PKC-Ɵ is Dispensable for G-BMDC-Induced TCR-Independent Treg Proliferation

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THESIS

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DEDICATION

This thesis is dedicated to my Mother, Father, Wife, and Children; without your support I would have never accomplished this.

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LIST OF ABBREVIATIONS OR NOMENCLATURE

APCs: Antigen presenting cells

BM: Bone marrow

CD: Cluster of differentiation

CTLA4: Cytotoxic T-lymphocyte associated protein 4

DCs: Dendritic cells

DN: Double negative

DP: Double positive

Foxp3: Forkhead box P3

GM-CSF: Granulocytes Macrophages colony-stimulating factor

G-BMDCs: GM-CSF-induced bone marrow-derived dendritic cells

IFN-γ: Interferon gamma

II-KO-BMDCs: MHC Class II deficient G-BMDCs

IPEX: Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked

MHC: Major histocompatibility complex

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NOD mice: Non obese diabetic mice

OX40-KO: OX40 knockout

PKC-Θ: Protein kinase C-theta

PKC-Θ-KO: PKC-Θ knockout

RORγt: RAR-related orphan receptor gamma t

SP: Single positive

SpDCs: Splenic dendritic cells

TCR: T cell receptor

TGF-β1: Transforming growth factor beta 1

Tfh: Follicular helper CD4+ T cells

Th: T helper cells

TNF: Tumor necrosis factor

TRAF: TNF receptor associated factor

Treg: Regulatory T cell

WT-BMDCs: Wild type G-BMDCs

SUMMARY

GM-CSF-induced bone marrow derived dendritic cells (G-BMDCs) have been shown by our lab to be able to induce preferential Treg proliferation when co-cultured with $CD4^+$ T cells. Interestingly, this Treg proliferation was shown to be TCR-independent but OX40L/OX40-dependent. We studied the signaling that drives the G-BMDC-induced Treg proliferation.

Firstly, we confirmed the critical role of OX40L/OX40 signaling in Treg expansion by using $CD4^+$ T cells from OX40 deficient mice. We also showed, using suppression assay and treatment of NOD mice, that these induced Tregs maintained their phenotype and suppressive function ex vivo and in vivo.

PKC-Ɵ was shown to play an important role downstream of OX40 signaling in the absence of TCR stimulation in earlier studies, therefore we investigated the role of PKC-Ɵ in G-BMDC-induce Treg expansion. Interestingly, $CD4^+$ T cells from PKC-Ɵ deficient mice, upon co-culture with WT G-BMDCs, show impaired Treg proliferation. However, supplementation of the co-culture with exogenous IL-2 rescued this impairment of Treg proliferation, suggesting that G-BMDC-induced Treg proliferation was PKC-Ɵ-independent. Our data further suggests that PKC-Ɵ is likely required for optimum IL-2 production by T effector cells.

Finally, our data showed an upregulation of TRAF1 mRNA in Tregs compared to T effector cells. Our data also showed co-localization of TRAF1 and OX40 which suggested that OX40 mediated downstream signaling in Tregs is likely to involve TRAF1.

1. INTRODUCTION

1.1 Background

The origin of immunology as a science is typically linked to Edward Jenner, an 18th century English physician and scientist (1). He observed that inoculation with cowpox (or vaccinia), which typically causes a mild disease, could protect against the fatal disease of smallpox (1). This experiment, termed vaccination, demonstrated for the first time the concept of what we know today as the adaptive immune response. This immune response is the mechanism by which the host develops and improves its skills to fight against infectious diseases or malignancy. Therefore, the main function of the immune system is to provide protection against infectious diseases and to eliminate any potential cellular malignant development. This protection is achieved by non-specific means called innate immunity, and/or by specific responses with memory capability called adaptive immunity (1).

The immune system executes its functions via four immunological tasks (1). The first task is recognition. In this stage the detection of infectious agents or abnormal cells takes place. The detection process involves phagocytic cells from the innate immunity and lymphocytes from the adaptive immunity. The second task is effector function. This task achieves the elimination of infected or malignant cells by means of blood proteins, antibodies, and the cytotoxic capability of lymphocytes. The third immunological task is the regulation process. This task is critical to maintain control of immune response to avoid self-damage which would cause allergy or autoimmunity. And, finally, the last task is memory development. This is the fundamental capacity of the immune system that makes vaccination possible. This memory function allows the host to protect itself from recurring disease from a pathogen that has been encountered previously (1).

1.1.1 T cells and Immunity

T lymphocytes or T cells represent one of the main subtypes of lymphocytes which are essential for the adaptive immunity. T cell precursors generated in the red bone marrow migrate to the thymus to undergo development and maturation (1, 2). These T cell precursors and their development stages are recognized by the expression of two surface molecules, cluster of differentiation 4 (CD4) and CD8. At first they lack the expression of CD4, CD8, and T cell receptor (TCR) when they migrate into the thymus, and hence are termed double negative (DN) ($CD4^-CD8^-$) cells (1, 2). CD4 is a surface co-receptor molecule, expressed on helper and regulatory T cells ($CD4^+$) that binds to major histocompatibility complex (MHC) Class II during antigen recognition. It consists of four immunoglobulin-like domains in a single protein chain (1, 2). CD8 is also a surface co-receptor molecule, expressed on cytotoxic T cells ($CD8^+$), but it binds MHC Class I during antigen recognition. Structurally it is a heterodimer assembled with two protein chains; α and β which are linked together via disulfide bond (1, 2). TCR is the antigen receptor on the surface of mature T cells, and it is composed of α and β protein chains (1, 2).

The DN precursors undergo cycles of proliferation and differentiation to pass through four stages of development (Fig-1) divided based on the expression of CD44, an adhesion molecule, and CD25, the α-subunit of IL-2-receptor (1, 2). The first stage is DN1 which is phenotypically described as $CD44^+CD25^-$. DN2-stage is phenotypically described as $CD44^+CD25^+$, DN3-stage phenotypically described as $CD44^-CD25^+$, and DN4-stage phenotypically described as $CD44^-CD25^-$ (1, 2). During the progression of these stages, precursors express and assemble pre-mature TCR (pre-TCR), consisting of non-rearranged α-chain and rearranged β-chain (1, 2). After successful expression of

the pre-TCR during the DN4-stage, precursors go through extensive proliferation and transition to become double positive (DP) precursors with a rearranged TCR α-chain, replacing the pre-TCR and yielding a complete αβ TCR (αβ-TCR$^+$CD4$^+$CD8$^+$) (1, 2).

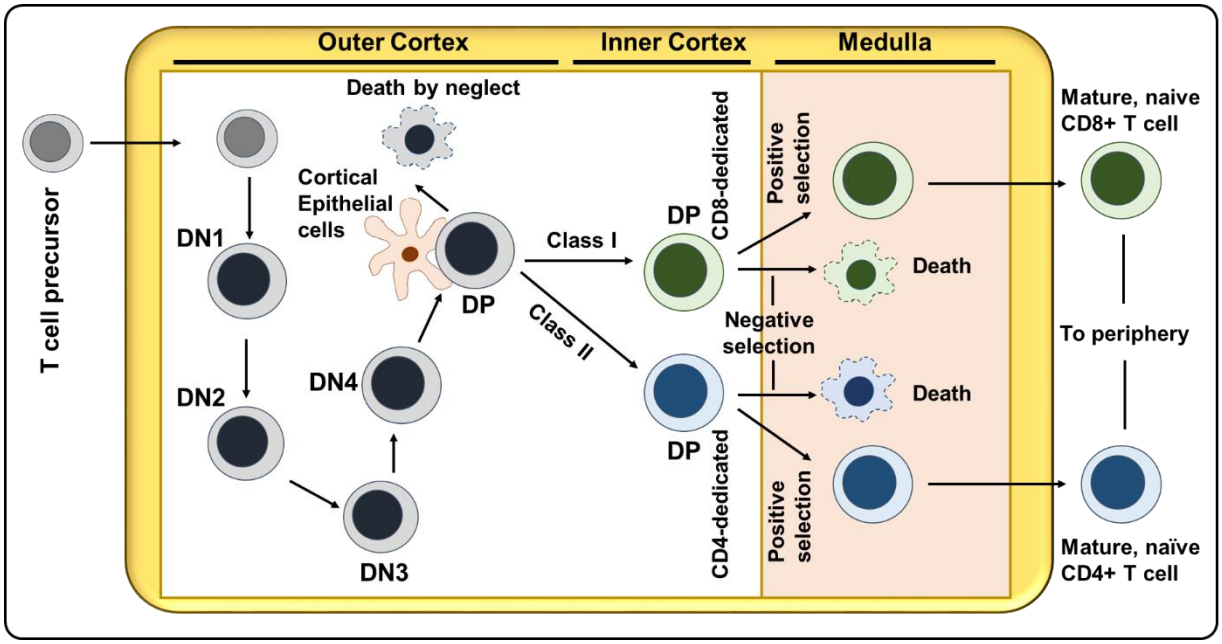

Fig. 1: Development of T cell precursors in the thymus: Precursors from the bone marrow migrate to thymus where they undergo maturation to become T cells. Starting as double negative (DN), to double positive, then becoming mature as single positive (CD4$^+$ or CD8$^+$) naïve T cells. (Source: adopted from Germain, 2002)

Subsequently, the fate of the αβ-TCR$^+$CD4$^+$CD8$^+$ DP thymocytes is dependent on the interaction of the thymocyte TCR with the self-peptide MHC ligands presented by epithelial cells in the thymic cortex that express self-peptide presented on MHC Class I and MHC Class II molecules (1, 2). Too weak of a signal induces delayed apoptosis of the αβ-TCR$^+$CD4$^+$CD8$^+$ DP thymocytes (1, 2). Too strong of a TCR-signal to the DP precursors can induce acute apoptosis or negative selection (1, 2). Suitable ranges of TCR signaling is required for maturation or positive selection. Precursors that bind MHC

Class II molecules or MHC Class I molecules in that suitable range, mature into single positive (SP) $CD4^+$ T cells and SP $CD8^+$ T cells, respectively. (1, 2).

Once development is completed, these mature naïve T cells are exported from the thymic medulla to peripheral lymphoid sites. In the periphery, these naïve mature T cells can become activated via a two signal mechanism (1, 3, 4). The first main signal is triggered when the naïve T cell encounters its cognate antigen of their TCR, expressed on the MHC molecules, on the surface of other cells (1, 3). $CD4^+$ T cells recognize antigens presented by professional antigen presenting cells (APCs) (1, 3). These antigens need to be presented by MHC class II, which are expressed only on the surface of APCs (1, 3). $CD8^+$ T cells, however, recognize antigens presented by MHC class I which is expressed by all nucleated cells (1, 3). The second signal comes from the ligation of co-stimulatory molecules, like CD28, expressed on the surface of T cells (1, 3-7). In the presence of these signals, T cells become activated, proliferate, and become effector T cells (Teffs) initiating pathogen destruction and clearance. A subset of these cells form the memory T cells, providing the host with protection against re-infection from the same pathogen (1, 3-7).

There are several subsets of T cells with their own functional attributes. Two major subsets of T cells that are generated as a result of T cell activation are cytotoxic $CD8^+$ T cells, specifically capable of destroying cells infected with intracellular pathogens (1, 3-7) and $CD4^+$ Helper T cells that facilitate the activation of other immunological cells like B lymphocytes (1, 3-7).

Amongst the CD4⁺ T cells there are several subsets and they include regulatory T cells (Tregs), and effector Th1, Th2, Th9, Th17, Th22, and Tfh cells (Fig-2) (8). The identification of Th1 and Th2 cells is based on their cytokine production profile (1, 8-11).

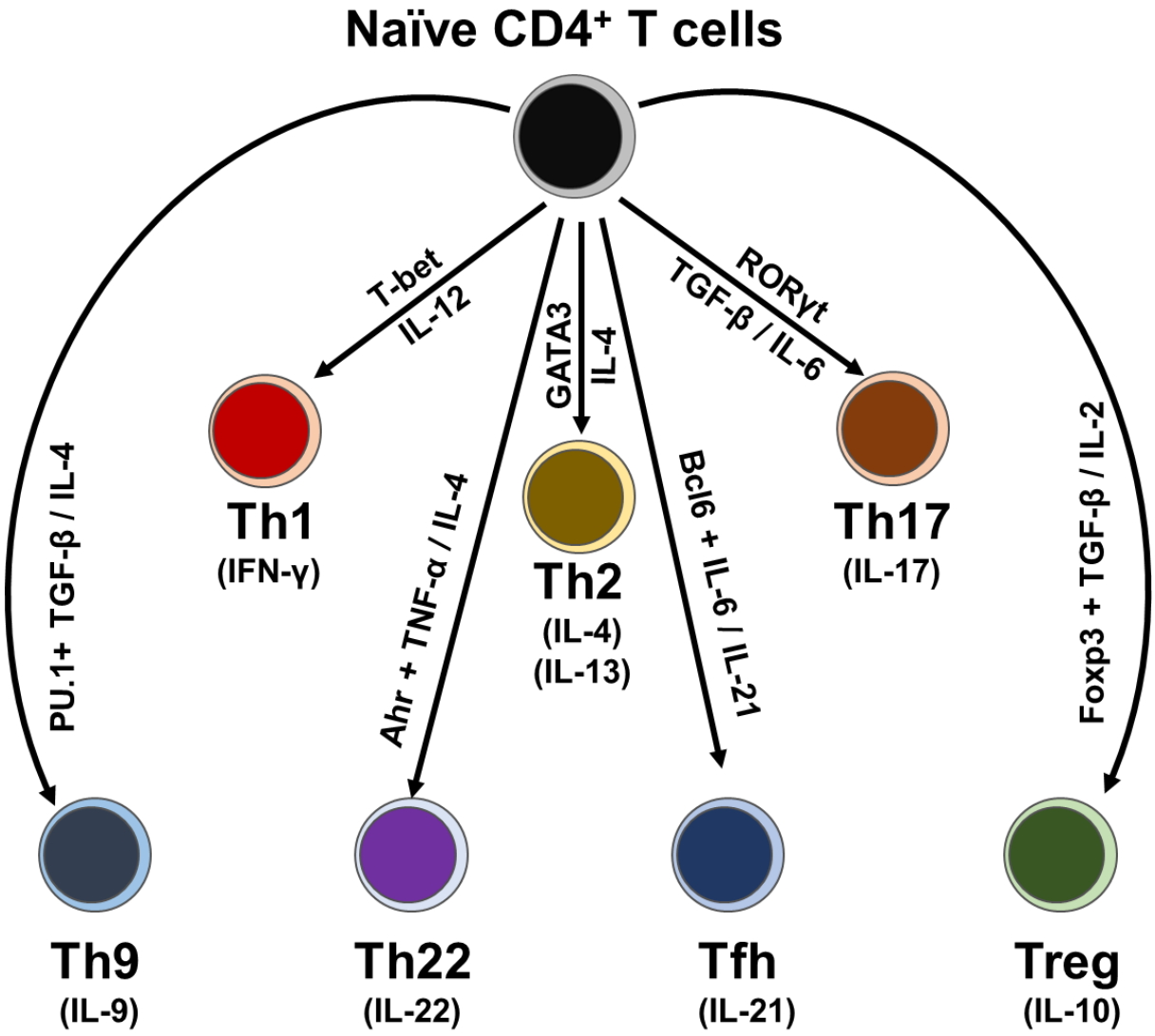

Fig. 2: CD4⁺ T helper cells induction and development: Naïve CD4⁺ T cells when encounter activation environment in the periphery give rise to different subsets of cells dependent on the available cytokines and/or activated transcription factors. (Source: adopted from Sun and Zhang, 2014).

Th1 induction is critically based on the transcription factor T-bet and the cytokine IL-12 (1, 8-11). However, Th2 development requires the activation of the transcription factor

GATA3 and the cytokine IL-4 (1, 8-11). Fully developed Th1 cells produce interferon gamma (IFN-γ) and typically target intracellular infections (1, 8-11). On the other hand, activated Th2 cells produce IL-4 and IL-13 and target extracellular pathogens and could cause allergic inflammation (1, 8-11). Th9 cells, first described by Dardalhon et al. and Veldhoen et al. in 2008, requires activation of the transcription factor PU.1 in the presence of IL-4 and Transforming growth factor beta 1 (TGF-β1), Th9 cells produce IL-9 and it is implicated in skin homeostasis and tissue inflammation (8, 12-15). Th17 polarization is induced by the transcription factor ROR-γt in the presence of TGF-β1 and IL-6 (8, 16, 17). Th17 cells produce IL-17 and target extracellular bacteria and fungi; they are also implicated in autoimmunity (8, 16, 17). Th22 phenotype requires the activation of genes associated with Th1 and Th17 phenotype, like IFN-γ, IL17A, T-bet and RORγt, and these cells produce IL-22 (8, 18). IL-6 and tumor necrosis factor alpha (TNF-α) have been found to promote the development of Th22 cells (8, 18). Ahr transcription factor is critically involved in IL-22 production by Th22 cells (8, 18). Th22 cells are believed to be involved in tissue inflammation (8, 18). Tfh (follicular helper $CD4^+$ T cells) cells were first isolated from human tonsils. Bcl6 is the critical transcription factor for Tfh development in the presence of IL-6 and IL-21. Tfh helps follicular B cell in antibody production (8, 19).

Unlike above described T effector cells, regulatory T cells (Tregs) are $CD4^+CD25^{high}$ subset of T cells, first described by Sakaguchi et al. in 1995, which are capable of suppressing immune response (20). The surface marker CD25, IL-2 receptor α-subunit, is also upregulated on activated T cells which makes it a non-specific marker to be used for Treg identification (3, 8, 21). The need for Treg-specific marker, led to the discovery of the transcription factor forkhead box P3 (Foxp3) which was found to be

critical for Treg function and a specific marker for Tregs (8, 21-24). The importance of Foxp3 for sustaining Treg functional capacity which is to maintain immune tolerance is clearly demonstrated in scurfy mice and Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome in humans both of which are caused by a mutated nonfunctional Foxp3 (25). Both scurfy mice and IPEX patients suffer from severe autoimmune disorders (25, 26). It has been shown that Tregs could develop in the thymus and are referred to as thymic/natural Tregs (nTregs) (8, 21, 27). This thymic development of Tregs has been shown to be dependent on high-avidity of TCRs to self-antigens (8, 21, 27, 28). Additionally, in the periphery, Tregs can be induced from naïve T cells and are called induced/peripheral Tregs (iTreg) (8, 21). The iTreg-induction could be achieved upon TCR stimulation accompanied by co-signaling through CD28 in the presence of TGF-β (8, 21). Several molecules have been identified as markers for Treg suppressive function and stable phenotype such as CTLA-4 (29, 30), Eos (31), CD39 (21, 32, 33), and CD44 (34). Treg suppression function could also be achieved via the secretion of immunomodulatory proteins such as IL-10 (21). Furthermore, IL-2 consumption by Tregs has been shown to be a mechanism of suppression used by Tregs (21, 35, 36). Treg production of IL-10 and TGF-β and their inhibition of the immune response are critical components required for the maintenance immunological self-tolerance.

1.1.2 Immune Tolerance and Autoimmunity

Autoimmunity that can lead to self-destruction is prevented mainly due to the ability of the immune system to differentiate between self and non-self. This ability is usually referred to as self-tolerance which is classified into central and peripheral tolerance (1).

Central tolerance refers to the mechanism of eliminating self-reactive lymphocytes in the thymus in the case of T cells, and in the bone marrow in the case of B cells (1, 28, 37, 38). The peripheral tolerance is a mechanism by which self-reactive lymphocytes (T or B cells) that escape central tolerance are kept in check from causing disease in the periphery (1, 28, 37, 38). For the purposes of this study, self-tolerance will be explained in the context of T cells.

Central tolerance is achieved in the thymus, during the maturation and development of T cell precursors (1, 2, 28, 37-39). Collectively, this central tolerance process involves two major steps; positive and negative selection. The DN T cell precursors arrive into the thymus. In the cortex region of the thymus, DN precursors undergo differentiation and proliferation, during which the rearrangement of the TCR β gene locus (Tcrb) takes place (1, 2, 28, 37-39). After successful rearrangement of the TCR β, TCR α gene locus (Tcra) rearrangement begins and continues during the DP ($CD4^+CD8^+$) stage (1, 2, 28, 37-39). DP precursors move from within the cortex into the medulla during which their fully assembled and functional TCR start to interact with self-peptides presented on MHC molecules by cortical and medullary epithelial cells, and by dendritic cells (1, 2, 28, 37-39). During this interaction, precursors that bind strongly to self-peptide MHC complex will be eliminated due to their strong self-reactivity (negative selection). Interestingly, some degree of low reactivity is required for precursors to avoid elimination and achieve the positive selection (1, 2, 28, 37-39). Additionally, some precursors undergo receptor editing, a process by which they edit their self-reactive TCR to avoid negative selection (40). After negative and positive selection, precursors

differentiate into SP ($CD4^+$ or $CD8^+$) cells and are released to the periphery as mature/naïve T cells (1, 2, 28, 37-39).

According to the classical affinity model of thymocyte selection, the fate of T cell precursors is determined by the reactivity-strength of their TCRs to self-peptide presented on MHC molecules (28). Positive selection requires weak reactivity from the TCR, indicating an ability to detect self-peptides presented on MHC molecules (28). On the other hand, TCRs that strongly react to self-peptides on MHC molecules induce activation induced cell death (apoptosis) resulting in negative selection of those T cells (28). However, previous research has shown a broad range of TCR affinities were capable of inducing Treg development. For example, high affinity, strong self-reactive, TCRs have been shown, using TCR-transgenic mice, to also lead to the development of self-reactive Tregs (28, 41). It has been shown that the probability of Treg induction increases as affinity increases. Furthermore, there has been evidence that there is an overlap between the TCRs specificities of nTregs and naïve T cells (28, 41). Therefore, a modified version of the classical affinity model in which Treg development is induced within a range of weak to strong reactivity of TCRs (28, 41) has been proposed.

Peripheral Tolerance is a mechanism to regulate self-reactive effector lymphocytes which escape central tolerance. Failure of this peripheral tolerance mechanisms leads to the activation of self-reactive T cells and the development of autoimmune disease. Tolerance in the periphery is achieved via cell-intrinsic, deletion and anergy, or cell-extrinsic mechanisms such as tolerogenic dendritic cells (DCs) and Tregs (1, 42).

