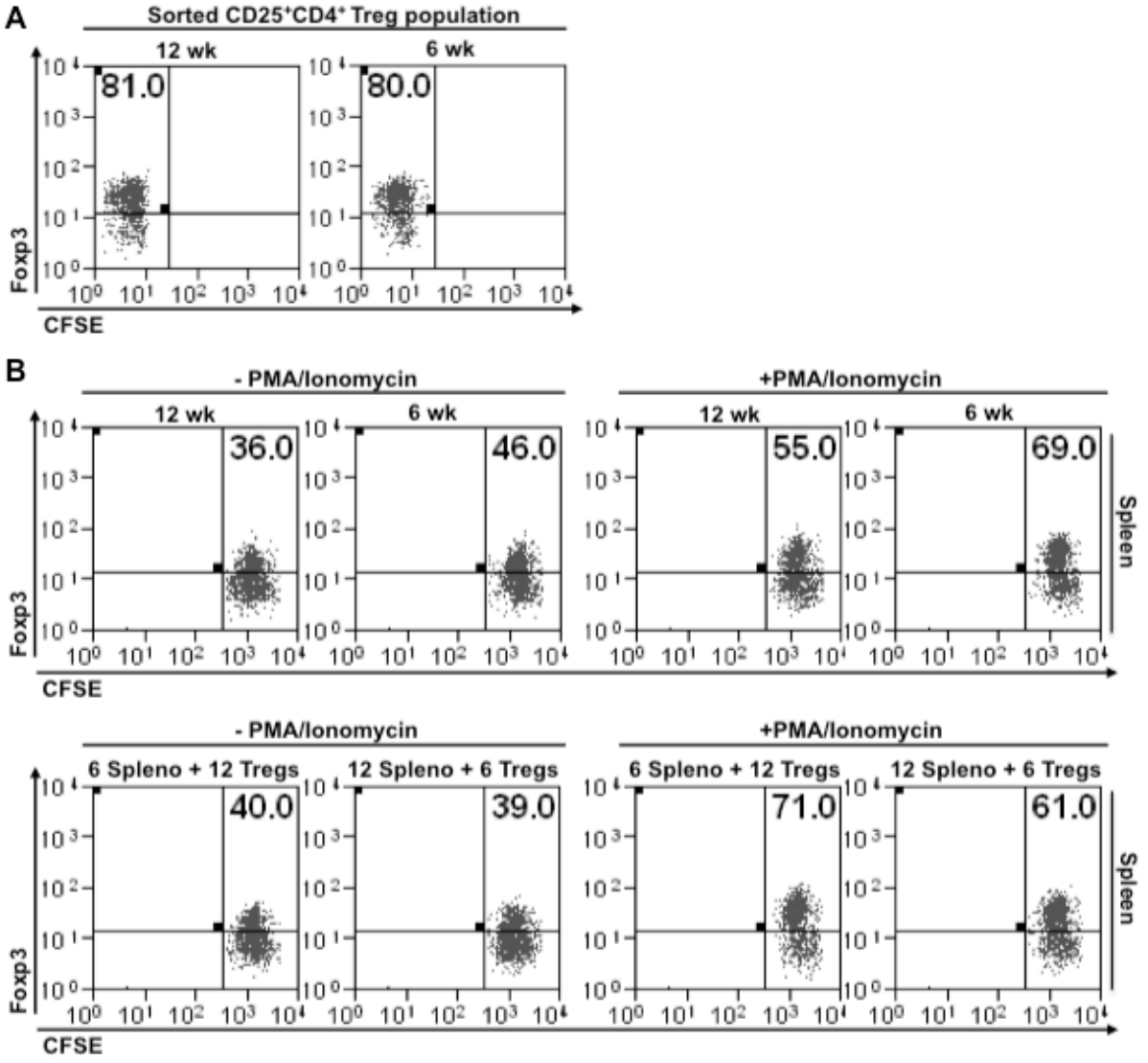

Fig. 23. Local cytokine environment plays a critical role in the conversion of Tregs *ex-vivo*: A) sorted $CD25^+$ $CD4^+$ Tregs were stained with Foxp3 to determine the purity of the sorted population. B) Sorted Tregs were CFSE labeled and then 6-week Tregs were co-cultured with 12-week total splenocytes,(bottom) and vice versa, in the presence or absence of PMA/Ionomycin. After 24 hrs, cells were analyzed for Foxp3 Expression in the $CFSE^+$ population. Co-culturing of 12-wk Tregs with 6-wk splenocytes prevents loss of Foxp3 expression.