Clonal deletion results when naïve T cells encounter their cognate antigen presented on MHC molecules by APCs under sub-optimal co-stimulatory environment (42). Optimum stimulation of naïve T cells requires encountering the cognate antigen presented by APC along with strong co-stimulation induced by molecules such as CD80/CD86 expressed on the surface of mature APCs (42). Maturation of APCs results from signaling molecules like pattern recognition receptors that are activated by pathogen-derived molecules. Therefore, in the absence of pathogens, T cells that bear self-reactive TCRs would recognize the cognate antigen presented by immature APCs but in the absence of the required co-stimulation, undergo deletion (42).

Anergy is an unresponsiveness state in T cells despite being stimulated via their TCRs (1, 42-45). The conditions that stimulate anergy are similar to those that induce deletion; recognition of cognate antigen in the absence of required co-stimulation (1, 42-45). The induction of deletion or anergy is decided by several factors including TCR affinity, antigen concentration, and antigen exposure time (1, 42-45).

Cell-extrinsic mechanisms refer to the suppression of activated Teffs by regulatory cells, which include Tregs, Th3, and Type 1 regulatory T cells (1, 42-45). The mechanisms by which regulatory cells suppress Teffs include contact-dependent and contact-independent mechanisms (1, 42-45). Contact-dependent mechanism includes binding of CTLA-4, expressed on surface of Tregs, to CD80/CD86 on APCs which inhibit generation of co-stimulation signals required for effector T cell activation (42-45). Contact independent mechanisms include the release of anti-inflammatory cytokines like IL-10, IL-35, and TGF-β or the consumption of IL-2 causing IL-2- deprivation of Teffs (42-45).

Autoimmunity represents one category of disease conditions that could result from a disturbance of immune system function (1). The first category of the immune diseases is caused by insufficient immune-response or failure of immune-response mechanisms (1). The mechanism by which the immune system fails to respond could be a method used by invading pathogens to hide themselves from the immune system and therefore preventing appropriate immune response. Furthermore, the failure could result from congenital defects in the immune system that leads to immunodeficiency (1). The second category of immune related disease conditions results from the immune system sometimes over-responding to antigens from non-infectious agents, like antigens from pollen, food, and drugs, leading to an immune disease called hypersensitivity reaction (1). The third immune condition that leads to disease is autoimmunity which results from an immune-response to self-antigens. The mechanisms that prevent autoimmunity and restore self-tolerance can be considered a chain of checkpoints. These checkpoints prevent parts of the autoimmune attack individually, and they work synergistically to avoid the occurrence of autoimmune diseases (1, 46, 47). The prevention of autoimmune diseases by these checkpoints, however, do not inhibit the immune system from mounting strong and effective responses against invading infectious pathogens (1, 46, 47). Negative selection mechanism in the central tolerance, for example, is the first checkpoint required for self-tolerance. Peripheral tolerance mechanisms are other examples of these checkpoints (1, 46, 47). Each checkpoint contributes to preventing autoimmunity. The exact mechanisms by which autoimmune diseases develop are not well defined. However, it is believed that autoimmune diseases are multifactorial and the susceptibility

to them could result from a mixture of genetic predisposition, impairment in tolerance mechanisms, and environmental triggers like infections (1, 46, 47).

1.1.3 Regulatory T cells and Autoimmunity

Regulatory T cells. In the late 1960s, the importance of the thymus for immune tolerance was shown via neonatal thymectomy in mice which led to autoimmunity (48, 49). In the next decade, IL-2 was found to be important for immune response; however, during the early 1990s it was found that IL-2 and IL-2 receptor (IL-2R); (IL-2Rα (CD25) and IL-2Rβ (CD122) knockout mice suffered from autoimmune diseases (49). The description of $CD4^{+}CD25^{high}$ subset of T cells, in the mid-1990s, with regulatory function could explain these findings. After the first description of $CD4^{+}CD25^{high}$ by Sakaguchi which were called regulatory T cells (Tregs), Tregs have been studied intensively (20, 49). IL-2 has been found to be essential for the survival of a subset of $CD4^{+}$ T cells with regulatory function (20, 49). Since then, several subset of cells with regulatory function have been described, such as Th3, Tr1, $CD8^{+}$ regulatory cells, and regulatory B cells (1, 49, 50).

However, our focus here will be on Tregs ($CD4^{+}CD25^{+}Foxp3^{+}$). Tregs and regulatory cells in general, represent the dominant or cell-extrinsic mechanisms for gaining self-tolerance, compared to recessive mechanisms, like receptor editing and negative selection, that occur in the cell-intrinsic manner (51). IL-2 is a crucial cytokine for Tregs, as CD25 (IL-2Rα) has been shown to be functionally critical for Treg differentiation (51).

The cellular and molecular aspects of Treg function regulation is achieved by the involvement of homo-oligomer of Foxp3 molecules which interact with other molecules, such as nuclear factor of activated T cells (NFAT), acute myeloid leukemia-1/run-related transcription factor (AML1/Runx1), histone acetyl transferase/histone diacetyl transferase (HAT/HDAC), and perhaps NF-kB (51).

It has been shown that Foxp3 and NFAT interaction require cooperative binding to DNA. Disruption of the amino acid sequence of Foxp3-domain that is responsible for its interaction with NFAT causes impairment of Foxp3 functions. Furthermore, NFAT-subtypes (NFATc2 and NFATc3) deficient mice develop severe lymphadenopathy spontaneously with selective induction of Th2 cells associated with elevated levels of IgE, which may indicate that the NFAT subset deficiency interrupts the role of Foxp3 leading to interruption of Treg function (51).

Treg activation and execution of suppression function in the context of TCR stimulation, in TCR-transgenic mice, requires 10-100 fold less concentration of their specific peptides, suggesting that Tregs could be activated by immature DCs which cannot activate naïve T cells. This could explain the ability of Tregs to suppress self-reactive naïve T cells and prevent autoimmunity (51). Despite their being refractory to proliferation by antigenic stimulation in vitro, Tregs in vivo proliferate through antigenic stimulation and sustain their functional efficiency (51).

Tregs achieve their regulatory function via four suppression mechanisms; i) affecting APC maturation and function, ii) destroying targeted cells, iii) disrupting metabolic pathways, and iv), secreting anti-inflammatory cytokines (1, 49, 50).

In the context of the first mechanism, CTLA-4 expression by Tregs was shown to induce down-regulation of CD80/CD86 expression on APCs. CD80/CD86 are important co-stimulatory molecules required for activation of naïve T cells after TCR stimulation (1, 49, 50). The reduction of CD80/CD86 expression limits the capability of APCs to activate naïve T cells and to induce immune response (1, 49, 50).

In the second mechanism, Tregs have been shown to be able to induce apoptosis on target cells (Teffs) (1, 49, 50) by releasing granzyme A and B and also perforin, which enter target cells and activate the caspase pathway leading to apoptotic cell death (1, 49, 50). Furthermore, despite the controversy, Tregs have been shown to express the member of the extracellular matrix modulators galectin family; Galectin-9 which binds and induces apoptosis in activated T cells expressing T cell immunoglobulin and mucin domain-3 (TIM-3) (1, 49, 50, 52). TIM-3 is a type 1 membrane protein that is expressed on activated Th1 cells, but not on naïve T cells (53).

The key molecule in the third mechanism is CD39 which is an ectonucleotidase (1, 49, 50). CD39 degrades ATP into ADP and AMP (1, 49, 50). The evidence for importance of CD39 came from studies with CD39 deficient mice. In CD39-deficient mice, Tregs showed 50-60% reduction in their suppressive capability when compared to Tregs from wild type mice (1, 49, 50). ATP/Adenosine hydrolysis cascade started by CD39 is believed to be involved in hindering Teffs activation and proliferation (1, 49, 50). Also, $CD39^{pos}$ Tregs are suggested to be specialized in suppressing IL-17 production (1, 49, 50).

For the last mechanism, Tregs have been shown to produce and secrete anti-inflammatory cytokines (1, 49, 50). These anti-inflammatory cytokines such as TGF-β, IL-

10, and IL-35 secreted by Tregs were shown suppress inflammation in organs like the gut, skin, and lungs (1, 49, 50).

The frequencies of circulating Tregs in autoimmune diseases is controversial. For example in SLE, MS, and RA, Tregs frequencies compared to healthy controls were reported to be decreased, normal, or increased in different studies (1, 49, 50). In the case of IBD majority of the reports showed numerical defects in Tregs (1, 49, 50). The reason behind this inconsistency is not clearly understood, however, the lack of standardization of Treg identification markers between studies could explain the discrepancies between these studies (1, 49, 50). Also, the time of sampling is important, because Tregs frequencies in autoimmune diseases are affected by the stage of the disease and the treatment regimen in use (1, 49, 50). Impaired Tregs frequencies and function were found in draining pancreatic lymph nodes (PLN) of type 1 diabetes (T1D) which may suggest the importance of studying Tregs in localized target organs rather than the peripheral blood which might help address the discrepancy regarding Treg frequency and function in autoimmune diseases (1, 49, 50).

1.2.1 Current Autoimmunity treatments, Maintenance not a Cure

According to the American Autoimmune Related Diseases Association (AARDA), the number of Americans suffering from autoimmune diseases is about 50 million (54, 55). Autoimmune diseases are diverse and include more than 100 diseases, and represent the third most common disease category after heart diseases and cancer (54, 55). Also, in American women, autoimmune diseases are the leading cause of morbidity (54, 55). Autoimmune diseases could affect specific tissue or they could be systemic

affecting multiple targets (54, 55). Almost all cells, tissues, or organs could be targeted by autoimmune diseases, however, the most common tissue targets of autoimmune diseases include blood vessels, red blood cells, connective tissues, endocrine organs, skin, muscles, and joints (54, 55). According to National Institute of allergy and Infectious Diseases the autoimmune diseases treatment consumes annually more the $100 billion (54, 55). Therefore, autoimmune diseases aggravate significantly the cost of health care and the morbidity and mortality annually (54, 55).

Currently, global immune-suppressive medications are the backbone of autoimmune disease treatment regimens (54, 55). However, these global immuno-suppressive drugs render patients more susceptible to fatal opportunistic infections and malignancy in the long term when used in high doses (54, 55). Additionally, the use of these drugs is associated with toxicity and severe undesirable side effects (54, 55). Collectively, these current modalities of autoimmune disease treatment are aimed primarily to manage, maintain and control autoimmune responses and avoid fatal complications. In other words they are only maintenance regimens and none of these treatments has any potential of curing the disease. Therefore, it is very important to develop a new modality of treatment that could have the potential of curing autoimmune disease or restoring the loss of self-tolerance (54, 55).

The objective of new modalities of treatment should be to achieve the following four goals; 1) high specificity and targets only the pathogenic components or cells while sparing normal immunological functions (54, 55), 2) Restore long-term tolerance or self-tolerance to avoid the need for continuous treatment (54, 55), 3) Reduce toxicity and

undesirable side effects to a minimum (54, 55), and 4) Must meet the cost-effectiveness compared to the other regimens (54, 55).

An example of a potential new modality of treatment is the use of Treg therapy, which involves induction in vivo or ex vivo expansion of purified Tregs isolated from a patient followed by adoptive transfer to the same patient (54, 55). Additionally, a potential approach is the use of an allergen-specific immuno-therapy (allergen-SIT) in the case of autoimmune diseases with known self-antigen(s) (54, 55). This approach uses the specific administration of allergens to inhibit immune responses rather than induce them which has been in clinical practice as the only curative treatment for allergic diseases (54, 55). Furthermore, co-signal blockade represents another potential new treatment. Naïve T cell activation requires a co-signal in addition to the signal derived from TCR when it encounter its cognate antigen presented on MHC molecules (54, 55). Without the co-signal T cells do not undergo activation, and these co-signals are received via surface expressed molecules like CD28 when it binds to its ligand (CD80/CD86) expressed by APCs. Targeting these co-signals could induce anergy in autoreactive T cells and suppress autoimmunity (54, 55). However, this approach is non-specific since its effect is to cause general blockade and not specific targeting of self-reactive/pathogenic T cells (54, 55).

1.2.2 Potential Application of Regulatory T cells in Autoimmunity Treatment

The expansion of Treg is gaining increasing importance because of the potential use of Tregs to manage autoimmune disease and transplantation-rejection, and several approaches have reached Phase I/Phase II in clinical trials (55-57). The use of Tregs in

clinical trials started based on the successful application of Tregs-based therapies in the treatment of animal models of autoimmune diseases and transplantation rejection (55-57). In clinical trials to treat T1D, autologous Tregs expanded ex vivo were used. Treg-based cell therapy has also been used in clinical trials to treat graft-versus-host diseases (GVHD) in which Tregs were isolated from either cord blood or peripheral blood. Additionally, autologous Tregs were used to treat kidney transplant rejection (55-57). Furthermore, it has been shown that ectopic over-expression of WT Foxp3 in Foxp3-mutant-$CD4^+$ T cells isolated from IPEX patients led to the development of Treg-like functional cells that exhibited efficient suppressive function ex vivo and in vivo, and demonstrated the capability to control autoimmune manifestations of IPEX patients (58). This ability of inducing Tregs ex vivo would add an important layer to the applications of Treg-based cell therapies especially in patients suffering from the deadly IPEX autoimmune disease. Furthermore, in the case of IPEX patients, it has been proposed to use molecular techniques like CRISPR/Cas9 to replace the mutated Foxp3 with a WT Foxp3 gene in the hematopoietic stem cells isolated from IPEX patient, and subsequently re-transfused back into the same patient (58). Finally, because Tregs exist in low numbers in physiological conditions and are hard to maintain in cell culture, the expansion of Tregs in vivo has gained more importance in an attempt to overcome these obstacles (55-57).

1.3 Expansion of Regulatory T cells, Approaches and Pitfalls

The majority of the approaches using Treg-based cell therapy is based on the isolation of Tregs from patients or donors, expanding them ex vivo through the use of anti-CD3/anti-CD28 stimulation, and supplementing with other factors like IL-2 and

retinoic acid, then re-transfusing the expanded Tregs back to patients aiming to achieve suppression of self-reactive Teffs and treat autoimmunity (55-57).

In order to expand Tregs ex vivo for subsequent re-transfusion, we first need to isolate them, which is not an easy task considering that Tregs are in low frequency and lack a unique surface marker (55-57). The use of magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) provides a method of Treg isolation based on the surface expression of $CD4^+CD25^+$ in mice, and $CD4^+CD25^+CD127^{lo/-}$ in human (note that Foxp3 is not used because it is a transcription factor i.e. not expressed on the cell surface) (55-57). Although most of the Tregs express CD25, since CD25 is also expressed on activated effector T cells, it does not serve as a unique marker for Tregs. This highlights the need to further study of Treg biology and identify potential unique surface markers that could be used for the isolation of pure Tregs.

Unfortunately, these approaches of Treg-based cell therapy is progressing slowly because of the required accumulation of knowledge regarding the fundamental biology of Tregs (1, 55). Current Treg therapies used in clinical trials encounter serious obstacles because of the technical challenges such as the refractory nature of Tregs to proliferation ex vivo and the difficulty of producing enough numbers of Tregs to achieve therapeutic effect (51, 55-57). Furthermore, the high cost of these current Tregs therapies used in clinical trials is estimated to be $48,000 for a single treatment of a patient (55-57). For these reasons, it is of great importance to investigate potential alternative approaches for the selective expansion of Tregs in vivo as a treatment for autoimmune diseases. Interestingly, our lab has shown earlier a TCR-independent preferential Treg expansion

protocol that has the potential to be the basis for investigation that could subsequently lead to use in humans.

The TCR-independent Treg expansion in our lab began with the observation that treatment of mice with low dose GM-CSF prevented the development of multiple autoimmune disease in animal models (59-65). GM-CSF treatment protected NOD mice from developing T1D (59), CBA mice from developing experimental autoimmune thyroiditis (EAT) (60-62), and prevented the development of experimental autoimmune myasthenia gravis (EAMG) in C57Bl/6 mice (63-65). Furthermore, GM-CSF administration was used to successfully treat ongoing EAT and EAMG (60, 63). Upon investigation of the GM-CSF-treatment, it was found to mobilize “tolerogenic” dendritic cells (DCs) which expressed little or no pro-inflammatory cytokines, but produced large amounts of TGFβ. Moreover, it resulted in an increase in IL-10-producing Tregs that suppressed the Teff cells (61, 62).

On the other hand, ex vivo treatment of bone marrow (BM) precursors with GM-CSF induced the development of a DC subset, GM-CSF-induced BM derived DCs (G-BMDCs). Co-culture of these G-BMDCs with naïve splenic $CD4^+$ T cells caused a preferential expansion of Tregs. However, DCs isolated from spleen (SpDCs) and further treated with GM-CSF ex vivo failed to induce a similar selective expansion of Tregs (66). Additionally, by using G-BMDCs derived from MHC Class II deficient mice our lab had shown that the Treg expansion induced upon G-BMDC-$CD4^+$ T cell co-culture is independent of the canonical TCR stimulation, but required IL-2 supplementation and G-BMDC-$CD4^+$ T cell contact (66).

Further, the ex vivo expansion of Tregs was found to be dependent on expression of high levels of OX40L, a TNF superfamily member, on G-BMDCs (66). Using anti-OX40L blocking antibodies G-BMDC-induced Treg expansion could be blocked, and using OX40, TNF-receptor superfamily member and the exclusive receptor for OX40L, agonist, the blockade of G-BMDC-induced Treg expansion could be rescued (66). Furthermore, we have shown that Jagged-1, a notch ligand, expressed on G-BMDCs is required for Treg expansion induced by G-BMDCs (67). In support of our data, it has been shown previously that Tregs were decreased in $OX40^{-/-}$ mice and increased in OX40L-over-expressing mice (68), and it has been shown also that Jagged-1 expressed by mesenchymal stromal cells is involved in Treg expansion (69). Collectively, these data suggested that OX40L and Jagged-1 expression on G-BMDCs have a critical role in their capability to induce Treg expansion (66).

Recently, we have shown that soluble OX40L and Jagged-1 ex vivo treatment supplemented with IL-2 induced Treg expansion in $CD4^{+}$ T cells, and these expanded Tregs sustained their suppressive phenotype (70). Further, treatment of 12-week old, pre-diabetic NOD mice, with soluble OX40L and Jagged-1 increased Treg and delayed the onset of hyperglycemia when compared to untreated control mice (70).

Based on these data we believe we are approaching the potential of developing a therapeutic, OX40L and Jagged-1, that could induce Treg expansion preferentially in the absence of canonical TCR stimulation which could be used to treat autoimmune diseases. This potential therapeutic was developed and investigated, so far, in mice especially NOD mice, therefore, our future plan is to continue investigating this potential therapeutic in

two parallel direction. First, optimizing the dosing and frequency of OX40L and Jagged-1 administration and testing their applicability to induce Treg expansion and manage other animal models of autoimmune diseases like EAE and EAMG. Additionally, our plans include investigating the capability of human OX40L and Jagged-1 to induce Treg expansion in humanized NSG mice.

1.4 Foxp3 and Regulatory T cell Phenotypic and Functional Markers

Tregs were identified first as a subset of $CD4^+$ T cells that express $CD25^+$ (20). CD4, as we mentioned earlier, is a co-receptor molecule expressed on the surface of conventional (Teff) and regulatory T cells and binds major MHC Class II during its engagement with TCR (1, 2). The function of CD4 molecule as a co-receptor that facilitates the interaction between MHC Class-II on APCs and TCR on $CD4^+$ T cells is not specific for Tregs, but common to all $CD4^+$ T cells (1, 2). CD25 is one of three chains of a heterotrimeric complex that forms the IL-2R; these chains are IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132) (71). CD25, or IL-2Rα, is the specific receptor chain for IL-2; CD122 and CD132 receptor chains are common between IL-2 and other cytokines such as IL-15 (72, 73). Since IL-2 is critical for Treg homeostasis and activation, therefore, the expression of its receptor is essential to Tregs (72). CD25 expressed on the surface of Tregs (74) and activated, but not naïve, conventional $CD4^+$ T cells (3, 8).

Foxp3 is a member of forkhead proteins, these proteins resemble a large family of transcription factors that were first discovered in drosophila, and they possess diverse functions and are involved in multiple cellular processes (75). It was found to be required and sufficient to induce Treg phenotype and function and, therefore, Tregs are usually

referred to as $CD4^+Foxp3^+$ T cells (8, 21-24, 76). Foxp3 gene is located on the X chromosome and its mutated variant was first cloned in 2001 by Brunkow et al. from scurfy mouse. Scurfy in mice were known to be caused by an X-linked recessive mutation that is lethal in hemizygous males (77).

CD28, ICOS, and CTLA-4 are members of B7-CD28-superfamily costimulatory molecules representing a critical and best-characterized co-signaling pathway in T cell activation and tolerance (78). In the context of Tregs CD28 has been suggested to be involved in controlling the thymic differentiation and peripheral maintenance of Tregs (79, 80). ICOS has been suggested to regulate the development and function of Tregs in CTLA-4-dependent manner (81). Also, it has been shown to control the proliferation, homeostasis, and IL-10 production in Tregs in the context of helminth infection (82).

CTLA-4 has been shown to be expressed constitutively on Tregs, but only upon activation on conventional T cells; it has been suggested that Foxp3 induces the expression of CTLA-4 on Tregs (29). However, it is worth mentioning here that, because of the discrepancy in the data regarding Treg function and CTLA-4, CTLA-4 has been suggested to function in a non-Treg compartment. However, Tregs have the ability to use mechanisms of suppression in both CTLA-4-dependent and CTLA-4-independent manners (83).

TIGIT, also known as Vstm3, is co-inhibitory receptor that involved in a pathway closely parallels the CD28/CTLA-4 pathway (84). Subset of Tregs express TIGIT and its expression is upregulated upon activation of conventional T cells and (85-88). It has been

shown that Tregs expressing TIGIT were capable of suppressing pro-inflammatory Th1 and Th17 cell responses, however failed to suppress Th2 cell response (88).

The signaling through the TNFRSF molecules; TNFR2, GITR, and OX40 were suggested to provide a critical role in the differentiation of Treg from thymic precursors after strong TCR signals that could otherwise lead to negative selection of these precursors (89). TNFR2 (TNFRSF1B or CD120b) has been shown to be constitutively expressed by Tregs, but its expression is upregulated rapidly on conventional T cells upon TCR stimulation (90, 91). Also, TNFR2 has been suggested to play an essential role in the stability of Tregs phenotype and function within the inflammatory environment (92). GITR (TNFRSF18 or CD357) expression is high on Tregs and upregulated on activated T cells compared to poor expression on naïve T cells, also GITR has been found to be involved in the enhancement of Treg proliferation and function (93). OX40 (TNFRSF4), also known as CD134, is believed to be expressed constitutively by Tregs, but conventional T cells express OX40 upon activation only (94). However, the role of OX40 in Tregs and Teffs will be further discussed later. 4-1BB (TNFRSF9), also known as CD137, another TNF-receptor superfamily member has been shown to play a role in regulating Treg development and function (95, 96), however, others have suggested that 4-1BB stimulation plays a negative regulation role in Tregs suppression (97).

CD39 is a phenotypic marker of Tregs that have been discussed in details previously in page 15 in this thesis. Briefly, it is been shown to be important for Treg suppression function (33, 50) and also suggested to give Tregs specialized ability to suppress IL-17 production (50, 98).

CD44, a transmembrane glycoprotein, acts as a cell adhesion molecule and is believed to be involved in cell signaling cascades (99). Its relative expression has been shown to correlate with Foxp3 expression by Tregs and their suppression function (34, 100, 101). In Tregs, CD44 promotes Foxp3 expression by inducing TGF-β expression in a partially IL-2-dependent context (34).

CD62L, also known as L-selectin, is a critical T cell homing receptor and a marker used to indicate T cell development stage; CD62L is highly expressed by naive T cells and rapidly down-regulated upon TCR stimulation. CD62L-expressing Tregs were shown to protect from lethal acute GVHD (102).

The zinc-finger protein family that includes Ikaros, Helios, Aiolos, Eos, and Pegasus were believed to be important for development of lymphocytes (103). Helios was proposed as a marker to identify Tregs developed in thymus from Tregs induced in periphery (103-105), but this proposal was challenged (103, 106). The expression of Helios in Tregs was mostly associated with the immune suppressive activity (103, 107).

In Tregs, Eos was found to cooperate with Foxp3 to promote chromatin modification that leads to gene silencing (31, 103, 108). Eos was shown to control the transformation of Tregs to helper T cells. Upon downregulation of Eos, not the loss of Foxp3 expression, a rapid induction of Treg reprogramming takes place as a response to inflammation (109). In this study, they used CD38, a surface antigen expressed on a subset of Tregs, and CD103, a maturation marker, as indicators proposed to be used to describe subsets of Tregs based on their plasticity to convert to Helper T cells (109).

Labile Treg phenotype are described as $CD4^+Foxp3^+CD38^+CD103^-$ and a stable Tregs phenotype $CD4^+Foxp3^+CD38^-CD103^+$ (109).

1.5 OX40L/OX40 Signaling and Regulatory T cells

OX40, TNF-receptor family (TNFRSF) member 4 (TNFRSF4) also known as CD134, was first described and named OX40 by Paterson et al. in 1987 (110). It is about 50 kD and is a type I transmembrane glycoprotein consisting of 249 amino acids of which 186 amino acids are in the extracellular domain and 49 amino acids are in the cytoplasmic domain (94). The gene encodes for OX40 is located on mouse chromosome 4 and human chromosome 1, which clusters with other TNFRSF molecules (94). OX40 is believed to be expressed by activated T cells, such as $CD4^+$ and $CD8^+$ T cells, along with Tregs (94). However, naïve and memory T cells do not express OX40 (94).

OX40L, TNFSF4, also known as CD252, was first identified in 1985 (111), and subsequently cloned for the first time as the ligand for OX40 in 1994 by Baum et al. (112). The OX40 is a 26-28 kD type II glycoprotein that consists of a total of198 amino acids, with 150 amino acids in the extracellular domain and 28 amino acids in the cytoplasmic domain (112). The gene encoding OX40L is located on chromosome 1 in both mouse and human (94). The cells that express OX40L is believed to include, but not exclusively, professional APCs, mature-conventional and plasmacytoid dendritic cells (cDCs and pDCs, respectively), Langerhans cells (94).

It has been shown that OX40L and OX40 can only bind to each other while other members of TNF family can bind several partners. In other words, OX40 is the exclusive

receptor for OX40L (94). OX40L in a trimeric configuration binds to OX40 molecules (94, 113).

In the context of naïve T cell activation via TCR signaling (Fig-3A), OX40 expression is detected starting after 12 hours of TCR ligation, however, memory T cells upon TCR stimulation start expressing OX40 within 1-4 hours of activation (94, 113, 114). The most significant function of OX40L/OX40 signaling is working as a co-signal with TCR stimulation leading to division and survival of conventional T cells (94). It is believed that OX40L/OX40 signaling in activated T cells prolong their clonal division and increases their cytokine production (114, 115).

OX40 signals through the TNF receptor associated factor (TRAF) family of phylogenetically conserved scaffold proteins (113, 116). OX40 signaling downstream events include the activation of MAPK, PI3K (117), AKT (117, 118), and NF-kB (119) (Fig-3A). TRAF family members that have been shown to associate with OX40 for signal transduction are TRAF2, TRAF3 and TRAF5 (119-124) (Fig-3A). In the absence of TCR signaling it was shown that OX40-OX40L activation leads to the formation of a ‘signalosome” that activates NF-kB (94, 125). In that signalosome protein kinase C-theta (PKC-θ) was shown to be critical for the level of NF-kB activation (94, 125) (Fig-3B).

In Tregs, it has been suggested that OX40L/OX40 signaling downregulates the expression of CTLA4 (120). It can also block the induction of IL-10-producing type 1 regulatory T (Tr1) cells (126) and other Foxp3$^+$ Tregs (127, 128). Furthermore, OX40 signaling has been shown to be critical for survival and proliferation of autoreactive effector T-cells (Teffs) and blocking OX40 signaling can ameliorate T1D (129),

experimental autoimmune encephalomyelitis (130), and inflammatory bowel disease (IBD) (131).

However, we and others have shown that OX40-OX40L interaction is critical for the proliferation and function of Tregs (66, 67, 132, 133). We have shown that G-BMDCs generated from bone marrow precursors were capable of inducing Treg proliferation in a TCR-independent, but contact-dependent manner (66), and it critically required high level expression of OX40L on G-BMDCs (66). Furthermore, others have shown that Treg numbers are decreased in $OX40^{-/-}$ mice and increased in OX40L-over-expressing mice Therefore, OX40L expressed on G-BMDCs is suggested to play a critical role in Treg homeostasis.

1.6 Objectives of This Study

The immune suppressive function of Tregs have gained tremendous attention as a tool to manipulate the immune system for control of autoimmune diseases and immune rejection after transplantation. The focus on Tregs has led to the development of ex vivo expansion protocols that have been used in clinical trials (55-57, 134, 135). However, these protocols require, purification of Tregs, TCR-stimulation by antiCD4/CD28, and re-introduction back to patients which require specialized facilities and high cost.

Interestingly, we believe, based on our published and unpublished data, that we can develop a potential therapeutic using OX40L and Jagged-1. This therapeutic could preferentially induce Treg proliferation in a TCR-independent manner and could be used to increase Tregs and manage autoimmune diseases.

Therefore, the aim of the current study in this broader project was to further investigate signaling proteins downstream of OX40 that participate/facilitate the induction of Treg expansion. Understanding these signals is critical for better preparation to

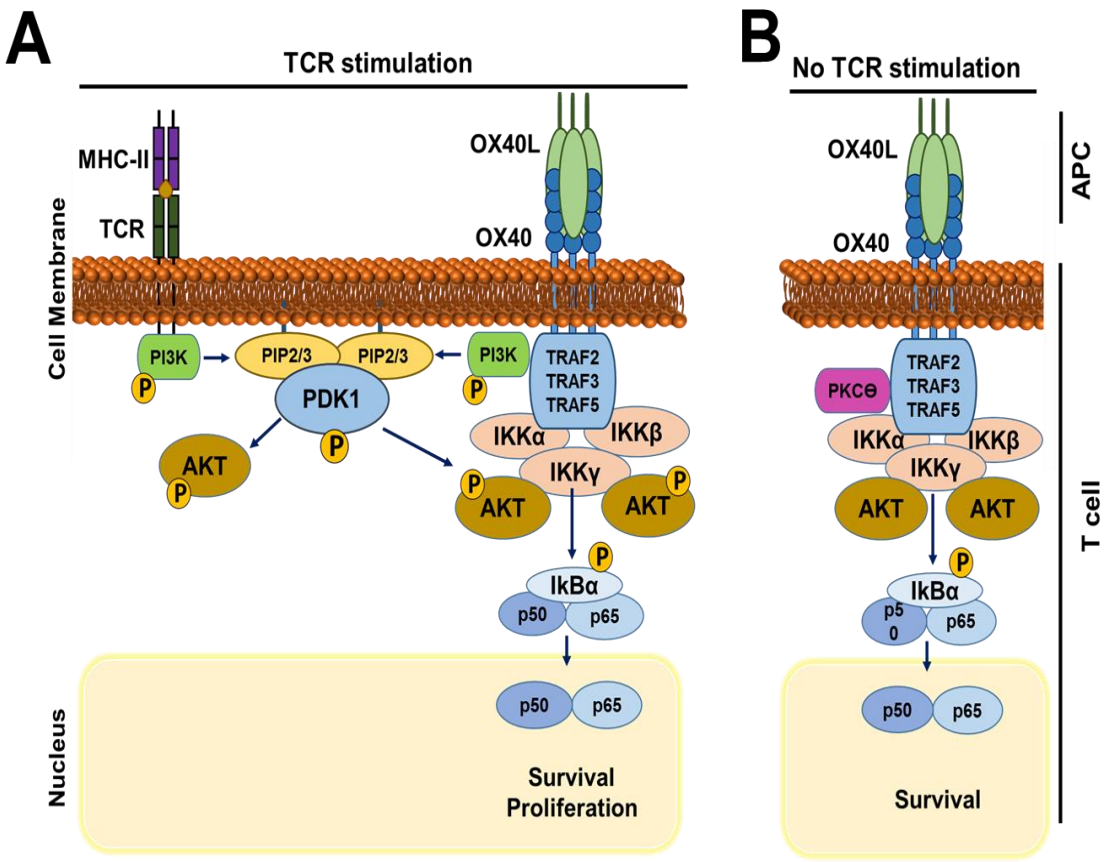

Fig-3: OX40L/OX40 signaling in the presence or absence of TCR stimulation. A) In the presence of antigen stimulation OX40 provides the co-signaling which synergizes the AKT activation induced by TCR-stimulation resulting in enhancement of T cell proliferation and survival. B) OX40 signaling in the absence of antigen stimulation form signalosome that involve PKC-Ɵ to induce activation of NF-kB and cause survival. (Source: adopted from M. Croft, 2010).

potential clinical utilization of Tregs proliferation, especially, in the management of autoimmune diseases. The significance of these signaling is due to the high demand for new modalities of autoimmune disease treatment. These high demands and the potential of our TCR-independent Tregs expansion protocol to be developed for in vivo usage

make the understanding of biological aspects of our protocol including OX40 signaling of great importance.

2. MATERIALS AND METHODS

2.1 Mice

Mice purchased from Jacksons Labs include C57Bl/6 wild type, OX40 deficient, and NOD. From Taconic we purchased MHC Class II deficient mice. Dr. Zuoming Sun, Department of Molecular Immunology, City of Hope, Duarte, CA, kindly provided us the PKC-Ɵ-deficient mice. Dr. Alan Epstein, Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, CA, kindly provided us the soluble OX40L. The infection free environment in the Biological Resources Laboratory (BRL) at the University of Illinois at Chicago (UIC) is used to keep all mice where food and water ad libitum is provided. All animal experiments described here were approved by the UIC animal care and use committee.

2.2 Mice in vivo treatment

C57Bl/6 WT, OX40-KO, and PKCƟ-KO treatment, OX40L was injected intraperitoneal three times (100µg/mouse/week). The week after the last OX40L treatment mice were euthanized and spleen, and thymus were harvested for analysis. For the NOD in vivo treatment, OX40L was administered intraperitoneal three times (200µg/mouse/week) into 6-weeks old NOD mice. Mice were either euthanized the week after the last treatment and spleen, thymus, and pancreatic lymph nodes were harvested for analysis or mice were kept alive and blood glucose was measured daily by tail vein bleed using Bayer Contour Blood Glucose Meter until 23 weeks age. At 23-weeks age mice were euthanized and pancreas were used for examination.

2.3 Antibodies, intracellular staining, and flow cytometry

Flow cytometry samples were analyzed using CyAn ADP analyzer (Beckman Coulter) in UIC, Research Resources Center (RRC), Flow Cytometry Service. Anti-Foxp3-APC, anti-CD39-PE, anti-OX40-APC, anti-pAKT-APC, anti-p-mTOR-PE, anti-pERK-APC, anti-BCL2-APC, anti-CD4-FITC, Anti-CD4-eFluor 780, Anti-Foxp3-PE, anti-Foxp3-PE-Cy5.5, anti-pIKBα-APC, and anti-OX40L-PE (eBioscience), Anti-phospho-RelB-PE and anti-phospho-p65-Alexa(R)488 (Cell Signaling technologies). Anti-CD44-FITC (Tonbobioscience). CellTrace violet from ThermoFisher Scientific was used as indication of cell division. Functional anti-CD3 was purchased from Tonbobioscience. Intracellular staining of cells were done using Foxp3 / Transcription Factor Staining Buffer Kit from Tonbobioscience.

2.4 Splenic T cell isolation

Isolation of total $CD4^+$ T cells was performed using mouse $CD4^+$ T cell Isolation Kit. Isolation of $CD4^+CD25^+$ T cells performed using mouse $CD4^+CD25^+$ Regulatory T cell Isolation Kit, from Miltenyibiotec. Cells were then counted, CellTrace labelled and used to setup co-cultures for ex vivo Tregs proliferation.

2.5 G-BMDCs generation and cells co-culture

G-BMDCs (both WT/II-KO-BMDCs) generation was described before (25). Briefly, from the mice femur and tibia bones, bone marrow was isolated, and cell suspension of bone marrow precursors were cultured in complete RPMI with 10% heat inactivated FBS and 20ng/ml GM-CSF for seven days, media were changed as required. WT-BMDCs

were generated using bone marrow from WT mice, and II-KO-BMDCs were generated using bone marrow from MHC Class II-deficient mice. Fresh splenocytes were used to isolate SpDCs. G-BMDCs to T cells ratio in the co-culture experiments was 1:2, and the media was complete RPMI containing 10% heat inactivated FBS. Dynabeads® Mouse T-Activator CD3/CD28 from Life Technologies were used according to the manufacturer recommendation. Unless stated elsewhere, all co-culture experiments were kept for five days.

2.6 Blocking antibodies

Total $CD4^+$ T cells were isolated from spleens of NOD mice. These isolated $CD4^+$ T cells were CellTrace violate labelled, and then co-cultured with G-BMDCs generated from bone marrow precursors isolated from NOD mice. To the co-cultures of $CD4^+$ T cells and G-BMDCs, blocking antibodies were added in 20 µg/ml final concentration. After five days of co-culture, cells were harvested and analyzed by flow cytometry for Treg proliferation. The blocking antibodies used were, anti-GITRL (MAB2177), anti-Neuropilin-1 (AF566), and anti-OX40L (AF1236), and have been purchased from R&D Systems (Minneapolis, MN).

2.7 Kinase inhibitors

NOD mice splenic $CD4^+$ or $CD4^+CD25^+$ T cells were isolated, CellTrace violate labelled, then incubated with Kinase Inhibitors (final conc.12.5µM) in 96 well plate. After six hours cells were washed and co-cultured with G-BMDCs for five days. In the case of

$CD4^+CD25^+$ T cells co-cultures, exogenous IL-2 is added. Cells were harvested and flow cytometry is used to investigate Treg proliferation.

2.8 Ex vivo suppression assay

Conventional $CD4^+$ T cells were isolated from diabetic NOD mice by depleting $CD4^+CD25^+$, the depletion was done using $CD4^+CD25^+$ Regulatory T cell Isolation Kit from Miltenyibiotec Inc. Isolated conventional $CD4^+$ T cells were CellTrace violet (ThermoFisher Scientific) labelled then seeded into round bottom 96-well plate with anti-CD3 (2µg/mL) and splenic APCs. OX40L-treated and untreated control mice were used to isolate Tregs ($CD4^+CD25^+$). Isolated Tregs were co-cultured with the CellTrace violet labeled conventional $CD4^+$ T cells at different ratios for 48 hours. Cells were harvested for flow cytometry investigation of the inhibition of division of conventional $CD4^+$ T cells.

2.9 Pancreatic Islets examination (H&E and confocal microscopy)

At the end of experiments mice were euthanized, pancreata were collected, fixed in formalin, and then embedded in paraffin. Tissue sections were prepared from the fixed embedded pancreases. Hematoxylin and eosin (H&E) staining of tissue sections is done using fixed-embedded pancreata followed by microscopic examination to evaluate the degree of islet infiltration. For confocal microscopy examination, tissue sections from the fixed-embedded pancreata were processed and then stained with anti-Insulin antibody (ab7842) from abcam, antibodies against Guinea Pig IgG coupled with TRITC (T7153), and DAPI (D9542) both from Sigma-Adlrich. Examination of slides was done using Zeiss

Laser Scanning Microscope; LSM 710, confocal microscopy in UIC, RRC, Core Imaging Facility.

2.10 OX40-TRAF1 co-localization (Confocal Microscopy & ImageJ analysis)

Isolated wild type (C57Bl/6) splenic $CD4^+$ T cells were cultured in the presence of IL-2 (10U/ml) with or without OX40L (7.5µg/ml) for 48 hours. Cells were harvested, fixed/permeabilized using Foxp3 Transcription Factor Staining Buffer Kit from Tonbobioscience, then transferred onto glass slides using Cytospin 2 centrifuge (SHANDON, ThermoFisher Company). Cells were stained using anti-Foxp3-Alexa Fluor 647 (sc-130666), anti-OX40 (sc-11403), and anti-TRAF1-FITC antibody (sc-6253) purchased from Santa Cruz (Dallas, TX). Anti-OX40-rabbit polyclonal antibodies were used for staining and the antibody binding was detected using TRITC-conjugated Anti-Rabbit IgG antibody (ab6718), purchased from abcam (Cambridge, MA). DAPI (D9542), purchased from Sigma-Adlrich (St. Louis, MO). Confocal microscopy examination was done using Zeiss Laser Scanning Microscope; LSM 710, in UIC, RRC, Core Imaging Facility. Images obtained were analyzed using ImageJ software (ImageJ 1.51h, National Institute of Health,USA). Foxp3 and DAPI Staining was used as identification markers for Tregs. On those identified Tregs OX40 and TRAF1 staining were merged and the median intensity of merged color from each cell was measured. The analysis includes at least ten cells in the OX40L/IL-2 treated group and eleven in the IL-2 alone treated group. Statistical analysis was done between the median intensity of merged color between the two groups.

2.11 RNA isolation and Quantitative Real-Time PCR

Tregs ($CD4^+CD25^+$) and Teffs ($CD4^+CD25^-$) were freshly isolated from spleen as described previously. Total RNA was isolated from Tregs and Teffs using RNeasy mini kit (Qiagen) and used for cDNA synthesis by iScript cDNA synthesis kit (BioRad). The cDNA synthesis and iQ SYBER Green Supermix used RT-PCR were done using CFX Connect Real-Time PCR Detection System (BioRad). The primers specific for TRAF1-7 Genes were listed in Table-I. Calculation of fold change of gene expression values was done using comparative ΔCt method following normalization to GAPDH then presented in bar graphs as fold change of TRAF gene in Treg over respective Teff.

2.12 RNA Microarray analysis

Total RNA was isolated from proliferating Tregs ($CD4^+CD25^+$) after five days of co-culture with G-BMDCs. Dilution of CellTrace violet was used as a marker of cell division to sort proliferating Tregs by flow cytometry (MoFlo Astrios, Beckman coulter). Affymetrix GeneChip Mouse Genome 430 2.0 microarray screening was done at UIC, RRC, Core Genomics Facility. In summary, using biotinylated dNTP to synthesize biotinylated cDNA was then used in the hybridization with microarrays. Following the array scanning only arrays that passed the quality control were used. Arrays were then normalized to housekeeping gene and subsequently gene expression analysis was carried out using R-package software. Student's T-Test was applied to find differential expressed genes. Microarray was submitted to NCBI-Gene Expression Omnibus database, and available publicly (Accession No. GSE81051). In heat map graphs colors represent the relative expression values compared to the average expression value per gene in the data. Blue

represent downregulation while red represent upregulation relative to average value. RStudio software (Version 1.0.44) was used to generate the heat map graphs.

2.13 Western blot

Soluble OX40L or IL-2 was used to stimulate thymocytes ex vivo. Cells were harvested, after the indicated time, washed with PBS, and then lysed with Laemmli buffer (BioRad). Cell Lysates were resolved in 10% SDS-PAGE gels, transferred to PVDF membranes (BioRad). Blocking was done at 4°C overnight using TBST containing 5% skimmed milk, 3% BSA. Anti-pp65/RelA rabbit antibodies, from Cell Signaling Technology, was used followed by anti-rabbit-HRP-conjugated antibodies. Development step were done using ECL detection kit (Pierce Scientific).

2.14 Statistical analysis

Statistical analysis was carried out using MS-Excel from MS-Office application software. These analyses include the calculation of average, standard deviation, p-values, and Chi-square. Student T-Test was used as the method of calculating p-values, and considered statistically significant when p-value is ≤ 0.05. Chi-square test was used for comparison of normoglycemia at 23 weeks of mice age.

3. OX40 SIGNALING IS REQUIRED FOR EX VIVO EXPANSION OF TREGS

3.1 Introduction

In the context of conventional T cells, upon TCR stimulation, OX40L/OX40 signaling has multiple roles: it acts as the co-signal required for T cell activation (Fig-3A), increases cytokine production by Teffs and prolongs their activation (136). This TCR-associated OX40 stimulation is known to involve the activation of MAPK, PI3K, and AKT (117, 118). Additionally, OX40 signaling in the presence of TCR stimulation has been shown to induce activation of the NF-kB signaling pathway (94). Therefore, it is thought that the activation of OX40 upon TCR signaling increases self-reactivity via increasing the survival and expansion of self-reactive Teffs and, conversely blocking OX40L/OX40 signaling could have the potential to prevent or treat experimental autoimmune diseases (129-131).