5.2.3. INCREASED IL-6 DOES NOT CONTRIBUTE TO THE CONVERSION OF Tregs IN THE PERIPHERY OF 12-WEEK-OLD NOD MICE

Labile Tregs have been shown to undergo conversion into "helper-like" cells under inflammatory setting. Therefore, to determine if OX40L treatment has different outcomes on 6-week vs. 12-week-old mice due to the presence of an inflammatory cytokine, such as IL-6, in the periphery of 12-week-old NOD mice, we compared the cytokine expression profile in the spleen of 6 and 12-week-old NOD mice (Fig. 24) using the mouse inflammatory cytokines and receptor RT^2 Profiler PCR Array (SABiosciences, CA). Interestingly, splenocytes of 12-week-old NOD mice showed higher production of inflammatory cytokines, namely IFN-γ and IL-17, upon stimulation with PMA/ionomycin compared to splenocytes from 6-week-old NOD mice. Additionally, splenocytes from 12-week-old NOD mice showed a slightly higher production of IL-6 upon stimulation compared to splenocytes from 6-week-old mice.

Tregs are known to differentiate from naïve $CD4^+$ T cells in the presence of TGF-β. However, in the presence of TGF-β and IL-6, this differentiation pathway is skewed towards Th17 cells (224, 225). Therefore, IL-6 is known to maintain the balance between Treg/Th17 (226). Furthermore, IL-6 has been shown to play a role the reprogramming of labile Tregs (213). Thus, we predicted that the increased levels of IL-6 in the periphery of 12-week-old NOD mice might be contributing to their conversion.

In order to determine whether IL-6 was involved in the conversion of Tregs, we conducted a pilot experiment (n = 3 mice/group) where 12-week-old NOD mice were treated with OX40L and anti-IL-6 blocking antibody. We suspected that blocking IL-6 would prevent the conversion of Tregs in the periphery and thus would prevent the

rapid onset of T1D seen upon OX40L. Surprisingly, co-administration of OX40L + anti-IL-6 did not prevent the rapid onset of hyperglycemia seen in mice treated with OX40L alone. Mice co-treated with OX40L/anti-IL-6 showed rapid onset of T1D (100% of mice became hyperglycemic within 1 week of 1^{st} administration of OX40L/anti-IL-6), as seen in the OX40L alone treatment group. Additionally, co-administration of OX40L/anti-IL-6 did not induce $Foxp3^{+}$ Tregs expansion in either the spleen (14.0 ± 0.7 %) or PLN (12.0 ± 1.2 %), compared to the PBS treated control mice (13.0 ± 1.5 %; *p*-value = 0.4 and 10.0 ± 1.5 %; *p*-value = 0.122). These data suggested that increased IL-6 was likely not responsible for the conversion of Tregs in the periphery of 12-week-old NOD mice.

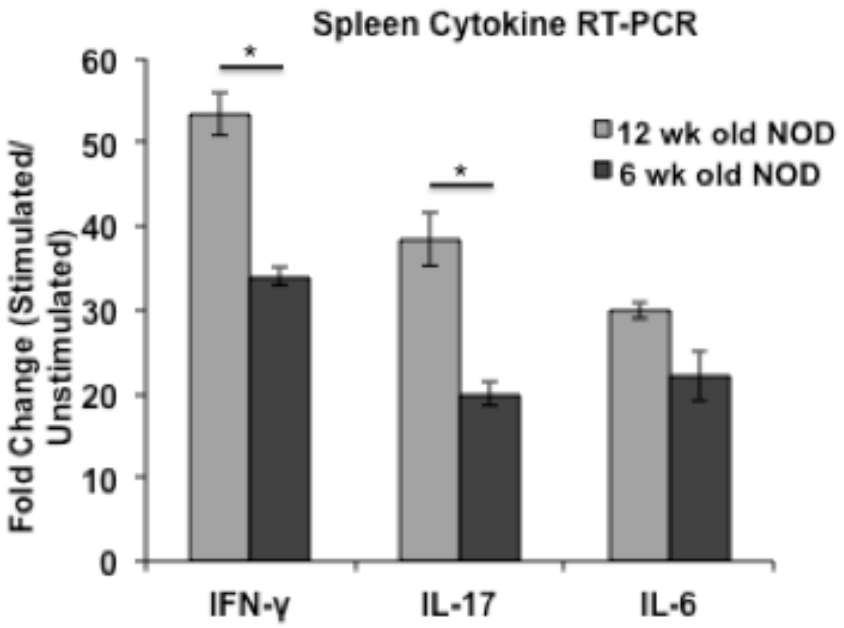

Fig. 24. Increased production of IFN-γ and IL-17 in splenocytes of 12-week-old mice: Splenocytes from 12 or 6-week-old NOD mice were isolated and cultured in the presence or absence of PMA/ionomycin for 4-6 hrs. RNA was then purified from each sample and reversed transcribed into cDNA. RT2-PCR was performed using the mouse inflammatory cytokines and receptor RT^2 Profiler PCR Array (SABiosciences, CA). Data were analyzed using the ΔΔCt method. The relative mRNA expressions for each cytokine in stimulated samples were normalized to the mRNA levels in unstimulated samples. (Data are representations of 3 technical replicates). (*) indicates p-value < .05.

5.2.4 IL-2 PREVENTS CONVERSION OF TREGS IN *EX-VIVO* CULTURES

Failure to sustain Treg expansion in the periphery of 12-week old NOD mice upon OX40L/anti-IL-6 co-administration suggested that another cytokine/mechanism is responsible for the enhanced conversion in 12-week-old mice. We noted that in the absence of cell stimulation (PMA/ionomycin), Tregs from both 12- and 6-week-old NOD mice were undergoing rapid conversion. This suggested that secretion of particular cytokine/s was required to sustain the $Foxp3^+$ population. IL-2 is known to be required for Treg cell survival and maintenance (144). Deficiencies in IL-2 production and IL-2 signaling have been implicated in the pathogenesis of T1D in the NOD mice (31, 146). Therefore, we tested whether the mere addition of IL-2 to the 12-week Tregs-splenocytes could prevent the conversion of $Foxp3^+$ Tregs (Fig. 25). Interestingly, addition of IL-2, to the stimulated cultures, maintained $Foxp3^+$Treg population in 12-week cultures, as indicated by the high percentage of $CFSE^+Foxp3^+$ T cells, compared to cultures stimulated in the absence of IL-2 (73 ± 1.5 vs. 57 ± 2.1; *p*-value = .0002). Furthermore, addition of IL-2 to even un-stimulated co-cultures prevented conversion of $Foxp3^+$ Tregs in both 12-week (73 ± 1.4 vs. 39.3 ± 3.1; *p*-value = .007) and 6-week cultures (73 ± 1.4 vs. 39.3 ± 3.1; *p*-value = .007) compared to controls lacking in exogenous IL-2. These data highly suggest that a decrease in IL-2 production in the 12-week splenocytes may be responsible for the inability of 12-week-old mice to sustain higher numbers of Tregs and/or Foxp3 expression.