In contrast, in the absence of antigen engagement (no TCR-stimulation), OX40L/OX40 interaction has been shown to activate NF-kB, without activation of the MAPK, PI3K, and AKT signaling pathways (Fig-3B) (94, 125). Additionally, OX40L/OX40-signaling have been shown by us and others to be critical for the proliferation and function of Tregs (66, 67, 132, 133). OX40 signaling is known to contribute to both Treg expansion and function based on the cytokine milieu (137). We have shown previously that G-BMDCs, generated from BM precursor cells cultured in GM-CSF, preferentially expands Tregs when co-cultured with splenic $CD4^+$ T cells (66). Futhermore, through transwell experiments and using G-BMDCs derived from MHC Class $II^{-/-}$ mice, we found this treg expansion to be TCR-independent, but contact-dependent and requires IL-2 supplementation (66). Furthermore, we observed G-BMDC-induced Treg expansion

critically required the expression of OX40L (66) and Jagged-1 (67) on G-BMDCs. In support of our finding, it has been reported that $OX40^{-/-}$ and OX40L-over-expressing mice have decreased and increased Tregs respectively (68). Recently, we have shown that soluble OX40L and Jagged-1 treatment ex vivo and in vivo were capable of inducing Treg expansion (70). Treatment of 12-week age, pre-diabetic NOD mice, with soluble OX40L and Jagged-1 not only increased Tregs, but also delayed the onset of hyperglycemia in treated compared to untreated control mice (70). These studies further showed that OX40L primarily promoted Treg proliferation while Jagged-1 facilitated sustained expression of FoxP3. Therefore, in this study, I investigated the role of OX40L/OX40 interaction and the phenotype and functionality of ex vivo and in vivo expanded Tregs.

3.2 Experimental results

3.2.1 OX40 signaling is indispensable for the induction of Treg proliferation by G-BMDC. In multiple experimental autoimmune diseases, our lab and others have shown that GM-CSF-treatment can prevent the development of autoimmune diseases (59, 60, 63, 138, 139). We found that tolerogenic dendritic cells, which are mobilized by GM-CSF treatment (59), induce the proliferation of Tregs. The increased IL-10 production by Tregs was found to cause the suppression of autoimmune diseases (61). Also, we have shown that G-BMDCs were capable of preferential induction of $Foxp3^{+}$ Tregs proliferation upon ex vivo co-cultures (66). Furthermore, we found this Tregs proliferation required OX40L/OX40 signaling (66).

To further investigate the critical role of OX40L/OX40 signaling in the G-BMDCs induced preferential expansion of Tregs, we isolated splenic $CD4^+$ T cells from WT mice and labelled these cells with CellTrace, a dye that is diluted by 50% following each cell division and is used as an indicator of cell division. We then subsequently co-cultured

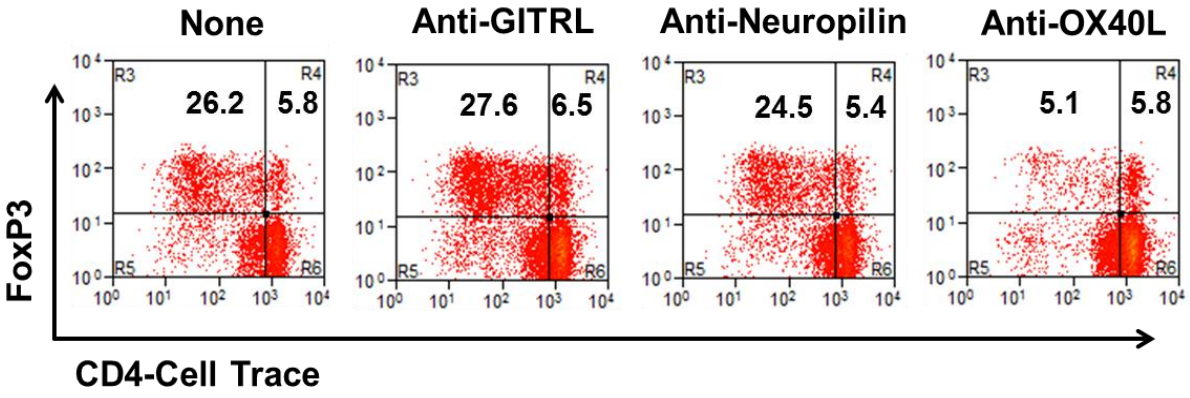

Fig-4: G-BMDC induced Treg proliferation was reduced in presence of anti-OX40L blocking antibodies. Splenic $CD4^+$ T cells were isolated, CellTrace-labeled, co-cultured with G-BMDCs in the presence or absence of the indicated blocking antibodies. After five days, cells were harvested and analyzed by flow cytometry for Treg proliferation.

these CellTrace labelled $CD4^+$ T cells with G-BMDCs, obtained from WT mice (WT-BMDCs) in the presence or absence of blocking antibodies (Fig-4) and assessed for Treg proliferation. We used blocking antibodies against OX40L and another TNF-Superfamily member, TNFSF18, also known as GITRL which is implicated in T cell co-stimulatory function (140). We also used blocking antibodies against neuropilin-1, a cell surface molecule implicated in immunological synapse maintenance (141). Our results showed that only anti-OX40L antibodies had substantial amelioration of Treg proliferation induced upon co-culture with G-BMDCs (Fig-4).

Subsequently, in order to determine the necessity of OX40L/OX40 signaling in G-BMDC induced Treg expansion, we co-cultured $CD4^+$ T cells isolated from the spleens of WT and $OX4O^{-/-}$ mice with WT-BMDCs (Fig-5A) and determind the effect on Treg proliferation. Contrary to the WT counterpart, Tregs isolated from $OX40^{-/-}$ mice showed a sever defect in proliferation capability (Fig-5A). As expected, when we analyzed the

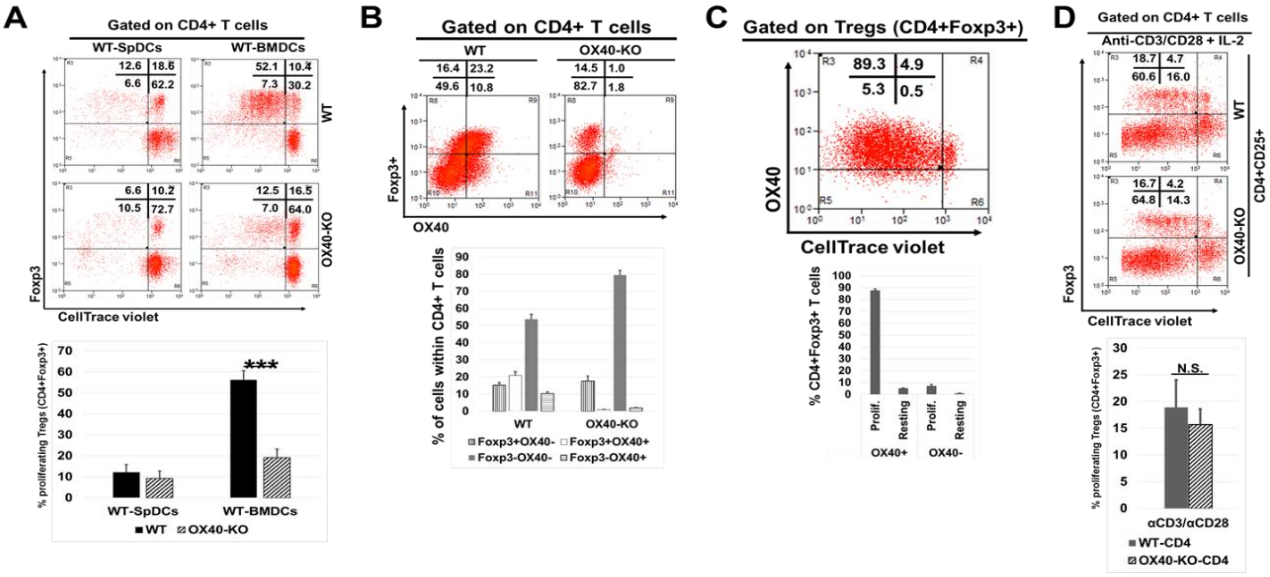

Fig-5: OX40-KO Tregs showed impaired G-BMDC-induced proliferation. A-C) Splenic $CD4^+$ T cells were isolated, CellTrace-labeled, and then co-cultured with G-BMDCs. On 5d cells were harvested and stained for FACS. A) Representative dot plots (top) and summarization bar graphs (bottom) show Treg proliferation. B) Representative dot plots (top) and summarizing bar graphs (bottom) show the expression of OX40 on WT and OX40-KO $CD4^+$T cells. C) Representative dot plots (top) and summarizing bar graphs (bottom) show the expression of OX40 on proliferating Tregs. D) Splenic $CD4^+CD25^+$ T cells isolated from WT and OX40-KO mice, cultured in the presence of exogenous IL-2, with microbeads coated with anti-CD3/28, after 4 days cells were harvested and analyzed by flow cytometry for Treg proliferation. Representative dot plots (top) and summarizing bar graphs (bottom). * =P<0.05, ** = p<0.005, and *** = p<0.0005.

expression of OX40 on $CD4^+$ T cells isolated from WT and $OX40^{-/-}$ mice, cells showed no expression of OX40 (Fig-5B) while almost all proliferating Tregs isolated from WT mice expressed OX40 (Fig-5C).

Furthermore, we investigated whether the failure of proliferation in Tregs isolated from $OX40^{-/-}$ mice upon co-culture with G-BMDCs could be overcome upon TCR-stimulation. Therefore, we stimulated Tregs ($CD4^{+}CD25^{+}$), isolated from both WT and $OX40^{-/-}$ mice, with microbeads coated with anti-CD3 and anti-CD28, a common method of TCR stimulation (Fig-5D). Upon TCR-stimulation, Tregs isolated from $OX40^{-/-}$ mice proliferated as the WT counterpart (Fig-5D). These results showed that OX40 signaling is essential for G-BMDC-mediated Treg proliferation and not TCR stimulated Treg expansion.

3.2.2 Ex vivo and in vivo expanded Tregs maintain their suppression phenotype and function. To investigate whether the induced proliferating Tregs sustained their suppression capability we studied the expression of known Treg phenotype markers on the G-BMDC-induced proliferating Tregs and compared the expression to resting Tregs. We used gene expression microarray to investigate the differential expression of targeted genes between proliferating and resting Tregs (NIH GEO; Accession No. GSE81051) (70). Interestingly, genes whose proteins are known to correlate with Treg phenotype and function (e.g. Foxp3, CTLA-4, CD39, and CD44) were found to be upregulated in proliferating Tregs compared to resting (Fig-6A) (70). Also, all IL-2R (α, β and γ-chains) genes were upregulated in the proliferating Tregs indicating activation of IL-2 signaling, and we found increased expression of negative regulators of STAT5 activation such as SOCS1, SOCS3 and CISH, suggesting tight regulation of STAT5 expression, (Fig-6B) (70).

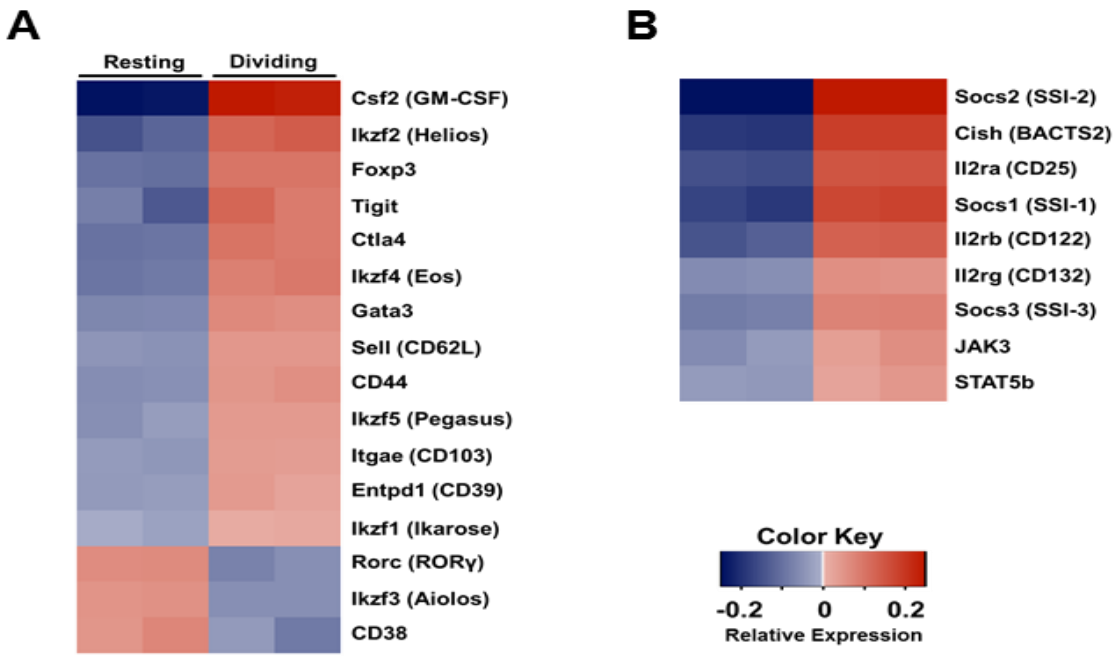

Fig-6: Proliferating Tregs showed upregulation of genes suggesting its functional and phenotype fitness. Gene Microarray analysis of relative expression of mRNA between proliferating and resting Tregs. Heat maps showing positive relative upregulation in red presented in related groups. A) Tregs functional and phenotypic markers. B) IL-2-related genes

Furthermore, to confirm the gene expression array finding, we used flow cytometry to analyze the expression of CTLA4, Eos, and CD39, markers indicative of the suppressive phenotype of Tregs (Fig-7) (31, 32, 51, 83, 142). Compared to the resting Tregs, an increased proportion of proliferating Tregs sustain the expression of these markers (Fig-7). These results indicated that Tregs proliferated upon G-BMDCs co-culture maintained their suppressive phenotype and, hence, likely their suppressive function.

Secondly, to investigate whether this mechanism of Treg proliferation with increased suppressive markers could be reproduced *in vivo*, we treated 6-week old NOD

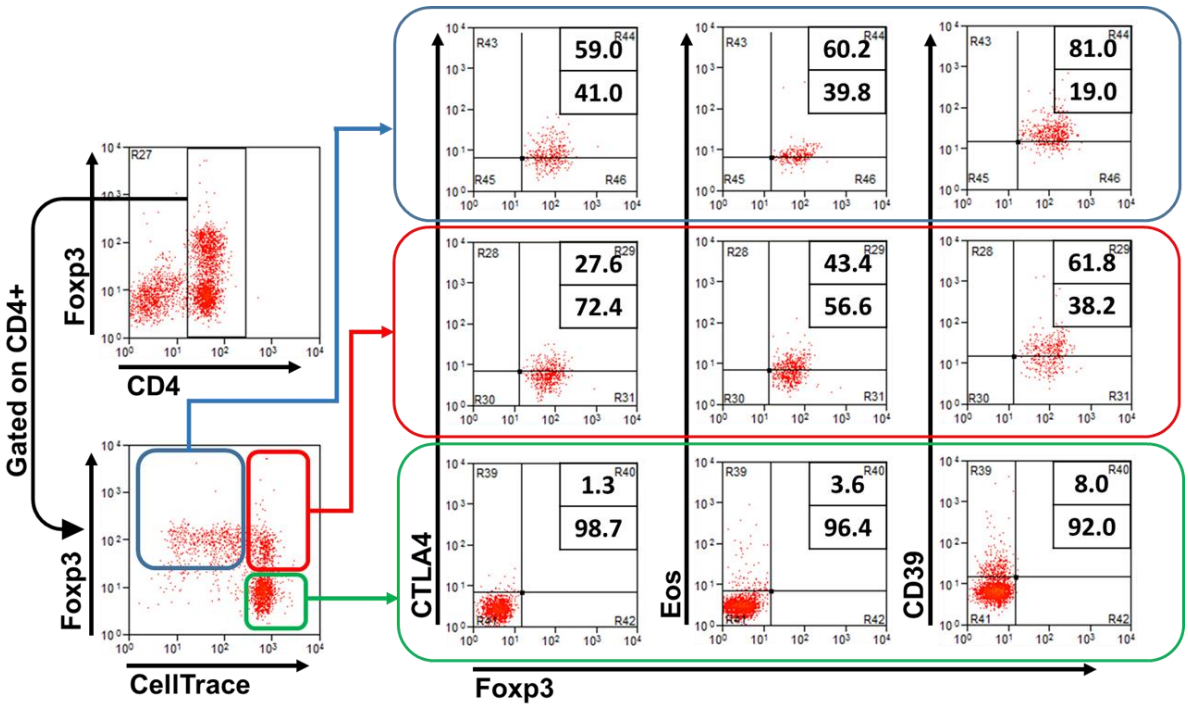

Fig-7: Ex vivo expanded Tregs maintain the phenotypic suppression function markers. Splenic $CD4^+$ T cells isolated for NOD mice, CellTrace-labeled, then co-cultured with G-BMDCs for 5 days then harvested and analyzed by flow cytometry for the indicated phenotypic markers.

mice with soluble OX40L or PBS, once a week for three weeks. After the fourth week mice were euthanized and organs harvested for analysis of Treg phenotype (Fig-8A). Our analysis of the expression of suppressor function markers on Tregs showed that in the OX40L treated mice there is a substantial increase in Tregs that were positive for CD39 and CD44, indicative markers of Treg stability and function (34, 143) (Fig-8B). To study the suppressive capability of *in vivo* expanded Tregs, we performed a Treg suppression assay using Tregs from OX40L treated mice. First, we isolated Teffs ($CD4^+CD25^-$) from untreated diabetic NOD mice, labelled them with CellTrace violet, and stimulated them with splenic APCs in the presence of anti-CD3. Tregs from the OX40L treated or untreated control mice were added to the Teffs-APCs mix in different ratios (Fig-8C & D) and the

suppression of Teff proliferation was assessed. As a result, we found that expanded Tregs isolated from OX40L treated mice showed comparable suppressive function to that of Tregs isolated from untreated control mice (Fig-8C & D).

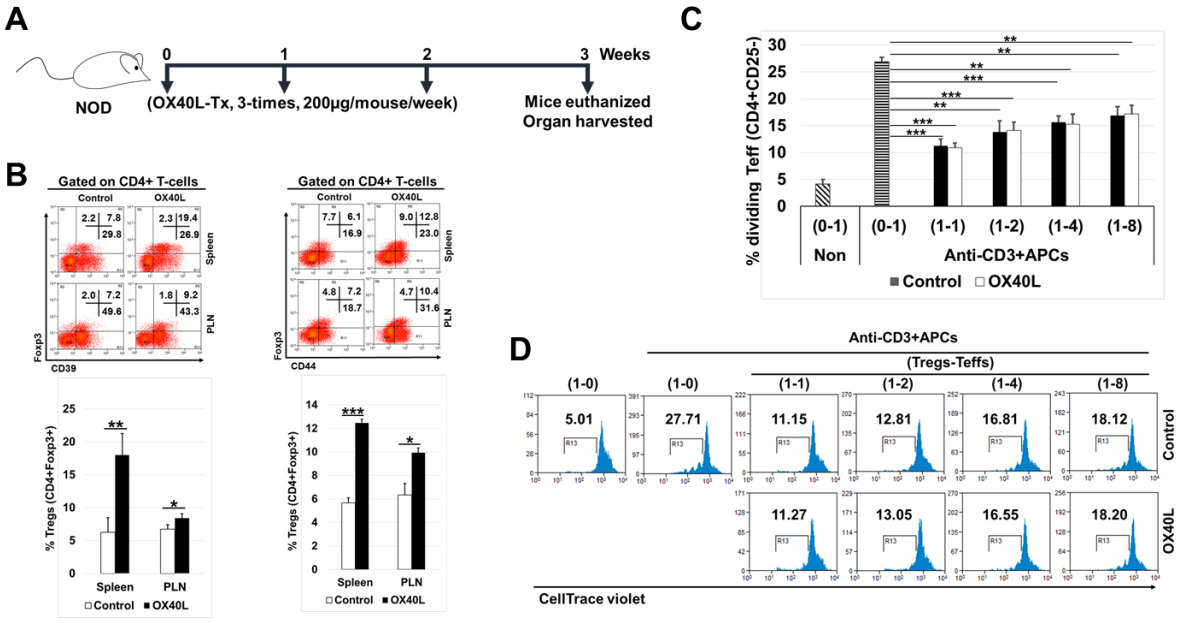

Fig-8: Tregs induced by OX40L-treatment in vivo maintained their suppressive function. NOD mice were injected i.p. with PBS or OX40L. In the fourth week mice were euthanized and organs harvested for investigation. A) Treatment scheme. B) Representative dot plots (top) and summarizing bar graphs show the percentage of Tregs and their CD39 (left) and CD44 (right) expression. C&D) Splenic Tregs from OX40L-treated and control mice were isolated then used in ex vivo suppression assay in different ratio with CellTrace-labelled Teff isolated from diabetic mice in the presence of anti-CD3 and splenic DCs. C) summarizing bar graphs show the suppression of Teff-division. D) Representative histograms from data summarized in C are shown. * =P<0.05, ** = p<0.005, and *** = p<0.0005.