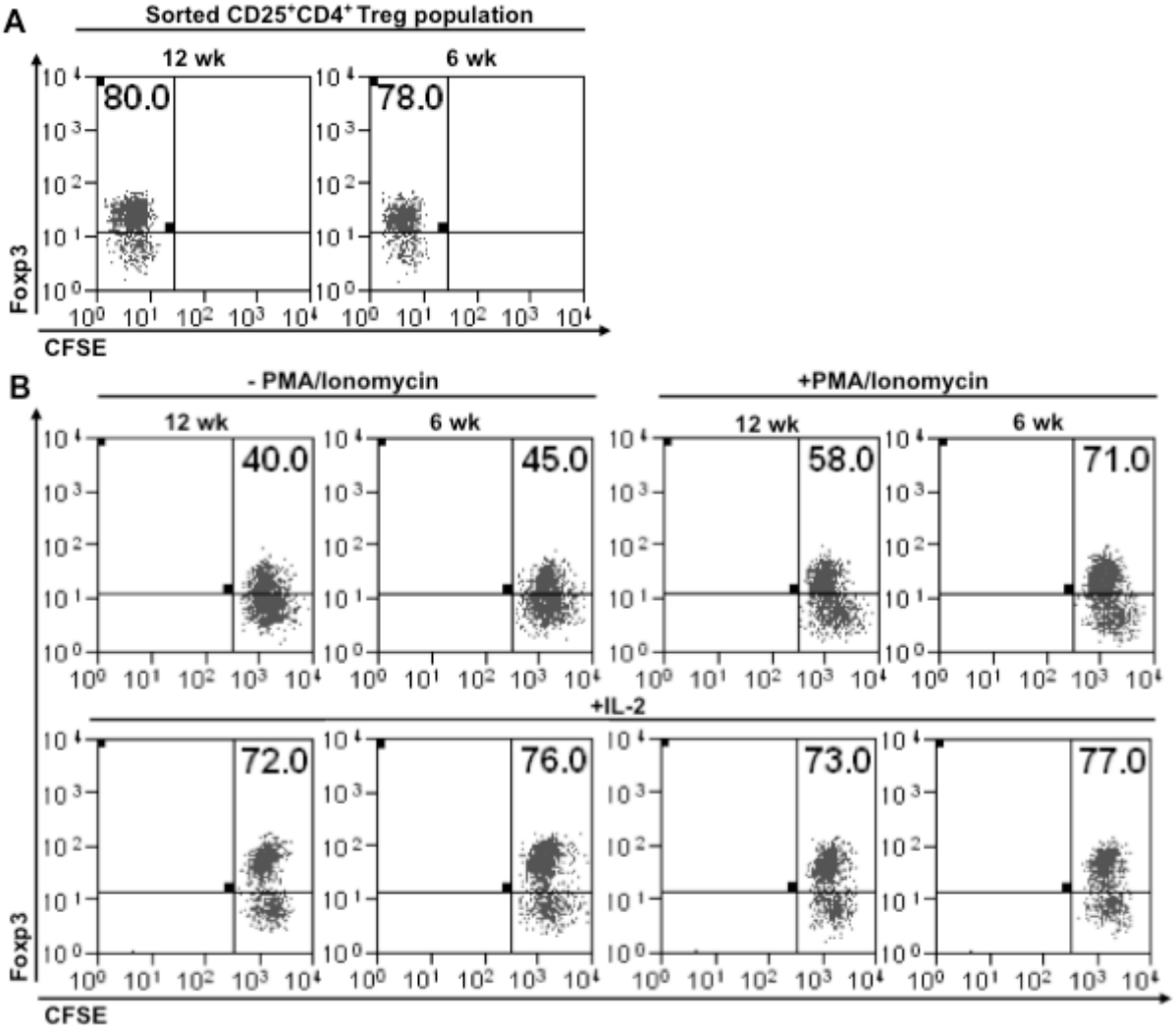

Fig. 25. IL-2 prevents the conversion of Tregs in *ex-vivo* cultures: A) sorted $CD25^+$ $CD4^+$ Tregs were stained with Foxp3 to determine the purity of the sorted population. B) Sorted Tregs from 12- or 6-week old mice were CFSE labeled and co-cultured with respective total splenocytes, in the presence (top) or absence (lower) of IL-2. After 24 hrs, cells were analyzed for Foxp3 Expression in the $CFSE^+$ population. Addition of IL-2 sustains Foxp3 expression in 12-wk cultures.

5.2.5 IL-2/OX40L CO-TREATMENT INDUCES SUSTAINED Treg EXPANSION IN THE PERIPHERY OF 12-WEEK-OLD MICE

To further determine the role of IL-2 in sustaining Foxp3$^+$Tregs, we tested whether the co-administration of OX40L and IL-2 could prevent the rapid onset of T1D induced by OX40L treatment alone and lead to sustained increase in Foxp3$^+$ Tregs in the periphery of 12-week old NOD mice. 12-week-old NOD mice received 3 treatments (1 treatment/week X 3 weeks) of OX40L (200ug/mouse) and IL-2 (25,000 U/mouse). Following the three treatments, mice were sacrificed and the spleens, PLNs and thymus were analyzed for Foxp3 expression. Interestingly, co-administration of OX40L/IL-2 increased Foxp3$^+$ Tregs in the spleen (26.7 ± 6.0 %) and PLN (19.2 ± 0.8%) compared to OX40L alone group (Spleen = 13.3 ± 1.0 %, *p*-value = .033; PLN = 13.0 ± 1.4 %, *p*-value = 0.004) and the control group (Spleen = 14.5 ± 0.7 %, *p*-value = 0.04; PLN = 11.1 ± 1.2 %, *p*-value = .0003). Additionally, mice that received OX40L/IL-2 showed an increase in thymic Tregs (6.0 ± 2.1 %), similar to the increase seen upon OX40L treatment alone (4.5 ± 0.5 %; *p*-value 0.14), compared to the control group (1.5 ± 0.7 %; *p*-value = 0.04). Administration of IL-2 alone did not have any proliferative effects on Tregs, as the frequency of Foxp3$^+$ in the thymus (1.8 ± 0.4 % vs. 1.5 ± 0.7 %; *p*-value = 0.34), spleen (13.5 ± 0.7 % vs. 14.5 ± 0.7 %; *p*-value = 0.15) and PLN (11.1 ± 1.2 % vs. 12.8 ± .4 %; *p*-value = 0.09), remained comparable to the control group. Finally, co-administration of OX40L/IL-2 prevented the rapid onset of hyperglycemia induced by OX40L alone. However, this experiment was performed using three mice only, therefore, repeating this experiment with a larger number of mice is needed to further confirm these data.

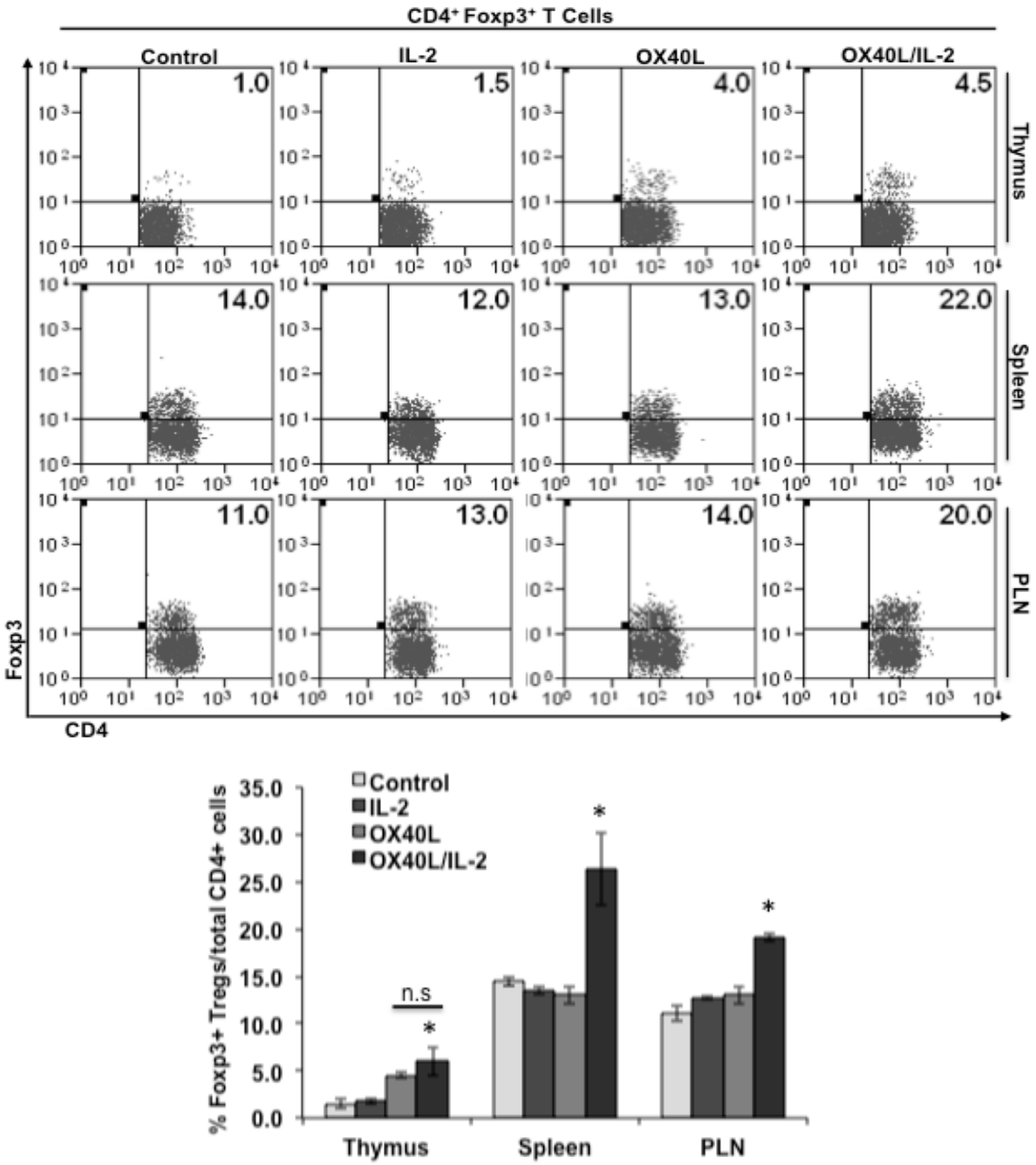

Fig. 26. OX40L/IL-2 co-administration induces sustained Treg expansion in 12-week-old NOD mice *in-vivo*: Thymus (top), spleens (middle), PLN (bottom) were isolated from 12-week-old NOD, treated with IL-2 (25,000 IU), OX40L (200 ug) or a combination of OX40L/IL-2. Cells were analyzed for Foxp3 expression by FACS. Mice co-treated with OX40L/IL-2 show increased frequency of Foxp3$^+$ Tregs in the central and peripheral lymphoid organs. Bar graphs are statistical representation of Treg percentages in different treatment groups; n=3/group. (*) Indicates *p*-value < 0.05.