Furthermore, we investigated the capability of expanded Tregs induced by soluble OX40L-treatment to achieve *in vivo* suppression of autoimmune disease. In order to test this, we injected six-week old NOD mice with soluble OX40L or PBS, once a week for

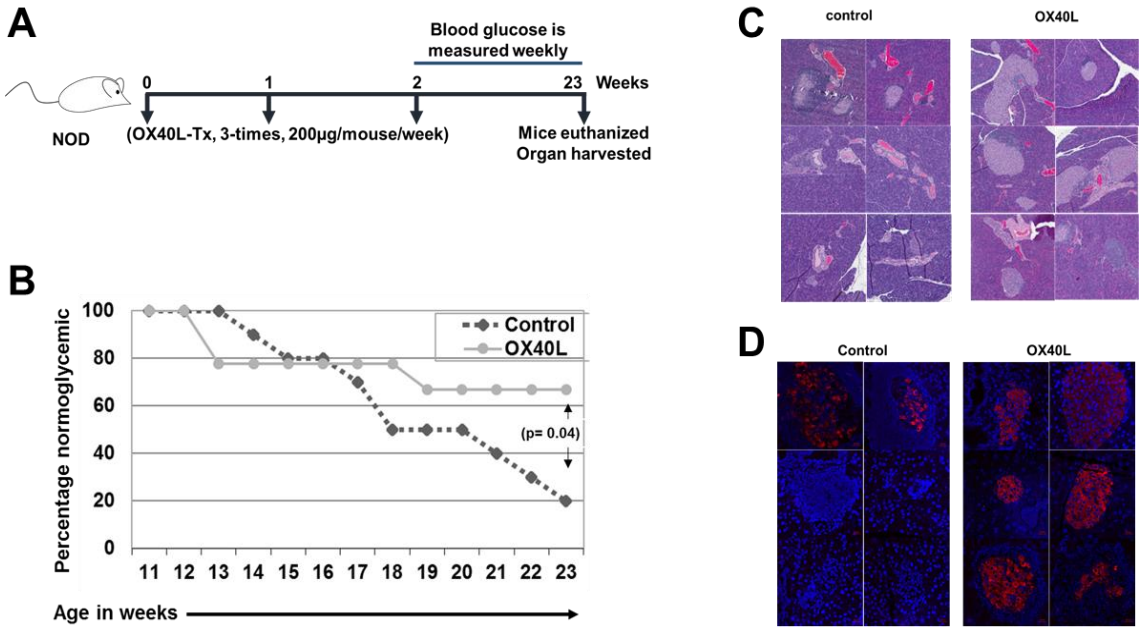

Fig-9: OX40L-treatment delayed the onset of hyperglycemia in NOD mice. 6-week old NOD mice were treated with PBS or OX40L and blood glucose was monitored each week. At 23 weeks of age mice were euthanized and organs harvested for analysis. A) OX40L treatment scheme B) Graph comparing development of hyperglycemia between OX40L treated and control groups. C) H&E stained pancreatic tissue sections from untreated and OX40L-treated NOD mice showing different degrees of lymphocytic infiltration and islet damage. D) Confocal microscopy of pancreatic tissue sections using anti-insulin antibody (Red=Anti-insulin, Blue=dapi).

three weeks and monitored for disease progression. Blood glucose was measured weekly beginning at 10 weeks of age. At 23 weeks of age mice were euthanized and organs harvested for analysis (Fig-9A). Interestingly, while 80% of untreated mice (n=10) became hyperglycemic by 23 weeks of age (Fig-9B), only 33.3% of OX40L treated mice became

hyperglycemic (p=0.04). H&E staining showed several intact islets in the OX40L treated group, however, the majority of the islets from the untreated control mice were severely infiltrated with lymphocytes (Fig-9C). Additionally, using confocal microscopy, we found that in the pancreata of OX40L treated mice there are several insulin positive islets, however, such islets were few in the untreated group (Fig-9D). Thus, our results suggest NOD mice treated with OX40L show an increase in Tregs (Fig-8B) which resulted in decreased lymphocyte infiltration and sustaining insulin production by pancreatic islets leading to delaying the onset of hyperglycemia (Fig-9). Collectively, these results suggested Tregs expanded *in vivo* through OX40L treatment did not lose their suppressive function.

3.3 Discussion

The importance of Treg expansion stems from the potential use of Tregs, which reached Phase I/Phase II in clinical trials, to treat autoimmunity and transplantation-rejection (55-57). However, this usage of Tregs is based on the ex vivo expansion of Tregs for a cell therapy (55-57), which presents huge challenges which I have discussed earlier. Therefore, our TCR-independent but OX40L/OX40 signaling-dependent Treg expansion protocol is important and represent a potential ex vivo and in vivo Treg expansion protocol. OX40, which is constitutively expressed on Tregs, is the cognate receptor for OX40L (144). Since IL-2 is a critical factor for Tregs viability (145), OX40 and IL-2 receptor (i.e. CD25) constitutive co-expression on Tregs could be used to provide the specificity for this mechanism of Treg expansion. In support of our findings, a decrease in Treg population has been reported in $OX40^{-/-}$ mice and the contrast has been

observed in OX40L over-expressing mice with an increase in Treg cells (68). Therefore, we used blocking antibodies against OX40L, GITRL (a TNF superfamily member (TNFSF18) that implicated in co-stimulatory function) (140), and neuropilin-1(implicated in immune synapse maintenance) (141) to further confirm the necessity of OX40L/OX40 signaling in Treg homeostasis. Anti-OX40L antibodies blocked Treg proliferation while both anti-GITRL and neuropilin-1 failed to do so which confirms the requirement of OX40L/OX40 interaction for Treg proliferation (Fig-4). Also, we used splenic $CD4^+$ T cells from $OX40^{-/-}$ mice, co-cultured them with G-BMDCs and further confirm that OX40L/OX40 signaling is critical for G-BMDC-induced Treg expansion, but not Treg proliferation induced by TCR stimulation (Fig-5). Using microarray analysis to investigate differential regulation of gene expression between proliferating, induced by G-BMDCs, and resting Tregs, we found that dividing Tregs upregulate the expression of genes encoding Treg functional phenotype markers (Fig-6). Using flow cytometry analysis we confirmed the upregulation of the expression of some of these protein markers on the surface of proliferating Tregs (Fig-7). Finally, we established that OX40L-treatment in vivo, is capable of expanding Tregs and those in vivo expanded Tregs sustained their suppression function (Fig-8).

4. IN TREGS, PKC-Θ IS DISPENSABLE, FOR EX VIVO EXPANSION

4.1 Introduction

In the presence of TCR stimulation, OX40 signaling in T cells can activate MAPK, PI3K (117), AKT (117, 118) and NF-kB signaling pathways (94). However, in the absence of TCR-signaling, OX40 stimulation can also result in NF-kB activation via the assembly of a signaling complex containing TRAF2 and PKC-Ɵ (125). PKC-Ɵ is a member of the family of serine/threonine kinases that participate in the regulation of differentiation and growth of a number of cell types (146). It was first cloned in 1993 from a cDNA library generated from human peripheral blood lymphocytes (146). Its expression is quite restricted and appears primarily in hematopoietic cells (146, 147). T cells, natural killer, mast cells, and platelets were found to express PKC-Ɵ; however, B cells, monocytes, macrophages, neutrophils, and erythrocytes did not show detectable expression (147-150). Based on homing organ, the expression level of PKC-Ɵ in T cells could be high in thymus and lymph nodes, and low to undetectable in spleen and bone marrow, respectively (147-149). It has been shown that PKC-Ɵ plays a critical role within the “immunological synapse”, during APC and T cell interaction that leads to TCR-induced T cell activation (151, 152). However, in the context of Tregs, PKC-Ɵ deficient mice showed a decrease in the frequency of Treg in the periphery (153). Treg reduction in PKC-Ɵ deficient mice could be explained to be possibly a consequence of Treg-intrinsic defect during their development in thymus and/or Treg-extrinsic defect because of IL-2 insufficiency due to Teff IL-2-production (153, 154). Therefore, we wondered if PKC-Ɵ has a role in G-BMDC-induced, TCR-independent Treg proliferation.

4.2 Experimental results

4.2.1 PKC-Θ$^{-/-}$ T cells showed defect in Tregs proliferation upon co-culture with G-BMDCs. In the absence of antigen stimulation, OX40L/OX40 stimulation in T cells has been shown to induce the formation of signaling complex, or "signalosome", which leads to activation of NF-kB signaling pathway (125). Among other components, TRAF2 and PKC-Θ were shown to be critical parts of that signalosome (125). Therefore, we hypothesized that PKC-Θ could be an important component of OX40-mediated downstream signaling that leads to NF-kB activation in the absence of TCR-activation. In light of these prior outcomes, we investigated the role of PKC-Θ in the Treg proliferation induced upon co-culture with G-BMDCs. If indeed PKC- Θ was critical to OX40 mediated

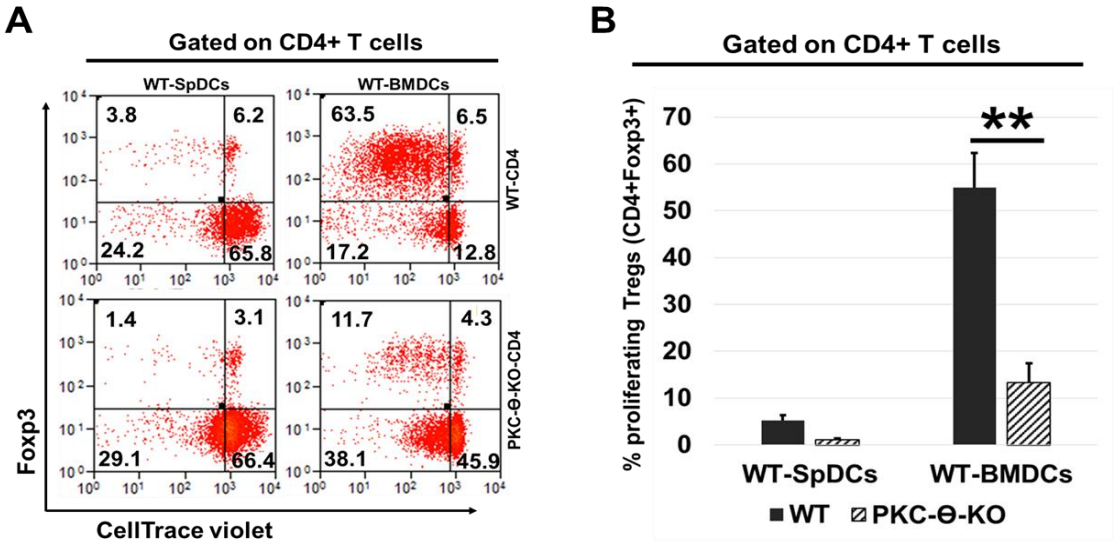

Fig-10: Tregs from PKC-Θ-KO mice showed impaired proliferation when Total CD4$^+$ T cells co-cultured with WT-BMDCs. Total CD4$^+$ T-cells were isolated from the spleens of WT and PKC-Θ-KO mice, CellTrace violet-labeled, then co-cultured with WT-BMDCs. Cells were harvested on day 5 and analyzed by flow cytometry for Treg proliferation. A) Representative dot plots of Treg proliferation. B) Summary bar graphs for proliferating Tregs. * =P<0.05, ** = p<0.005, and *** = p<0.0005.

downstream signaling in Tregs, we would expect to see no Treg proliferation in G-BMDC co-cultures in the absence of PKC-Θ. We isolated splenic $CD4^+$ T cells from both PKC-$\Theta^{-/-}$ and WT mice and co-cultured them with G-BMDCs generated from WT mice (Fig-10A & B). As expected, PKC-$\Theta^{-/-}$ Tregs showed defective proliferation compared to their WT counterparts (55±7.4% vs 13.2±4.2% respectively), supporting our hypothesis that PKC-Θ might have a role in Treg proliferation induced by G-BMDCs co-culture (Fig-10A & B).

4.2.2 PKC-$\Theta^{-/-}$ Tregs regain G-BMDCs-induced proliferation when supplemented with exogenous IL-2. Earlier we have shown that G-BMDCs generated from MHC Class $II^{-/-}$ mice bone marrow precursors were capable and efficient in inducing Treg proliferation implying that the process did not require canonical TCR-stimulation per se; but the induction of Treg proliferation required the supplementation with exogenous IL-2 (66).

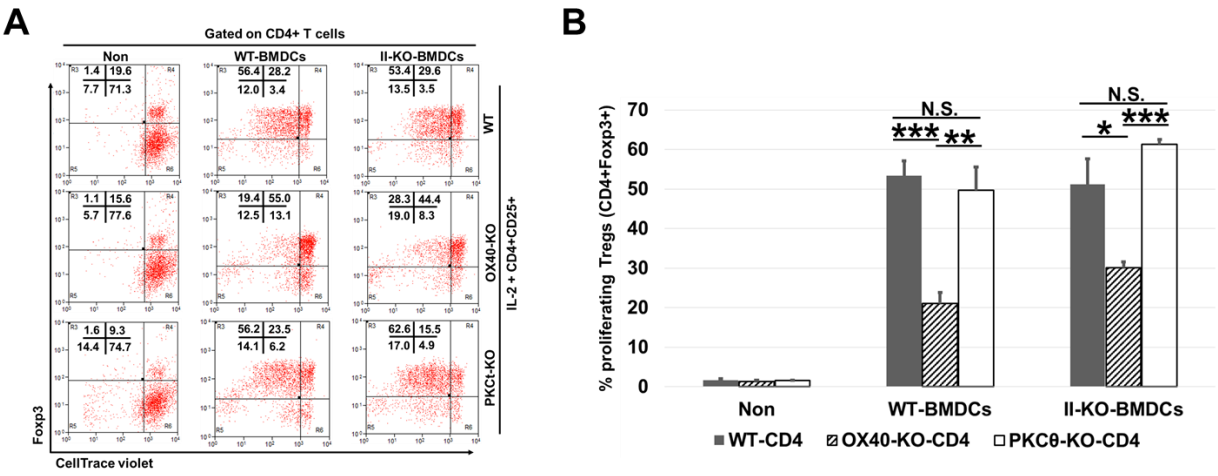

Fig-11: Exogenous IL-2 restore the capability of PKCΘ-KO Tregs to proliferate ex vivo. Splenic $CD4^+CD25^+$ Tregs isolated from WT, OX40-KO, and PKCΘ-KO mice, cultured alone, with WT-BMDCs or II-KO-BMDCs in the presence of exogenous IL-2. Cells were harvested after 4 days and analyzed by flow cytometry. A) Representative dot plots of Treg proliferation. B) Summary bar graphs for proliferating Tregs. * =P<0.05, ** = p<0.005, and *** = p<0.0005.

Since PKC-Θ has been implicated in IL-2 production (155-157), we wanted to investigate whether a deficit in IL-2 production was the reason behind the markedly reduced proliferation of PKC-$\Theta^{-/-}$ Tregs. We isolated splenic Tregs ($CD4^+CD25^+$) from WT, PKC-$\Theta^{-/-}$, and $OX40^{-/-}$ mice, and then co-cultured them separately with WT G-BMDCs, or MHC class $II^{-/-}$ G-BMDCs in the presence of exogenous IL-2 (Fig-11A & B). We found that proliferation of $OX40^{-/-}$ Tregs were significantly decreased when co-cultured with either WT or MHC Class $II^{-/-}$ G-BMDCs ($p<0.0005$, $p<0.05$, respectively) compared to WT Tregs (Fig-11A & B). These results suggested that the requirement of OX40 signaling in the TCR-independent Treg proliferation by G-BMDCs could not be resolved by the addition of exogenous IL-2. However, PKC-$\Theta^{-/-}$ Treg proliferation were rescued to WT levels when supplement with exogenous IL-2, for both WT and MHC Class $II^{-/-}$ G-BMDCs co-cultures (Fig-11A & B).

To further examine the role of IL-2 in PKC-$\Theta^{-/-}$ Treg proliferation phenotype, we isolated total splenic $CD4^+$ T cells from WT, $OX40^{-/-}$, and PKC-$\Theta^{-/-}$ mice then co- cultured them with WT or MHC class $II^{-/-}$ G-BMDCs with or without exogenous IL-2 (Fig-12A & B). As expected, the proliferation of Tregs from WT $CD4^+$ T cells co-cultured with WT G-BMDCs were efficient and comparable in the presence or absence of exogenous IL-2 (Fig-12A & B). Also, these WT Tregs upon co-culture with MHC Class $II^{-/-}$ G-BMDCs regained efficient proliferation when supplemented with exogenous IL-2 (Fig-12A & B). Expectedly, Tregs from $OX40^{-/-}$ $CD4^+$ T cells, maintained impaired proliferation compared to WT Tregs, when co-cultured with either WT or MHC class $II^{-/-}$ G-BMDCs in the presence or absence of IL-2 (Fig-12A & B). These data suggested that OX40 signaling is critical for Treg proliferation induced by G-BMDCs co-culture. On the other hand, Tregs from PKC-

Θ$^{-/-}$ CD4^{+} T cells co-cultured with WT or MHC Class II$^{-/-}$ G-BMDCs showed partially (24.8±3.1% vs 56.3±5.2% in WT) and fully (1.2±0.7% vs 18.0±8.6% in WT) impaired proliferation, respectively. Interestingly, this proliferation defect in PKC-Θ$^{-/-}$ Treg was

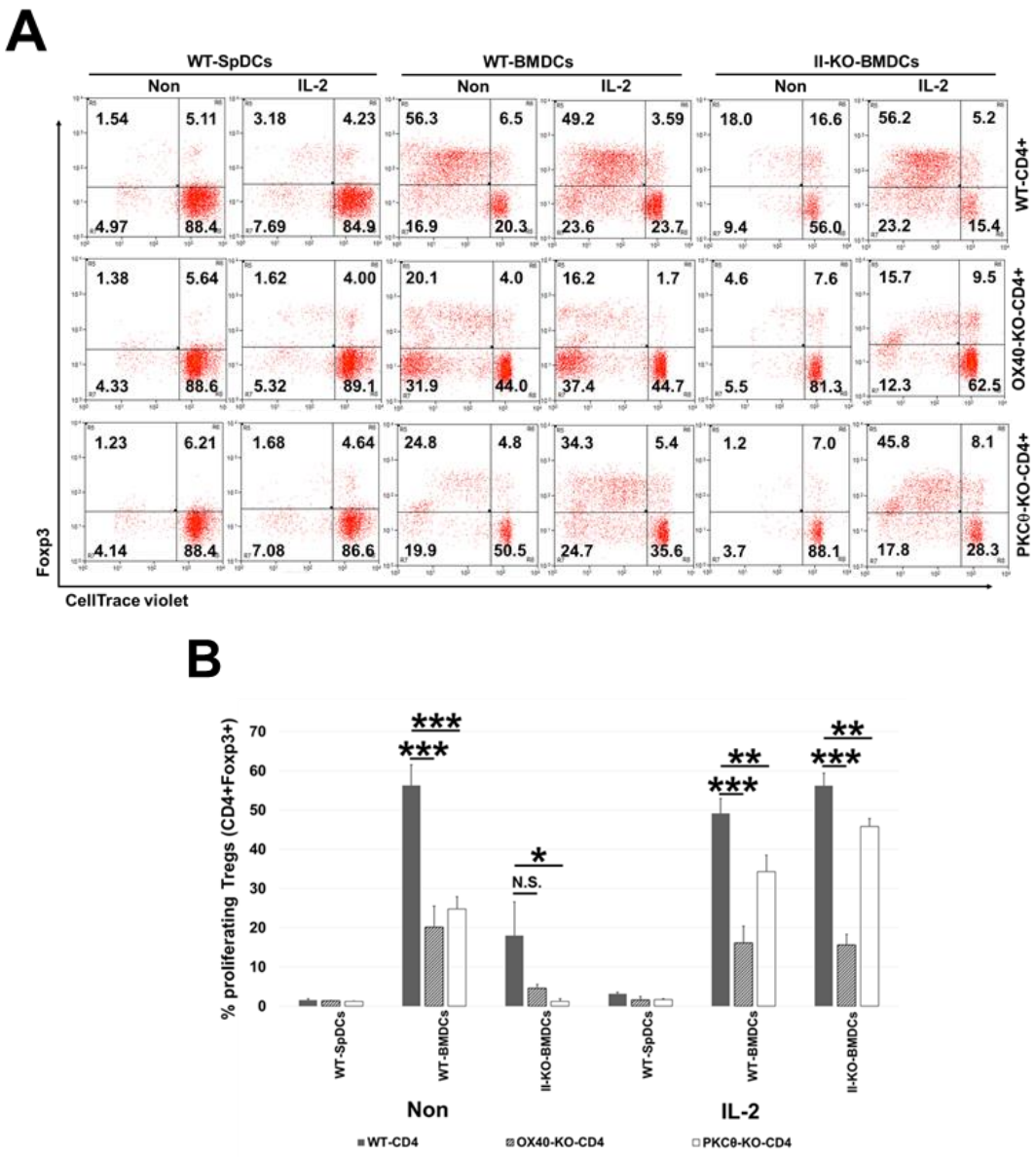

Fig-12: Addition of IL-2 to total CD4+ T cells co-cultured with either WT or class II-KO G-BMDCs rescue the PKCθ-KO Treg phenotype, restore their ex vivo proliferation. Total WT, OX40-KO and PKCθ-KO splenic CD4^{+} T cells, isolated and CellTrace-labeled, were co-cultured with either WT-BMDCs or II-KO-BMDCs in the presence or absence of exogenous IL-2. After 5 days cells were harvested and analyzed by flow cytometry for Treg proliferation. A) Representative dot plots of Treg proliferation. B) Summary bar graphs for proliferating Tregs. * =P<0.05, ** = p<0.005, and *** = p<0.0005.

mostly restored in both co-cultures when supplemented with exogenous IL-2 (Fig-12A & B). Collectively, these data suggested that PKC-Θ is not a critical requirement for the downstream signaling of OX40 in Treg proliferation.

4.2.3 The role of PKC-Θ in Treg proliferation induced by G-BMDCs co-culture is in IL-2 production by Teff cells. Since exogenous IL-2 restored the proliferation of PKC-$\Theta^{-/-}$ Tregs in G-BMDC co-cultures, we suspected that PKC-Θ deficiency may restrict IL-2 production by Teffs in these co-cultures. However, WT Teffs do not have such restrictions and therefore support the proliferation of WT Tregs without addition of exogenous IL-2. To verify this possibility we decided to see if a mix of WT Teff with PKC-$\Theta^{-/-}$ Tregs can support Treg proliferation in G-BMDC co-cultures without addition of exogenous IL-2. We therefore isolated splenic Tregs ($CD4^+CD25^+$) and Teffs ($CD4^+CD25^-$) from WT, $OX40^{-/-}$ and PKC-$\Theta^{-/-}$ mice, re-mixed them in various combinations, co-cultured them with WT or MHC Class $II^{-/-}$ G-BMDCs, and then harvested them for the measurement of Treg proliferation.