5.3 DISCUSSION:

Several lines of evidence have suggested that, upon repeated proliferation, Tregs tend to lose their suppressive function and expression of Foxp3 (200, 204, 227). Additionally, recent reports have described the presence of a “labile” subset of Tregs that has the capacity to undergo reprogramming and gain “helper-like” function under certain pro-inflammatory conditions (213). The age-dependent dichotomous effects of OX40L and the failure to expand Tregs in the periphery of 12-week-old NOD mice highly suggested that expanded Tregs may in fact convert and lose Foxp3 expression upon reaching the periphery of 12-week-old mice. Indeed, in ex-vivo assays, we show that Tregs from 12-week-old mice show rapid loss of Foxp3 expression compared to 6-week-old mice. This loss of Foxp3 was driven by the local cytokine microenvironment induced by 12-week-old splenocytes, as co-culturing 12-wk Tregs with 6-wk total splenocytes prevented the loss of Foxp3 expression. Further studies revealed a critical role for IL-2 in preventing the rapid conversion of 12-week-old Tregs. The mere supplementation with IL-2 to the splenocytes-Treg co-cultures supported sustained Foxp3 expression in 12-week cultures, even in the absence of cell stimulation. Subsequently, 12-week-old NOD mice were co-treated with OX40L/IL-2 to determine if IL-2 can reverse the effects of OX40L. Interestingly, co-administration of OX40L/IL-2 induced Treg expansion in the thymus and peripheral lymphoid organs and prevented the rapid onset of hyperglycemia induced by OX40L. Administration of IL-2 alone did not expand Tregs in the periphery, suggesting that co-signaling from OX40L and IL-2 is required to expand and sustain Treg survival in the periphery of 12-week-old NOD mice.

OX40 signaling is known to drive T-cell proliferation in the context of TCR-ligation. However, in the absence of TCR, OX40 can form a signalosome and drive T cell proliferation and survival. Based on our data, we propose that OX40L/OX40 can drive Treg proliferation in a TCR-independent manner in thymocytes, where thymic APCs and thymic stromal cells may further contribute thymic Treg proliferation. Once the expanded Tregs migrate to the periphery, they require cytokines, such as IL-2, to sustain their Foxp3 expression and for their survival. Defects in IL-2 signaling have been described and linked to disease pathogenesis in NOD mice (31, 145, 146). Our data suggest that the defect occurs in an age-dependent manner, which may explain why diabetes begins to occur starting at 12-weeks in NOD mice. Although it is possible that IL-2 production is optimal, but the defect is due to a loss of IL-2 sensitivity in Tregs, culturing 12-week Treg with 6-week splenocytes resulted in sustained Foxp3 expression. In contrast, when Tregs from 6-week-old mice were co-cultured with splenocytes from 12-week-old mice, there was a higher loss of $Foxp3^{+}$ compared to when 6-week Tregs were co-cultured with splenocytes from 6-week-old mice, suggesting a central defect is in IL-2 production by effector T cells. Although we did not measure IL-2 levels in 12-week vs. 6-week-old NOD mice, we suspect that there is decreased production of IL-2 in the periphery of 12-week-old NOD mice. Effector T cells require activation by APCs to produce IL-2. DC defects in NOD mice have been reported previously. Thus, the defects in IL-2 production could be either due to defective APC function or defective response by T cells. Our future work will focus on determining the levels of IL-2 in 12 vs. 6-week-old NOD mice, and determining whether decreased IL-2 production is due to defect in APCs or effector T

cells. Additionally, expanded Tregs may be undergoing apoptosis in the periphery of 12-week-old NOD mice, therefore, we will test for increased Treg apoptosis in these mice. Finally, we will carry adoptive transfer studies to further confirm conversion of Tregs in-vivo in the 12-week-old NOD mice.

6. CONCLUSION

6.1 GENERAL DISCUSSION:

Type 1 diabetes mellitus (T1D) is a T-cell- mediated disease characterized by destruction of pancreatic beta cells, resulting in life-long dependence on insulin therapy. Unfortunately, the only current treatment option for T1D is insulin therapy, which helps maintain euglycemia in these patients but does not cure the underlying autoimmune defects. Although different immunotherapies have been proposed as a potential treatment for T1D, most had no promising outcomes. $Foxp3^+$ Regulatory T cells play a crucial role in maintaining peripheral tolerance and suppressing unwanted autoimmune responses. Many studies have suggested a therapeutic potential for Tregs in autoimmune diseases, including T1D. Unfortunately, as Tregs are a subset of $CD4^+$ T cells, specific targeting of Tregs without contamination with effector T cells has remained a big challenge. Additionally, inducing large numbers of Tregs without loss of their suppressive function is another big challenge of the field. Thus, finding therapeutic targets that can specifically enhance Tregs numbers/function is of great clinical value.

We have previously shown that treatment with GM-CSF can increase $Foxp3^+$ Tregs *in-vivo* and protect against different autoimmune diseases, such as MG, EAT and T1D (152, 154, 155). GM-CSF acted mainly on bone marrow precursor and administration of GM-CSF induced the mobilization of a specialized subset of DC,that was $CD11c^+CD11b^+B220^-CD8\alpha^-$. Later work showed that GM-CSF differentiated bone marrow dendritic cells, but not splenic dendritic cells (SpDCs), were capable of expanding Tregs *ex-vivo* (156). GM-BMDC-induced Treg expansion was TCR-independent, IL-2 dependent, and required signaling through OX40L/OX40 (157).