First we studied the proliferation of WT Tregs co-cultured with WT-BMDCs in the presence of Teffs from WT, $OX40^{-/-}$ or PKC-$\Theta^{-/-}$ mice. In the presence of $OX40^{-/-}$ Teffs, the proliferation of WT Tregs were comparable to that of WT Tregs/WT Teffs combination (Fig-13A). This suggested that OX40 signaling in Teff did not play any role in IL-2 production. However, the proliferation of WT Treg was significantly ($p<0.05$) decreased in the presence of PKC-$\Theta^{-/-}$ Teffs compared to WT Tregs and WT Teffs co-cultures (Fig-13A). These data suggested the involvement of PKC-Θ in the IL-2 production by Teffs (Fig-13A).

To further investigate and establish that the main defect in PKC-Θ Teffs was related to IL-2 production by Teff cells, we co-cultured Teffs isolated from WT, OX40$^{-/-}$, or PKC-Θ$^{-/-}$ mice with WT Tregs and MHC Class II$^{-/-}$ G-BMDCs in the presence of exogenous IL-2 (Fig-13B). Interestingly, Tregs isolated from WT mice, co-cultured with

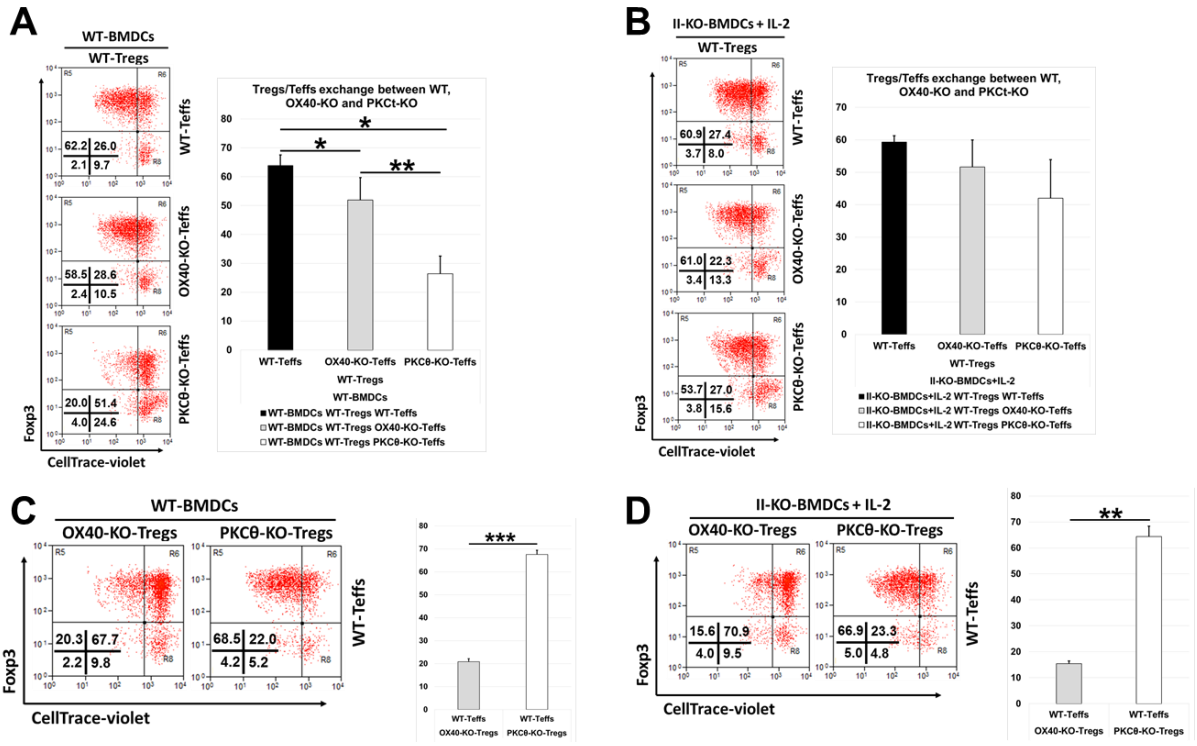

Fig-13: PKCθ-KO Tregs proliferate robustly when co-cultured in the presence of WT Teffs despite the absence of exogenous IL-2. Splenic Tregs ($CD4^+CD25^+$) were isolated from WT, OX40-KO, and PKCθ-KO mice then labeled with CellTrace violet followed by remixing with Teffs ($CD4^+CD25^-$) from their own counterpart and the other two cell subtypes as indicated. Subsequently, remixed T-cells were co-cultured with WT-BMDCs or II-KO-BMDCs + IL-2, after 4 days cells were harvested and analyzed by flow cytometry. A&B) representative dot plots (left) and summary bar graphs (right) show the percentage of proliferating Tregs when WT Tregs remixed with either WT Teffs, OX40-KO Teffs or PKC-θ-KO Teffs then co-cultured with WT-BMDCs (A) or II-KO-BMDCs (B). C&D) representative dot plots (left) and summarizing bar graphs (right) show the percentage of proliferating Tregs when OX40-KO or PKC-θ-KO Tregs remixed with WT Teffs then co-cultured with WT-BMDCs (C) or II-KO-BMDCs (D). * =P<0.05, ** = p<0.005, and *** = p<0.0005.

MHC Class $II^{-/-}$ G-BMDCs in the presence of exogenous IL-2, proliferated efficiently and were comparable despite the presence of either WT, $OX40^{-/-}$ or PKC-$\Theta^{-/-}$ Teffs in the co-culture (Fig-13B). These data supported our prior hypothesis and suggested that the main involvement PKC-Θ in G-BMDC induced Treg proliferation is related to IL-2 production by Teffs (Fig-13B).

In a complementary experiment, we investigated the ability of Tregs isolated from PKC-$\Theta^{-/-}$ mice to proliferate efficiently when co-cultured in the presence of WT Teffs (Fig-13C & D). PKC-$\Theta^{-/-}$ Tregs proliferate efficiently and comparable to Tregs isolated from WT mice in the presence of either WT (Fig-13C) or MHC Class $II^{-/-}$ G-BMDCs supplemented with IL-2 (Fig-13D). This data demonstrated that PKC-$\Theta^{-/-}$ Tregs are intrinsically competent to proliferate upon TCR-independent induction by G-BMDCs co-culture. In contrast, Tregs isolated from $OX40^{-/-}$ mice failed to proliferate efficiently and showed a large reduction compared to PKC-$\Theta^{-/-}$ mice-derived Tregs proliferation. The $OX40^{-/-}$ Tregs proliferation-failure was also observed upon co-cultured with WT Teffs in the presence of MHC-$II^{-/-}$ G-BMDCs with IL-2 supplementation (Fig-13D). Collectively, these data further suggested that G-BMDC-induced Treg-proliferation is OX40 signaling-dependent, and that dependence is intrinsic to Tregs; while PKC-Θ may be involved in the production of IL-2 by effs in G-BMDC co-cultures which in turn is critical for Treg proliferation.

4.2.4 PKC-Θ is dispensable for OX40L treatment mediated Treg-expansion in vivo.

To further investigate the role of PKC-Θ in OX40 signaling in the in vivo context, we injected (i.p.) soluble OX40L three times—one dose per week—into WT, $OX40^{-/-}$ and

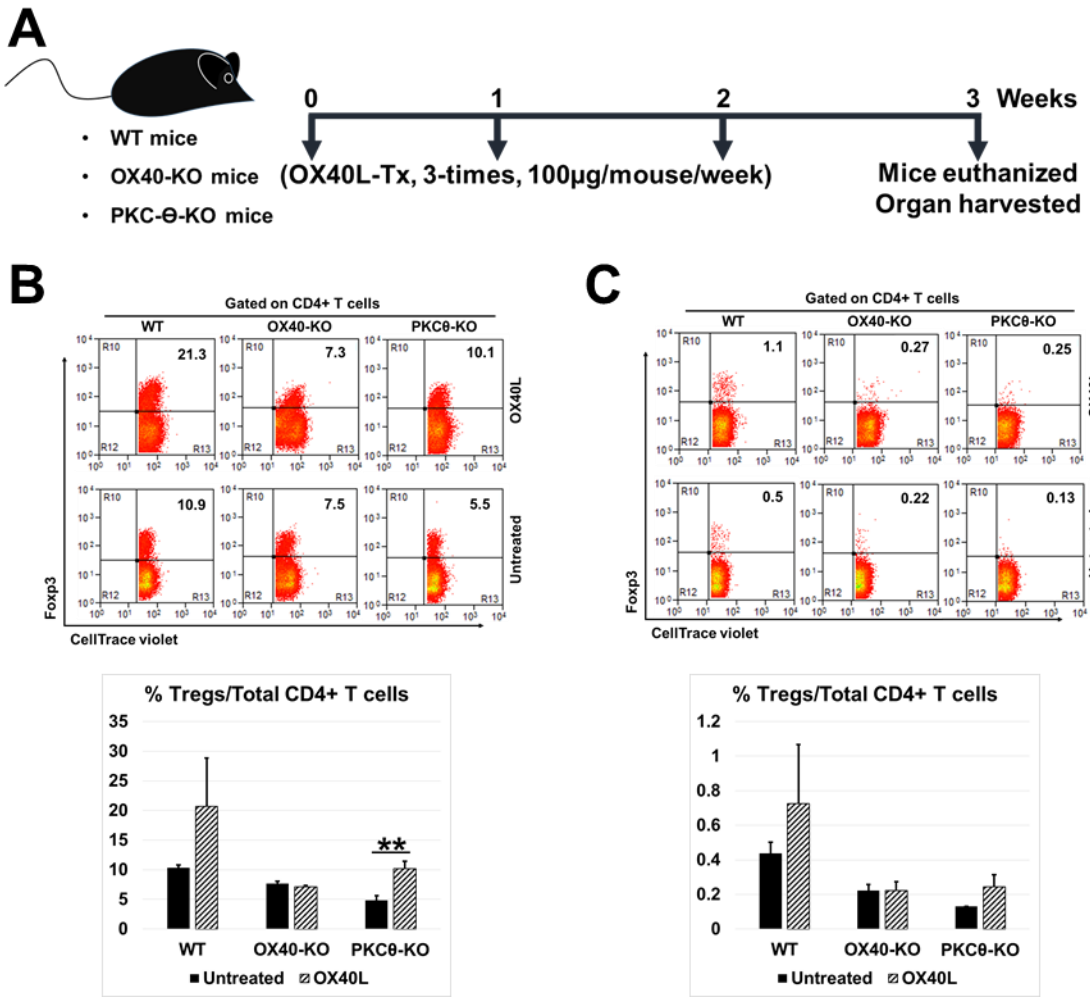

Fig-14: Treatment of PKCθ-KO mice with soluble OX40L increased Tregs. WT, OX40-KO, and PKCθ-KO mice were injected i.p. with PBS or OX40L. In the fourth week mice were euthanized and organs harvested for investigation. A) OX40L treatment scheme. B) Representative dot plots (top) and summary bar graphs (bottom) show the percentage of splenic Tregs from total CD4+ T cells. C) Representative dot plots (top) and summarizing bar graphs (bottom) show the percentage of thymic Tregs from total CD4+ T cells. * =P<0.05, ** = p<0.005, and *** = p<0.0005.

PKC-$\Theta^{-/-}$ mice, one week after the last treatment mice were euthanized and organs were harvested for analysis (Fig-14A). WT and PKC-$\Theta^{-/-}$ mice treated with OX40L showed an increase in Treg percentage among splenic CD4+ T cells compared to their untreated

control counterparts (Fig-14B). However, OX40L-treated OX40$^{-/-}$ mice showed no change in Treg percentage (Fig-14B). Furthermore, we found an increase in Treg percentage among thymic CD4^{+} T cells in WT and PKC-Θ$^{-/-}$ mice treated with OX40L compared to their untreated control counterparts, but that similar increase in Treg percentage were absent in the case OX40$^{-/-}$ treated mice (Fig-14C). Therefore, the shortfall in IL-2 requirement in vitro is not a limiting factor for the proliferation of Tregs in vivo in PKC-Θ$^{-/-}$ mice; likely due to some unknown compensatory mechanism. In conclusion, these data are in agreement with our ex vivo data and further suggest that OX40L/OX40-signaling-induce Treg proliferation is OX40-dependent but PKC-Θ-independent.

4.3 Discussion

In contrast to the well-established concept of adaptive Treg induction via TCR/TGF-β dependent manner (158), Treg development and T cell activation in MHC Class II-TCR-independent manner have been reported (159). Also, stimulation of CD28 (160) in the absence of TCR signal, the most investigated co-stimulatory molecule associated with TCR signaling, has been shown to enhance T cell viability (161) and cytokine production (162). Interestingly, CD28 activation in the absence of TCR signaling is able to induce NF-kB activation (163). Similarly, TCR-independent OX40 activation has been shown to induce assembly of a signalosome, in which PKC-Θ is a critical component. This signalosome leads to NF-kB activation and enhancement of T cell viability and proliferation (125). Therefore, we tested whether a similar mechanism was involved in G-BMDC/OX40L induced Treg proliferation.

We isolated splenic $CD4^+$ T cells from PKC-Θ deficient mice and investigated if they had exhibited defective Treg proliferation. Although PKC-$Θ^{-/-}$ Tregs in total CD4-BMDC co-culture showed inability to proliferate efficiently (Fig-10), isolated PKC-$Θ^{-/-}$ Tregs could be efficiently proliferate when supplemented with IL-2 (Fig-11 and Fig-12). We therefore concluded that PKC-Θ may not be intrinsically required by Tregs for their proliferation, but may be involved in the production of IL-2, a critical survival factor for Tregs which is generally produced by Teffs. While PKC-$Θ^{-/-}$ Tregs proliferated at comparable levels to WT Tregs in the presence of WT Teffs or with the addition of exogenous IL-2, $OX40^{-/-}$ Tregs failed to proliferate even in the presence of WT Teffs or exogenous IL-2 (Fig-13). Additionally, in vivo treatment of WT and PKC-$Θ^{-/-}$ mice with soluble OX40L resulted in a robust increase in Treg percentages, however, the same treatment failed to induce any increase in Treg percentages in $OX40^{-/-}$ mice (Fig-14). These data suggested that PKC-Θ was dispensable for the OX40-dependent Treg expansion ex vivo and in vivo.

5. OX40 SIGNALING, IN TREGS, ACTIVATES NF-KB AND RECRUITS TRAF1

5.1 Introduction

OX40L is a T-cell co-stimulatory molecule and can induce the activation of different signaling cascades including MAPK, PI3K/AKT (117), and NF-kB (94) signaling pathways along with primary TCR-stimulation (Fig-3A). OX40-deficient $CD4^+$ T cells were shown to have impaired NF-kB activation associated with reduced levels of anti-apoptotic molecules like BCL-2 and poor survival as well. In addition, restoration of NF-kB signaling rescued their ability to sustain the expression of BCL-2 and survival (94, 164). Furthermore, OX40 signaling has been shown to regulate the activation of PI3K and AKT both of which are upstream activators of mTOR (94, 118) in the presence of TCR activation.

On the other hand, in the absence of TCR-ligation (Fig-3B) OX40 signaling induces formation of a signaling complex consisting of PKC-Ɵ, TRAF2/TRAF3/TRAF5, IKKα, IKKβ, and IKKγ that leads to the phosphorylation of IKKα and IKKβ and activation of NF-kB (94, 125). However, these studies were performed on effector T-cells, whereas we have observed selective activation of OX40 signaling in Tregs, but not on Teff cells. In addition, it is not clear whether OX40L can activate cell-signaling pathways other than NF-kB in the absence of TCR activation, which in turn can regulate the Treg proliferation. Therefore, we decided to study the downstream signaling mechanisms activated upon OX40L/OX40 ligation which leads to selective Treg expansion.

5.2 Experimental results

5.2.1 NF-kB signaling pathway is involved in G-BMDC-induced *ex vivo* Treg proliferation. At first, we investigated the key signaling pathways activated during G-BMDC-induced Treg proliferation. Total $CD4^+$ T cells or $CD4^+CD25^+$ Tregs from NOD mice were pre-treated with kinase inhibitors targeting the EGFRK, p38 MAPK, PKC, PI3K and IKK2 (NF-kB) signaling pathways then washed followed by co-culturing with G-

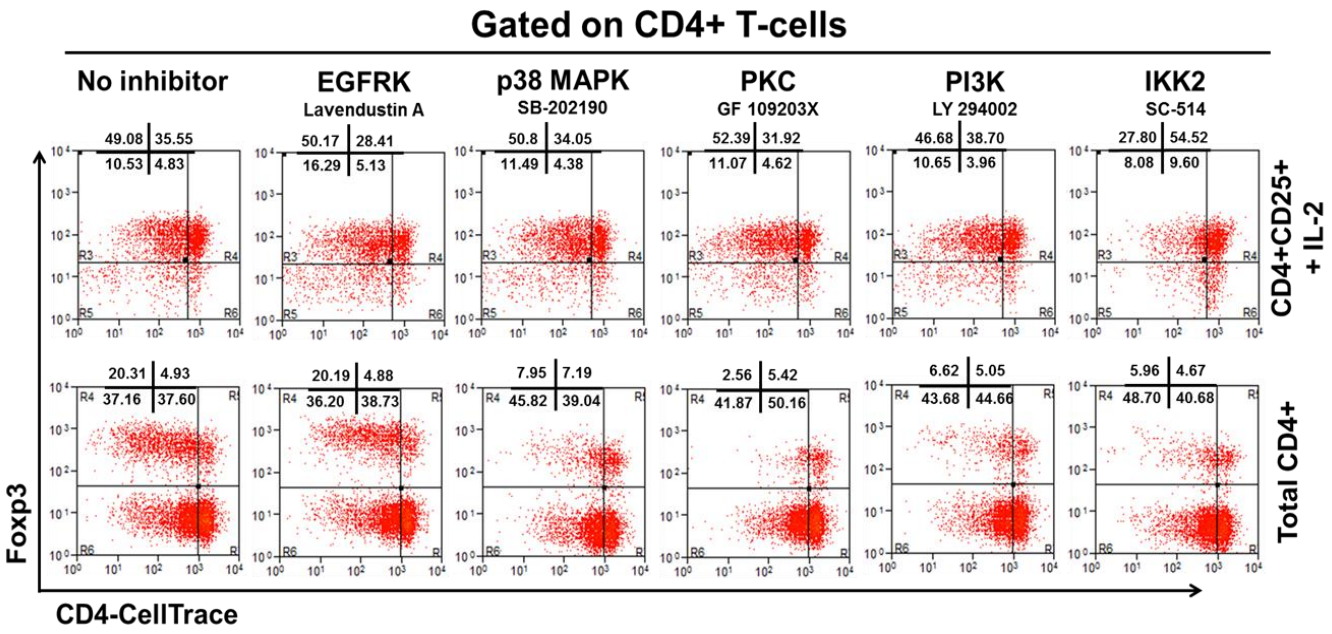

Fig-15: IKK2 kinase inhibitor ameliorates G-BMDC-induced Treg proliferation. Total $CD4^+$ (Bottom panel) or $CD4^+CD25^+$ T cells (Top panel) isolated from NOD mice spleen were CellTrace violet-labeled, pre-treated with indicated kinase inhibitors for six hours cells. Cells were washed, and co-cultured with G-BMDC for five days. Cells were harvested analyzed by flow cytometry for Tregs proliferation.

BMDCs. Treg co-cultures supplemented with exogenous IL-2 to compensate for lack of IL-2 production from Teff cells. As shown in Fig-15, among the various inhibitors tested, IKK2 kinase inhibitor ameliorated Treg proliferation in both $CD4^+$ T cells as well as $CD4^+CD25^+$ Treg co-cultures supplemented with IL-2. These data suggested that G-BMDC-induced Treg proliferation involved activation of the NF-kB signaling.

To further clarify the importance of NF-kB signaling in the G-BMDC-induced, TCR-independent Treg proliferation, we studied the phosphorylation of key components of NF-kB signaling such as RelA (pRelA) and RelB (pRelB) in WT, PKC-Θ$^{-/-}$ and OX40$^{-/-}$ Tregs upon co-culturing with MHC Class II$^{-/-}$ G-BMDCs and IL-2 supplementation. After 1, 2, 3,

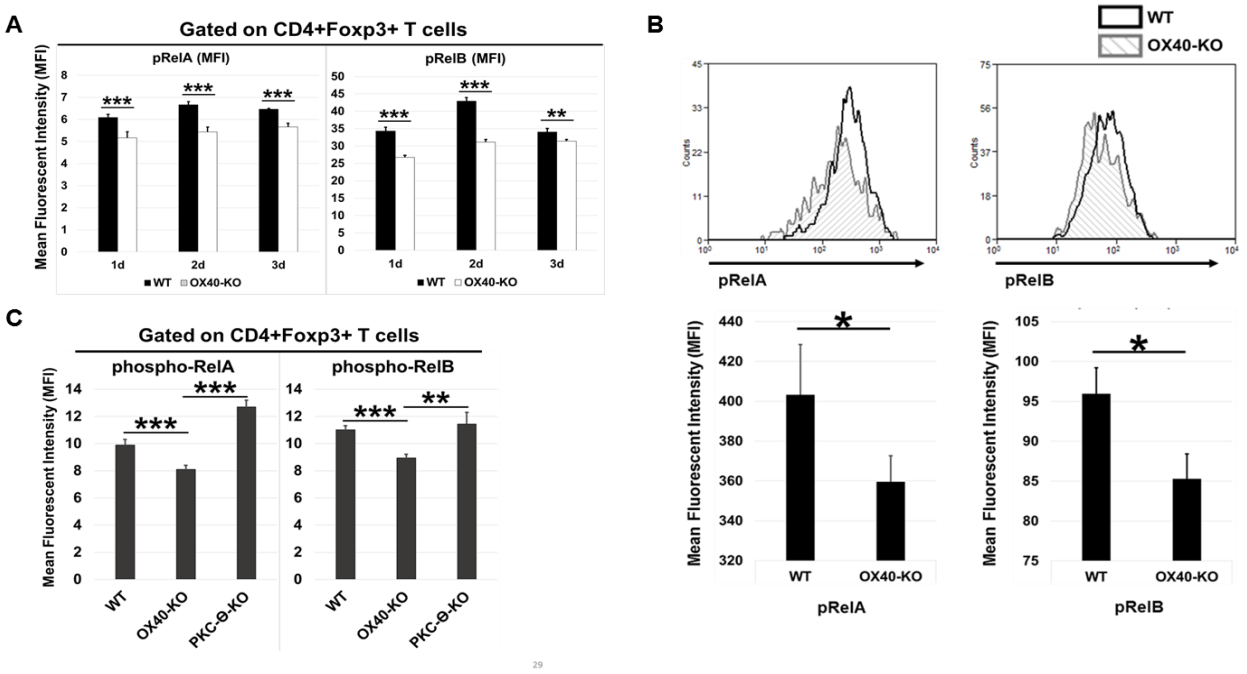

Fig-16: Involvement of NF-kB signaling pathway in Treg proliferation induced by G-BMDCs. A&B) WT and OX40-KO splenic CD4$^+$ T cells, isolated, CellTrace violet-labeled, and co-cultured with II-KO-BMDCs in presence of exogenous IL-2. A) After 1, 2, and 3 days cells were harvested and analyzed by flow cytometry for the MFI of the indicated molecules. B) After five days cells were harvested and analyzed by flow cytometry, top representative overlay histograms, bottom bar graphs summarizing the MFI of the indicated molecules. C) WT, OX40-KO and PKC-Θ-KO splenic CD4$^+$ T-cells, isolated, CellTrace-labeled, then co-cultured with II-KO-BMDCs in presence of exogenous IL-2. After 24hrs, cells were harvested and analyzed by flow cytometry. * =P<0.05, ** = p<0.005, and *** = p<0.0005.

and 5 days of cultures cells were harvested and analyzed for the levels of RelA and RelB phosphorylation by flow cytometry using mean fluorescence intensity (MFI). The MFI analysis showed substantial upregulation of pRelA, and pRelB in WT Tregs compared to OX40$^{-/-}$ Tregs in all the time points tested (Fig-16A & B). Additionally, we observed

significant down-regulation of RelA and RelB phosphorylation in OX40$^{-/-}$ Tregs when compared to WT and PKC-Θ$^{-/-}$ Tregs co-cultured in the presence of MHC Class II$^{-/-}$ G-BMDCs and IL-2 supplementation (Fig-16C). Taken together, these data suggest that OX40 signaling can induce NF-kB activation in absence of TCR-stimulation, which could be a critical driver of Treg proliferation.