Both OX40L and OX40 are members of the TNFRS. OX40 has been well characterized as a co-stimulatory molecule with a role in effector T cell proliferation, survival and function (158, 159). In contrast, the role of OX40 in Treg function is still controversial, with some studies suggesting that OX40 signaling blocks Treg function (160, 190, 195), while others showing that it can enhance Treg proliferation and function (163, 164, 199). In a model of EAE, OX40 signaling has been proposed to have two different outcomes on Treg function, depending on the local cytokine milieu (163). Thus, understanding the critical role of OX40L/OX40 signaling in Treg homeostasis and function is critical to either enhance or suppress Treg function in the context of autoimmunity or cancer, respectively.

In this context, this study aimed to 1) determine the full potential of using GM-BMDCs to expand Tregs in the NOD mice, 2) determine the role of OX40/OX40L signaling in this expansion, 3) determine the effects of soluble OX40L on Treg phenotype and function in vivo in NOD mice, 4) understand the mechanism underlying the divergent effects of OX40 signaling on Treg phenotype.

In chapter 3, we show that GM-BMDCs can expand Treg ex-vivo in NOD mice, and that OX40/OX40L was critical for that expansion. Expanded Tregs retained their functional capacity to suppressive effector T cell proliferation, which indicate that GM-BMDCs can be potentially used to expand large numbers of functional Treg *ex-vivo*, which can be used to suppress autoimmune responses *in-vivo*. Additionally, our data suggest that BMDCs can be directly adoptively transferred to induce Treg expansion *in-vivo*.

Given the challenges of cell-based therapy and the controversial role of OX40L on Treg function, we subsequently determined the effects of soluble OX40L on Treg function and disease phenotype *in-vivo* in the non-obese diabetic (NOD) mouse model. Interestingly, OX40L induced dichotomous effects depending on the time of administration. Treatment of 12-week-old NOD mice with OX40L induced rapid onset of hyperglycemia, with an increase in Tregs in the thymus but not the periphery. In contrast, treatment of 6-week-old NOD mice did not induce rapid disease onset, and resulted in a significant increase in Tregs in the thymus and the periphery. The failure to expand Tregs in the periphery of 12-week-old mice was specific to NOD mice, as treatment of 12-week-old Balb/c mice induced marked expansion of Tregs in the thymus and the periphery. Accordingly, treatment of 6-week-old thymectomized mice failed to expand Treg in the periphery, which suggested that OX40L acts primarily on cells in the thymus. Finally, OX40L-treated 12-week-old NOD mice showed an increase in the labile subset of Tregs in the thymus. Collectively, these data suggested OX40L induces expansion of thymic Tregs in both 6- and 12-week-old mice. However, upon migration to the periphery, expanded Tregs undergo rapid conversion resulting in loss of Foxp3 expression in 12-week-old NOD mice. As Tregs are known to self-reactive, the converted Tregs may in fact contribute to the increase in the autoreactive $InsB_{9\text{-}23}^{+}$ $CD4^{+}$ T cells and consequent rapid onset of hyperglycemia seen in treated 12-week-old NOD mice.

In this context, in chapter 5, we show that Tregs from 12-week-old mice under go rapid conversion into $Fop3^{-}$. This conversion was most likely driven by the local cytokine microenvironment as culturing 12-week Tregs with 6-week splenocytes

prevented the rapid loss of Foxp3 expression. Subsequently, addition of IL-2 to the cultures was sufficient to prevent the conversion of 12-week Tregs. As opposed to effector T cells, Tregs require IL-2 for their survival and maintenance. Additionally, defects in IL-2 production and signaling have been reported in NOD mice. Thus, we suspected that low levels of IL-2 may contribute to the inability to sustain Treg expansion in the periphery of 12-week-old mice. Interestingly, co-administration of OX40L/IL-2 prevented the rapid onset of hyperglycemia induced by OX40L alone and resulted in sustained Treg expansion in the periphery of 12-week-old mice.

OX40L/OX40 signaling is well known to induce T cell proliferation in the context of TCR signaling. Based on our findings, we propose that in the absence of TCR signaling, OX40L acts primarily on the thymus to induce Treg proliferation. This proliferation probably requires signaling through other molecules, expressed on thymic APCs and perhaps thymic stromal cells. This may explain why the mere addition of soluble OX40L, alone or in the presence of SpDCs, cannot expand Tregs *ex-vivo* (157).

Regulatory T cells require IL-2 for their survival and homeostasis, although they do not produce IL-2. Mice deficient in IL-2, IL-2R or CD25 have reduced Treg numbers and develop lethal autoimmune disease (220-222). Although some studies suggest that IL-2 is not necessary for the development of Tregs in the thymus as Foxp3 expressing Tregs are present in IL-2 KO and IL-2R KO mice (144, 222), others have shown that the level of Foxp3 protein expression as well as the frequency of Tregs are reduced in these mice, suggesting a role of IL-2 in thymic Treg

homeostasis (144, 228, 229). Thus, if the failure to sustain Tregs in the periphery of OX40L-treated 12-week-old NOD mice is due to a defect in IL-2 production/signaling, then why is there an increase in Foxp3+ Tregs in thymus of these mice? This may be due to a difference in the mechanism of IL-2 production in the thymus vs. the periphery. In the periphery, IL-2 is usually produced by effector T cells following antigen presentation. However, IL-2 producing cells have been detected in the thymus even in the absence of TCR signaling (229). Additionally, it has been suggested that IL-2 production in the thymus may occur in T cells undergoing selection (220). Alternatively, IL-2 production in the periphery requires activation of T cells by APCs. Thus, although IL-2 production in the periphery of 12-week-old NOD mice may be deficient, thymic IL-2 production may still be intact or sufficient. While our study has not conclusively demonstrated a defect in IL-2 production in the periphery of 12-week-old mice, we propose that the defect may be either in the function of the APCs or effector T cells. DCs defects in NOD mice have been reported in the literature (102, 230, 231). Thus, a defect in APC function may lead to poor T cell activation and thus poor production of IL-2. Alternatively, a defect in T cell response to activation by APCs, such as decreased IL-2 production by T cells, may be responsible for the suboptimal IL-2 production in NOD mice.

Collectively, our data show that OX40L/OX40 is required to drive thymic Treg proliferation, while IL-2 is required to sustain Treg expansion and survival in the periphery of NOD mice. Furthermore, our study is of high clinical relevance as it demonstrates that immunotherapies may have vastly different outcomes depending on the stage of disease progression. Thus, depending on the time of administration

during the course of disease progression, therapies may need to be modulated (i.e. co-adminstration of IL-2 and OX40L) in order to produce the desired outcomes in patients.

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Publications

Gopisetty, A., Bhattacharya, P., **Haddad, C.**, Bruno, JC Jr., Vasu, C., Miele, L., Prabhakar, BS. OX40L/Jagged1 Cosignaling by GM-CSF-Induced Bone Marrow-Derived Dendritic Cells Is Required for the Expansion of Functional Regulatory T Cells. J Immunol. 2013;190(11):5516-25

Elshabrawy, HA., Fan, J., **Haddad, CS.**, Ratia, K., Broder, CC., Caffrey, M., Prabhakar, BS. Identification of a broad-spectrum antiviral small molecule against severe acute respiratory syndrome coronavirus and Ebola, Hendra, and Nipah viruses by using a novel high-throughput screening assay. J Virol. 2014 Apr;88(8):4353-65.

Li, LC., Wang, Y., Carr, R., **Haddad, CS.**, Li, Z., Qian, L., Oberholzer, J., Maker, AV., Wang, Q., Prabhakar, BS. IG20/MADD Plays a Critical Role in Glucose-Induced Insulin Secretion. Diabetes. 2014 May;63(5):1612-23

Bhattacharya, P., Fan, J., **Haddad, C.**, Essani, A., Gopisetty, A., Elshabrawy, HA., Vasu, C., Prabhakar, BS. A novel pancreatic β-cell targeting bispecific-antibody (BsAb) can prevent the development of Type 1 diabetes in NOD mice. Clin Immunol. 2014 May. [Epub ahead of print]

Presentations

Haddad, CS. , Bhattacharya, P., Prabhakar, B.S. Expansion of Regulatory T-cells by GM-CSF Differentiated Bone Marrow Dendritic Cells in NOD Mice. Poster Presentation. UIC College of Medicine Research Forum. Chicago, IL: November 16, 2013

Haddad, CS. , Bhattacharya, P., Prabhakar, B.S. The Role of OX40/OX40L Signaling in the Expansion of Regulatory T cells in the NOD Mice. Poster Presentation. UIC College of Medicine Research Forum. Chicago, IL: November 20, 2013

Haddad, CS. , Bhattacharya, P., Prabhakar, B.S. The Role of OX40/OX40L Signaling in the Expansion of Regulatory T cells in the NOD Mice. Poster Presentation. ADA 74th Scientific Sessions. San Francisco, CA: June 13-17, 2014

Grants and Awards

Jan. 2013-Jan. 2014: American Heart Association MWA Summer 2012 Predoctoral Fellowship
Jan. 2014-Present: Center for Clinical and Translational Science (CCTS) Pre-doctoral Education for Clinical and Translational Scientists (PECTS) Fellowship