5.2.2 Convergence of NF-kB and STAT5 signaling likely leads to selective TCR-independent Treg proliferation. NF-kB activation has been shown to be an important mediator of OX40 signaling in T cells in the absence of TCR-stimulation (125), and our results (Fig-15 &16) also suggested an integral role for NF-kB activation in Treg proliferation induced by OX40L/OX40 signaling in the absence of TCR-stimulation. Furthermore, a TCR-independent pathway has already been suggested to be involved in the development nTregs (165, 166). Therefore, to further investigate the role OX40-NF-kB and IL-2/STAT5 signaling in Treg proliferation in the context of ex vivo treatment of thymocytes with soluble OX40L in the presence of exogenous IL-2, we stimulated WT thymocytes with soluble OX40L in the presence of IL-2 and studied Treg proliferation, activation of NF-kB signaling molecules (RelA and RelB), and activation of STAT5, a well-established signaling molecule activated by phosphorylation downstream of IL-2 signaling (Fig-17). We found that soluble OX40L significantly increased Treg proliferation compared to IL-2 alone control (Fig-17A) along with simultaneous increase in the phosphorylation of NF-kB subunits RelA and RelB (Fig-17B).

Further, our western blot analysis also confirmed time dependent increase in the phosphorylation of NF-kB-RelA induced by OX40L in thymocytes (Fig-17C). IL-2 induced STAT5 activation in thymic Tregs has been shown to play a key role in the regulation of

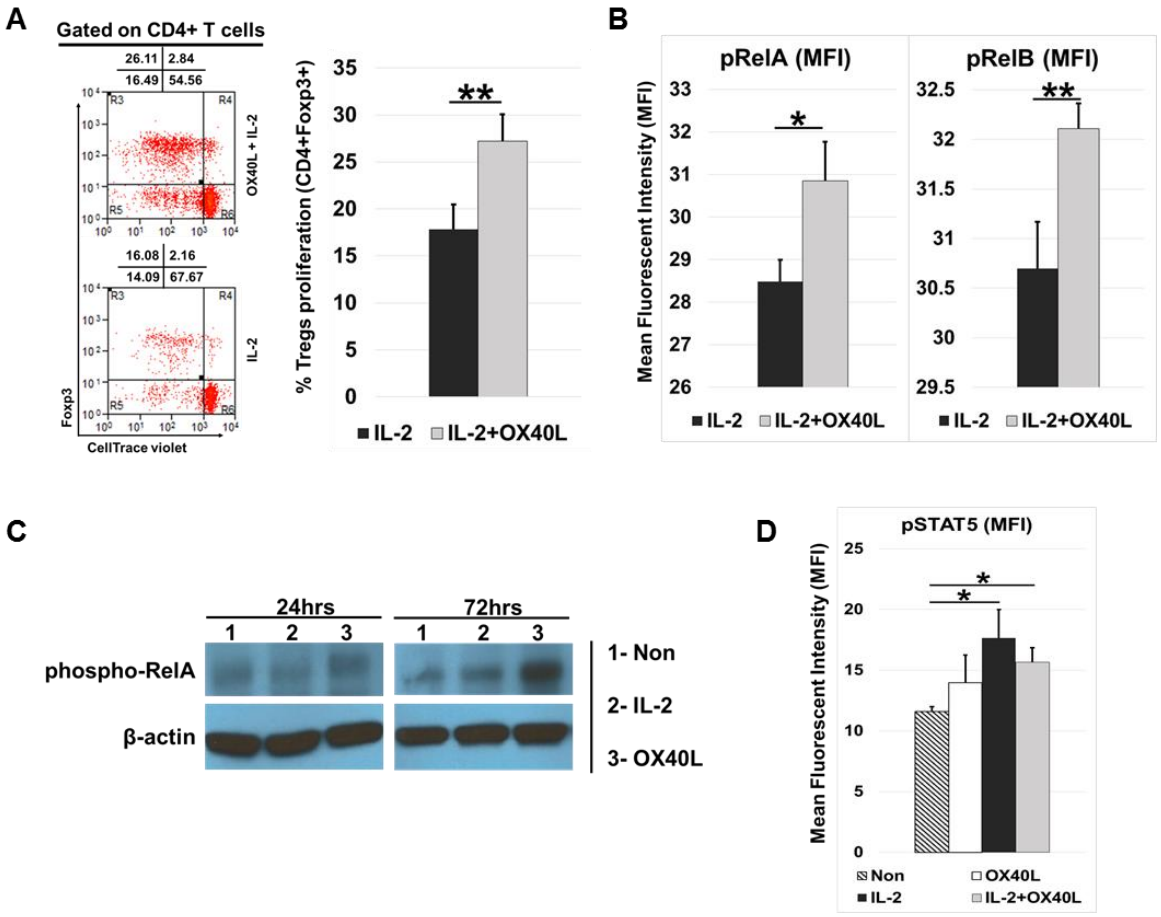

Fig-17: Ex vivo treatment with soluble OX40L induced Treg proliferation in thymocytes. A & B) WT thymocytes were CellTrace violet labeled then cultured with or without OX40L in the presence IL-2. Cells were harvested after 5 days and analyzed. A) Representative dot plots (left) and summarizing bar graphs (right) show the percentage of proliferating Tregs. B) Bar graphs show the Mean Fluorescent Intensity (MFI) of pRelA (left) and pRelB (right) in Tregs ($CD4^+Foxp3^+$). C) Western blot analysis of pRelA activation by OX40L in ex vivo thymocyte cultures. D) WT thymocytes were cultured in the presence of no stimuli, IL-2 alone, OX40L alone or IL-2+OX40L. After 24hr cells were harvested for analysis. Summarizing bar graphs show the MFI of pSTAT5 in Tregs ($CD4^+Foxp3^+$). * =P<0.05, ** = p<0.005, and *** = p<0.0005.

Foxp3 expression in thymocytes. Therefore, we analyzed STAT5 phosphorylation by flow cytometry and we found that the phosphorylation of STAT5 was substantially upregulated

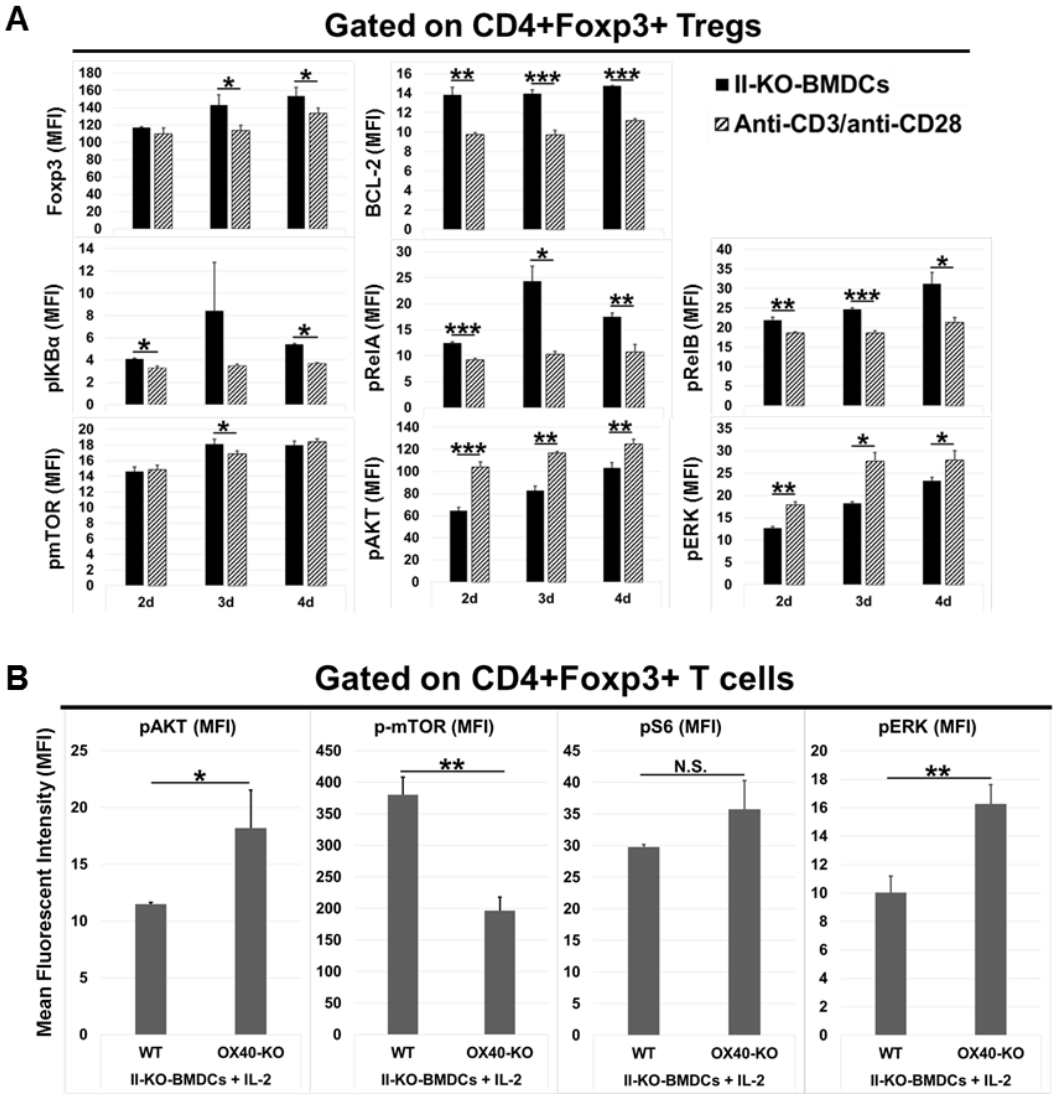

Fig-18: NF-kB signaling molecules phosphorylation found to be upregulated in Tregs upon G-BMDCs co-culture compared to TCR stimulation. A) Splenic $CD4^+$ T cells isolated from WT mice, co-cultured with either anti-CD3/anti-CD28-coated microbeads or II-KO-BMDCs in the presence of exogenous IL-2. Cells were harvested after 2, 3, and 4 days and analyzed by flow cytometry. B) Splenic $CD4^+$ T cells isolated from WT and OX40-KO mice, co-cultured with II-KO-BMDCs in presence exogenous IL-2. After 5 days cells were harvested and analyzed by flow cytometry. * =P<0.05, ** = p<0.005, and *** = p<0.0005.

by OX40L/IL-2 treatment compared to untreated thymocytes at 24h (Fig-17D). Taken together, these results suggested that activation of NF-kB and STAT5 signaling pathways induced by OX40L-IL-2 could act in synergy to drive Treg proliferation with sustained Foxp3 expression in the absence of TCR stimulation.

5.2.3 Increased NF-kB activation in G-BMDC-induced Tregs compared to TCR-stimulated Tregs. Previous studies have shown activation of MAPK, PI3K/AKT/mTOR (117, 167), and NF-kB signaling pathways (94) in T-cells upon TCR activation when CD28 co-stimulation provided. As shown in Fig-3B, OX40 signaling also forms a signaling complex which can lead to the phosphorylation of IKKα and IKKβ and activation of NF-KB in the absence of TCR stimulation (94, 125). Therefore, we setup an experiment to investigate the differential activation of MAPK, PI3K/AKT/mTOR, and NF-kB signaling pathway in Tregs expanded by TCR-stimulation (anti-CD3 + anti-CD28) vs TCR-independent G-BMDC stimulation. We isolated $CD4^+$ T cells from the spleen of WT mice and cultured with either MHC Class $II^{-/-}$ G-BMDCs or with microbeads coated with anti-CD3 and anti-CD28 monoclonal antibodies in the presence of exogenous IL-2 for 2-4 days. Interestingly, we observed increased Foxp3 expression in Tregs expanded with MHC Class $II^{-/-}$ G-BMDCs compared to TCR-stimulated Tregs (Fig-18A). In addition, phosphorylation of NF-kB signaling pathway molecules (IKBα, RelA, and RelB) were significantly upregulated in MHC Class $II^{-/-}$ G-BMDCs expanded Tregs compared to anti-CD3 and anti-CD28 expanded Tregs (Fig-18A) at indicated time points. Also, the expression of the anti-apoptotic molecule BCL-2 whose expression has been shown to correlate with the activation of NF-kB (94, 164) was upregulated in MHC Class $II^{-/-}$ G-BMDCs expanded Tregs (Fig-18A). These data suggested that the G-BMDCs were

capable of expanding Tregs and maintaining their viability (BCL-2), potentially through activating NF-kB and sustaining their Foxp3 expression in a more efficient manner compared to anti-CD3/CD28 induced TCR-stimulation.

Furthermore, we observed increased phosphorylation of ERK1/2 and AKT in anti-CD3/anti-CD28 expanded Tregs compared to G-BMDC-induced Tregs (Fig-18A) at indicated time intervals. However, our results showed no change in mTOR phosphorylation other than day 3, between anti-CD3/anti-CD28 stimulation and G-BMDC-induction (Fig-18A). To further investigate the MAPK and PI3K/AKT/mTOR pathways in the context of OX40 signaling, we isolated splenic $CD4^+$ T cells from WT and $OX40^{-/-}$ mice then co-cultured them with MHC Class $II^{-/-}$ G-BMDCs with exogenous IL-2 supplementation. After five days, cells were harvested and analyzed by flow cytometry (Fig-18B). Phosphorylation of ERK1/2 and AKT were substantially more in Tregs isolated from $OX40^{-/-}$ mice compared to WT Tregs (Fig-18B). On the other hand, phosphorylation of mTOR was upregulated in WT Tregs compared to $OX40^{-/-}$ Tregs (Fig-18B). However, the phosphorylation of S6, downstream target of mTOR complex 1 (mTORC1), showed no notable change between the WT and $OX40^{-/-}$ Tregs (Fig-18B). Collectively, these data suggested a possible regulatory role for OX40 signaling in the activation of MAPK and PI3K/AKT signaling pathways.

5.2.4 OX40 likely recruits TRAF1 to drive TCR-independent Treg proliferation in a Treg specific manner. To further investigate the signaling pathways activated in Tregs upon G-BMDC induction, we sorted resting and proliferating Tregs ($CD4^+CD25^+$) from G-BMDC co-cultures. Total RNA isolated from these Tregs were subjected to microarray analysis (NIH GEO; Accession No. GSE81051) (70). Interestingly, among the TNF-

receptor superfamily members, OX40, 4-1BB, and GITR showed substantial upregulation and TNFR2, showed moderate upregulation in proliferating compared to resting Tregs (Fig-19A; i). BCL10 and RIPK1, components of signaling complex downstream of OX40 signaling (94, 125), were also found to be upregulated in proliferating Tregs (Fig-19A; ii). Members of TNF receptor super family, including OX40, are known to form a signaling complex which can lead to the activation of NF-kB. TRAF-family members (TRAF1-7) play an important role in this NF-kB activation. Differential gene expression analysis showed substantial upregulation of TRAF1 in proliferating Tregs compared to resting Tregs (Fig-19A; iii). Among the cell cycle regulators, we found CDK1 (Fig-19B) and cyclin

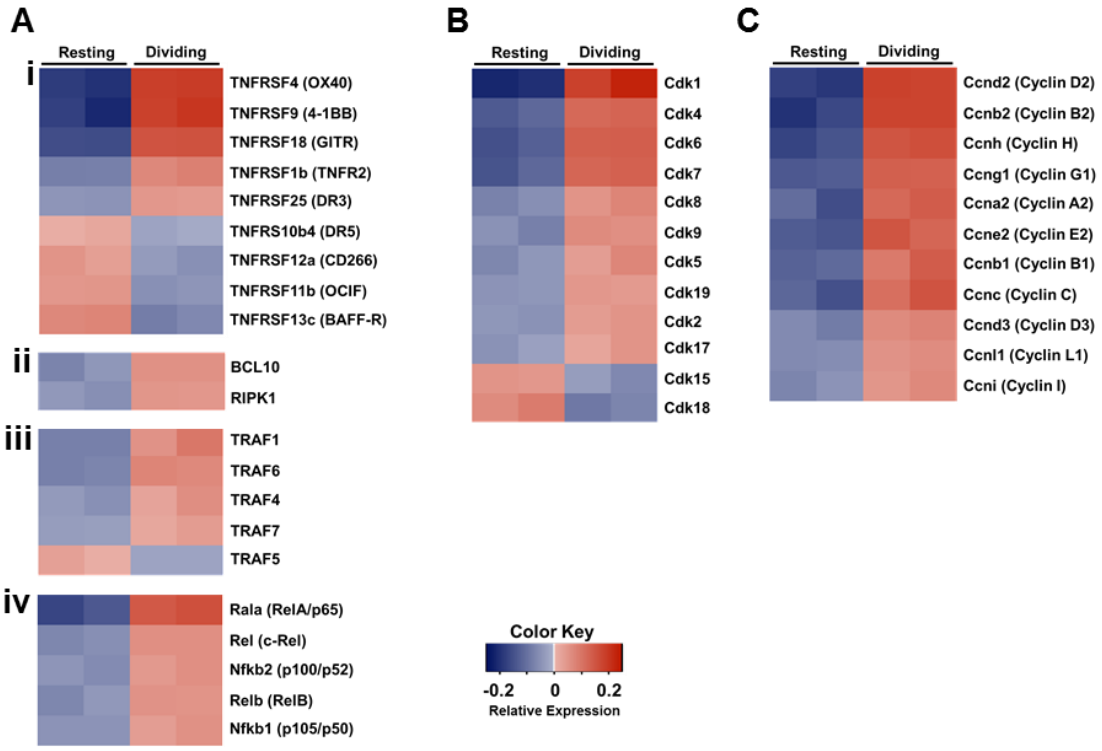

Fig-19: Proliferating Tregs showed upregulation of TNFRSF genes including OX40. Gene Microarray analysis of relative expression of mRNA expression between proliferating and resting Tregs. Heat maps showing positive relative upregulation in red presented in related groups. (First part of experiment shown in Fig-6). A) TNF-superfamily Receptors (i), OX40-signalosome molecules (ii), TRAFs (iii), and NF-kB pathway molecules (iv). B) CDKs C) cyclins.

D2 (Fig-19C) upregulation in proliferating Tregs. It is pertinent to mention here that, cyclin D2 has been shown to stimulate cell cycle progress in resting T cells after activation by NF-kB (168). Also, the ability of CDK1 to interact with all cyclins, including cyclin D2, and promote cell cycle progression despite the absence of other CDKs, has already been shown (169). Therefore, OX40 signaling could involve TRAF1 mediated NF-kB activation, followed by cyclin D2 expression and CDK1 activation leading to Treg proliferation.

TABLE-I PRIMER SEQUENCE FOR QRT-PCR

Target gene	primer	Sequence
TRAF1	Forward	5’- AGG GTG GAA TTA CAG CAA -3'
	Reverse	5’- GCA GTG TAG AAA GCT GGA GAG -3'
TRAF2	Forward	5’-AGA GAG TAG TTC GGC CTT TCC -3'
	Reverse	5’- GAG CAT CCA TCA TTG GGA CAG -3'
TRAF3	Forward	5’-AGA CCC GAG AAA ACC GTC G -3'
	Reverse	5’- CTC CTT TTG AGG AAC TGT AGC TG -3'
TRAF4	Forward	5’- CCC GGC TTC GAC TAC AAG TTC-3'
	Reverse	5’- TCA GGG CAT TTG AAG ACT CCT-3'
TRAF5	Forward	5’- TTT GAG CCC GAC ACC GAG TA -3'
	Reverse	5’- AGA GAC CGG ATG CAC TGC T -3'
TRAF6	Forward	5’- AAA GCG AGA GAT TCT TTC CCT -3'
	Reverse	5’- ACT GGG GAC AAT TCA CTA GAG C -3'
TRAF7	Forward	5’- GAC CAG GAT GGA AAC CTT T -3'
	Reverse	5’- AAT AGG CAA GGG TGC TAG AGG -3'

To further study the possibility of TRAF1 involvement in OX40 signaling and Treg proliferation, we analyzed the mRNA expression of TRAFs (1-7) using quantitative real-time-PCR (qRT-PCR) in freshly isolated WT splenic Tregs ($CD4^+CD25^+$) compared to

freshly isolated WT splenic Teffs ($CD4^+CD25^+$). Mouse TRAF1-7 specific primers used for the qRT-PCR are shown in (Table-I). Interestingly, and in consistence with what has been shown for human Tregs (170), TRAF1 was found to be preferentially overexpressed in Tregs compared to Teffs (Fig-20A).

Additionally, to investigate the association of TRAF1 with OX40 during OX40L/OX40 interaction, we isolated $CD4^+$ T cells from the spleen of WT mice then cultured them in the presence or absence of soluble OX40L with IL-2 supplementation. Cells were subsequently harvested and stained with fluorescent-coupled antibodies for Foxp3, OX40, and TRAF1 and were analyzed for the recruitment of TRAF1 in OX40 signaling complex. Using ImageJ co-localization software, we found that TRAF1 and OX40 were co-localized in $Foxp3^+$Tregs stimulated with OX40L compared to unstimulated Tregs (Fig-20B). Collectively, these data suggested that OX40 signaling complex is likely to involve TRAF1, leading to NF-kB activation and proliferation of Tregs.

5.3 Discussion

G-BMDC-induced Treg proliferation has two unique properties that distinguish it from canonical TCR activation and proliferation of T cells (3). Firstly, it preferentially induces the expansion of $Foxp3^+$ Tregs. Additionally, this expansion is induced in a MHC-II/TCR-independent manner. Furthermore, earlier we have shown that OX40 signaling is critical for this G-BMDC-induced Treg proliferation. OX40 and other TNFR superfamily members signaling involve TRAF family of proteins (113, 116). TRAF proteins family members TRAF2, TRAF3, and TRAF5 have been shown to associate with OX40 for signal transduction (119-124). In the context of TCR-independent signaling, OX40L/OX40

activation was shown to induce the formation of a signalosome which activate NF-kB signaling (94, 125). We therefore used kinase inhibitors (Fig-15) and OX40 deficient cells (Fig-16) to investigate NF-kB signaling activation downstream of OX40L/OX40 interaction

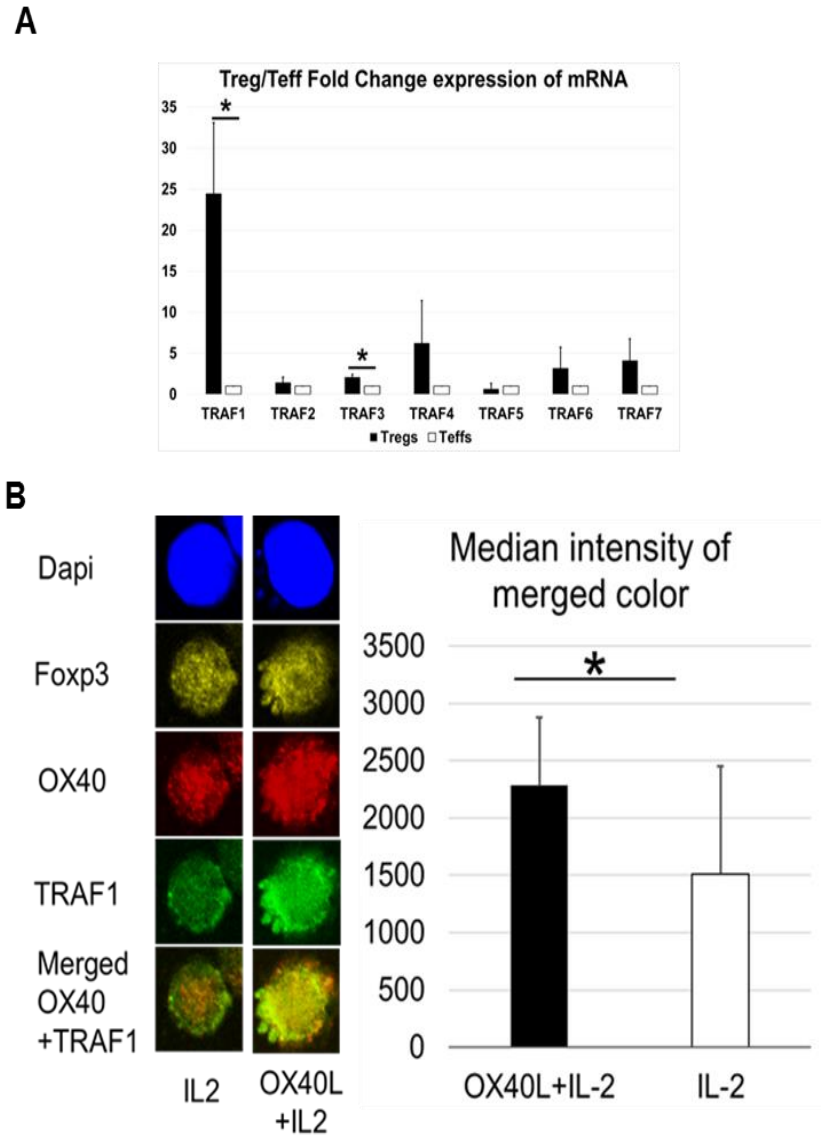

Fig-20: TRAF1 expression is upregulated in Tregs and is co-localized with OX40 upon OX40L treatment. A) Tregs ($CD4^+CD25^+$) and Teffs ($CD4^+CD25^-$) isolated from WT mice spleen. RNA isolated and subjected to cDNA synthesis followed by quantitative Real-Time-PCR for differential TRAFs mRNA expression. B) $CD4^+$ T-cells isolated from WT mice spleen, cultured in the presence of IL-2 with or without OX40L. After 48hr cells were harvested and stained for confocal microscopy analysis. ImageJ software is used to measure the median intensity of merged colors between OX40-red and TRAF1-green. * =P<0.05, ** = p<0.005, and *** = p<0.0005.

in G-BMDC-induced Treg expansion phenotype. Our results suggested that G-BMDCs induced Treg proliferation is mediated via OX40/OX40L induced NF-kB activation in the absence of TCR stimulation (Fig-15 and Fig16).

It is relevant to mention here that a TCR-independent mechanism of nTreg development in the thymus has already been suggested (165, 166). Autoreactive Treg progenitors have been suggested to be rescued from apoptosis in the thymic medulla by IL-2R/STAT5-dependent, but TCR-independent mechanism (171). To further investigate this possible mechanism, we cultured WT thymocytes supplemented with IL-2 with or without OX40L. Thymocyte cultures treated with OX40L in the presence of IL-2 showed substantial increase in Treg proliferation and also upregulation of p-RelA, p-RelB, and phospho-STAT5 compared to untreated control (Fig-17). These results suggested that NF-kB activation could be a conserved mechanism of TCR-independent proliferation between splenic and thymic Tregs.

In the presence of TCR activation, OX40 signaling induces the activation of MAPK, PI3K (117), AKT (117, 118), and NF-kB (119). However, in the absence of TCR- activation OX40-signaling has been shown to activate NF-kB (94, 125) to maintain the survival of T cells. However, it is not clear whether other signaling pathways such as MAPK and PI3K/AKT are also activated by OX40L in the absence of TCR activation. Therefore, we further investigated that by comparing signaling pathways activated between WT Tregs induced to proliferate either by G-BMDC co-culture or anti-CD3/anti-CD28 coated microbeads (Fig-18). Tregs co-cultured with G-BMDCs showed significant upregulation of Foxp3 and phosphorylation of NF-kB molecules, but downregulation of phosphorylation of AKT and ERK. Interestingly, in OX40 deficient Tregs, we found increased

phosphorylation of AKT and ERK compared to WT Tregs (Fig-18). These data suggest that, in the context of G-BMDC-induced Treg proliferation, OX40 signaling might down regulate AKT and ERK activation. However, it is not clear if down regulation of AKT/ERK might in turn regulate Treg proliferation or not.

Next, we examined the possible molecular mechanims underlying G-BMDC induced Treg proliferation. We compared the gene expression profile between prolferating and resting Tregs and found upregulation of TNF receptor family members OX40, 4-1BB, GITR, and TNFR2 mRNA expression in proliferating Tregs (Fig-19). Interestingly, OX40, 4-1BB, GITR, and TNFR2 are encoded by genes located on the same chromosome in both human and mouse (172, 173). It also appears that these genes coordinately upregulated via TNFR2 activation (174). Therefore, the OX40-activation downstream signaling in Tregs could be similar to the well-established 4-1BB/GITR/TNFR2 induced mechanisms involved in Treg expansion (89, 91). Furthermore, our microarray data showed upregulation of members of OX40 signalosome such as BCL10 and RIPK1, TRAFs, and NF-kB genes and as expected cell cycle-related molecules CDK1 and cyclin D2 (Fig-19).

During T cell activation, TRAF2 and TRAF5 are usually associated with OX40 signaling leading to NF-kB activation (119), however, in the case of 4-1BB (175) and TNFR2 (123, 125) NF-kB activation involved TRAF1. Interestingly, we found substantial upregulation of TRAF1 mRNA in Tregs compared to Teffs (Fig-20). Additionally, we observed an increase in the association between OX40 and TRAF1 in Tregs when supplemented with OX40L (Fig-20). Therefore, our results suggest a possible novel OX40/TRAF1 interaction might be involved in the G-BMDC-induced Treg proliferation.

6. CONCLUSION

6. Conclusion

Because of the potential use of Tregs in the treatment of autoimmune diseases and transplantation-rejection, investigating Tregs induction and proliferation became a very important area of research (55-57). However, these have been cell-based therapy approaches, which is based on purification and in vitro expansion of Tregs followed by re-transfusion back to patients (55-57). Therefore, these protocols face huge challenges such as the purity of Treg isolation in absence of Treg-specific surface markers. Further, these protocols are limited to in vitro use because Treg expansion is based on TCR stimulation, which could induce the proliferation of all T cells. The TCR-independent Treg-preferential expansion phenotype described by our lab is very important and could lead to the development of an ex vivo and in vivo Treg expansion protocol.

We have found that anti-OX40L blocking antibodies could block Treg proliferation induced by G-BMDCs compared to control, also, blocking antibodies against other TNF superfamily members failed to block Treg proliferation. This confirmed the critical role that OX40L/OX40 signaling has in G-BMDC-induced Treg expansion (Fig-4). Further, splenic $CD4^+$ T cells from $OX40^{-/-}$ mice co-cultured with G-BMDCs failed to proliferate like their WT counterpart, further confirming that OX40L/OX40-stimulation is vital for G-BMDC-induced Treg proliferation (Fig-5). Additionally, using TCR-induced proliferation we found that $OX40^{-/-}$ Treg proliferate to a degree comparable to WT counterpart (Fig-5). Microarray analysis of differential regulation of gene expression between proliferating and resting Tregs showed expression upregulation of genes encoding Treg functional phenotype markers in expanding Tregs (Fig-6). Further, flow cytometry analysis confirmed the protein expression upregulation of some of these markers on the proliferating Tregs (Fig-

7). Furthermore, using OX40L in vivo treatment, we established that the in vivo expanded Tregs maintained their suppression function (Fig-8 and Fig-9).

Treg induction and development of Treg through TCR/TGF-β dependent manner (158) and Treg development and T cell activation in MHC Class II-TCR-independent manner have been intensively studied (159). The induction of NF-kB activation by CD28, the most investigated co-stimulatory molecule associated with TCR signaling (161, 162), stimulation in the absence of TCR signaling has been reported (163). Comparable to that, in the absence of TCR signaling OX40 stimulation has been shown to form a signalosome with PKC-Θ as a critical component; this signaling complex leads to NF-kB activation and augmentation of T cell viability and expansion (125).

Using splenic $CD4^+$ T cells from PKC-$\Theta^{-/-}$ mice, we found that in total CD4-BMDC co-culture Tregs lost the ability to proliferate efficiently (Fig-10), however, IL-2 supplementation restored Treg proliferation (Fig-11 and Fig-12). Therefore, we hypothesized that PKC-Θ may be dispensable intrinsically for Treg proliferation, but may be involved in IL-2 production by Teffs. We found that PKC-$\Theta^{-/-}$ Tregs proliferate comparable to WT Tregs upon co-culture in the presence of WT Teffs or exogenous IL-2 (Fig-13). Contrarily, $OX40^{-/-}$ Tregs could not proliferate efficiently even in the presence of WT Teffs or exogenous IL-2 (Fig-13). Furthermore, in vivo treatment of WT and PKC-$\Theta^{-/-}$ mice with soluble OX40L induced substantial increase in Treg percentages, but the same treatment did not induce any increase in Treg percentages in $OX40^{-/-}$ mice (Fig-14). These data demonstrated that PKC-Θ was dispensable for the preferential OX40-dependent Treg expansion ex vivo and in vivo.

TNFR superfamily members, including OX40, signaling involve TRAF protein family members (113, 116). TRAF2, TRAF3, and TRAF5 have been shown to be involved downstream of OX40 signal transduction (119-124). In the absence of TCR-stimulation, OX40L/OX40 signaling was shown to cause the assembly of signaling complex that led to NF-kB signaling activation (94, 125).

In the context of G-BMDC-induced Treg proliferation, using kinase inhibitors (Fig-15) and $OX40^{-/-}$ cells (Fig-16) we found that OX40 signaling in the absence of TCR stimulation likely activates NF-kB. A mechanism of TCR-independent nTreg development in thymus has already been suggested (165, 166). Autoreactive Treg precursors have been suggested to be rescued from apoptosis in thymic medulla by an IL-2R/STAT5-dependent, but TCR-independent mechanism (171). We found that thymocytes cultures supplemented with OX40L showed substantial upregulation of Treg proliferation, phospho-RelA, phospho-RelB, and phospho-STAT5 compared to IL-2 alone (Fig-17). These data suggested that NF-kB activation is a conserved mechanism of TCR-independent proliferation between splenic and thymic Tregs.

OX40 signaling induces activation of MAPK, PI3K (117), AKT (117, 118), and NF-kB (119) in the presence of TCR stimulation. But in absence of TCR signaling, OX40 activation induces the assembly of signaling complex that activate NF-kB (94, 125). We found that Tregs from MHC Class II deficient mice co-cultured with G-BMDCs showed upregulation of Foxp3 and phosphorylation of NF-kB molecules, but downregulation of phosphorylation of AKT and ERK as compared to TCR-stimulated Tregs (Fig-18). Interestingly, OX40 deficient cells, when used in the same experimental setup, showed upregulation of AKT and ERK phosphorylation in deficient Tregs compared to their WT

counterpart (Fig-18). These data suggest that OX40 signaling induced by G-BMDCs might have downregulation role on AKT and ERK activation in proliferating Tregs.

The microarray analysis compared gene expression in proliferating versus resting Tregs showed upregulation of OX40, 4-1BB, GITR, and TNFR2 mRNA (Fig-19). OX40, 4-1BB, GITR, and TNFR2 are encoded by genes located on the same chromosome in both human and mouse (172, 173), and they also appear be coordinately upregulated through TNFR2 activation (174). Therefore, the downstream signaling of OX40 in Tregs could be similar to the well-established 4-1BB/GITR/TNFR2 induced mechanisms involved in Treg expansion (89, 91).

TRAF2 and TRAF5 are usually associated with OX40 signaling leading to NF-kB activation during T cell activation (119), but TRAF1 is involved in 4-1BB (175) and TNFR2 (123, 125) NF-kB activation. Interestingly, we found that TRAF1 mRNA in Tregs were substantial upregulated compared to Teffs (Fig-20). Additionally, cultures supplemented with OX40L showed increase in the association between OX40 and TRAF1 in Tregs compared to non-supplemented (Fig-20). Therefore, these data suggest a possible novel OX40/TRAF1 interaction might be involved in the G-BMDC-induced, TCR-independent Treg proliferation.

In summary, using cells from $OX40^{-/-}$, we confirmed the important role of OX40L/OX40 interaction in Treg expansion. We demonstrated that PKC-Ɵ is dispensable for OX40L-induced TCR-independent Treg expansion ex vivo and in vivo. Finally, our data indicated a possible involvement of TRAF1, downstream of OX40, in Tregs.

REFERENCES

REFERENCES

1. Murphy K, Weaver C. 2016. *Janeway's immunobiology*. New York, NY: Garland Science/Taylor & Francis Group, LLC. p. pp.
2. Germain RN. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* 2: 309-22
3. Abbas AK, Murphy KM, Sher A. 1996. Functional diversity of helper T lymphocytes. *Nature* 383: 787-93
4. Bretscher PA. 1999. A two-step, two-signal model for the primary activation of precursor helper T cells. *Proc Natl Acad Sci U S A* 96: 185-90
5. June CH, Ledbetter JA, Gillespie MM, Lindsten T, Thompson CB. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol Cell Biol* 7: 4472-81
6. Mueller DL, Jenkins MK, Schwartz RH. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* 7: 445-80
7. Chen L, Flies DB. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* 13: 227-42
8. Sun B, Zhang Y. 2014. Overview of orchestration of CD4+ T cell subsets in immune responses. *Adv Exp Med Biol* 841: 1-13
9. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. 2005. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. 1986. *J Immunol* 175: 5-14
10. Zhang Y, Zhang Y, Gu W, Sun B. 2014. TH1/TH2 cell differentiation and molecular signals. *Adv Exp Med Biol* 841: 15-44
11. Zhang Y, Zhang Y, Gu W, He L, Sun B. 2014. Th1/Th2 cell's function in immune system. *Adv Exp Med Biol* 841: 45-65
12. Jia L, Wu C. 2014. Differentiation, regulation and function of Th9 cells. *Adv Exp Med Biol* 841: 181-207
13. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, Mitsdoerffer M, Strom TB, Elyaman W, Ho IC, Khoury S, Oukka M, Kuchroo VK. 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol* 9: 1347-55
14. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, Martin B, Wilhelm C, Stockinger B. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9: 1341-6
15. Stassen M, Schmitt E, Bopp T. 2012. From interleukin-9 to T helper 9 cells. *Ann N Y Acad Sci* 1247: 56-68
16. Song X, Gao H, Qian Y. 2014. Th17 differentiation and their pro-inflammation function. *Adv Exp Med Biol* 841: 99-151
17. Pourgholaminejad A, Aghdami N, Baharvand H, Moazzeni SM. 2016. Is TGFbeta as an anti-inflammatory cytokine required for differentiation of inflammatory TH17 cells? *J Immunotoxicol* 13: 775-83
18. Jia L, Wu C. 2014. The biology and functions of Th22 cells. *Adv Exp Med Biol* 841: 209-30
19. Qi H, Chen X, Chu C, Liu D, Ma W, Wang Y, Wu L, Yan H, Yan J. 2014. Tfh cell differentiation and their function in promoting B-cell responses. *Adv Exp Med Biol* 841: 153-80

20. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-64
21. Piccioni M, Chen Z, Tsun A, Li B. 2014. Regulatory T-cell differentiation and their function in immune regulation. *Adv Exp Med Biol* 841: 67-97
22. Fontenot JD, Gavin MA, Rudensky AY. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4: 330-6
23. Hori S, Nomura T, Sakaguchi S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057-61
24. Plitas G, Rudensky AY. 2016. Regulatory T Cells: Differentiation and Function. *Cancer Immunol Res* 4: 721-5
25. Wildin RS, Freitas A. 2005. IPEX and FOXP3: clinical and research perspectives. *J Autoimmun* 25 Suppl: 56-62
26. Kim JM, Rasmussen JP, Rudensky AY. 2007. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* 8: 191-7
27. Weissler KA, Caton AJ. 2014. The role of T-cell receptor recognition of peptide:MHC complexes in the formation and activity of Foxp3(+) regulatory T cells. *Immunol Rev* 259: 11-22
28. Klein L, Kyewski B, Allen PM, Hogquist KA. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol* 14: 377-91
29. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322: 271-5
30. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192: 303-10
31. Pan F, Yu H, Dang EV, Barbi J, Pan X, Grosso JF, Jinasena D, Sharma SM, McCadden EM, Getnet D, Drake CG, Liu JO, Ostrowski MC, Pardoll DM. 2009. Eos mediates Foxp3-dependent gene silencing in CD4+ regulatory T cells. *Science* 325: 1142-6
32. Li P, Gao Y, Cao J, Wang W, Chen Y, Zhang G, Robson SC, Wu Y, Yang J. 2015. CD39+ regulatory T cells attenuate allergic airway inflammation. *Clin Exp Allergy* 45: 1126-37
33. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, Hopner S, Centonze D, Bernardi G, Dell'Acqua ML, Rossini PM, Battistini L, Rotzschke O, Falk K. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110: 1225-32
34. Bollyky PL, Falk BA, Long SA, Preisinger A, Braun KR, Wu RP, Evanko SP, Buckner JH, Wight TN, Nepom GT. 2009. CD44 costimulation promotes FoxP3+ regulatory T cell persistence and function via production of IL-2, IL-10, and TGF-beta. *J Immunol* 183: 2232-41
35. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* 8: 1353-62
36. Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, Gasteiger G, Feng Y, Fontenot JD, Rudensky AY. 2016. An essential role for the IL-2 receptor in Treg cell function. *Nat Immunol* 17: 1322-33
37. Sprent J, Kishimoto H. 2001. The thymus and central tolerance. *Philos Trans R Soc Lond B Biol Sci* 356: 609-16
38. Schwartz RH. 1989. Acquisition of immunologic self-tolerance. *Cell* 57: 1073-81

39. Hogquist KA, Baldwin TA, Jameson SC. 2005. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* 5: 772-82
40. McGargill MA, Derbinski JM, Hogquist KA. 2000. Receptor editing in developing T cells. *Nat Immunol* 1: 336-41
41. Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY. 2006. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol* 7: 401-10
42. Hamilton-Williams EE, Bergot AS, Reeves PL, Steptoe RJ. 2016. Maintenance of peripheral tolerance to islet antigens. *J Autoimmun* 72: 118-25
43. Medzhitov R. 2001. Toll-like receptors and innate immunity. *Nat Rev Immunol* 1: 135-45
44. Redmond WL, Hernandez J, Sherman LA. 2003. Deletion of naive CD8 T cells requires persistent antigen and is not programmed by an initial signal from the tolerogenic APC. *J Immunol* 171: 6349-54
45. Smith TR, Verdeil G, Marquardt K, Sherman LA. 2014. Contribution of TCR signaling strength to CD8+ T cell peripheral tolerance mechanisms. *J Immunol* 193: 3409-16
46. Goodnow CC, Sprent J, Fazekas de St Groth B, Vinuesa CG. 2005. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* 435: 590-7
47. Goodnow CC. 2007. Multistep pathogenesis of autoimmune disease. *Cell* 130: 25-35
48. Nishizuka Y, Sakakura T. 1969. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science* 166: 753-5
49. Kasper IR, Apostolidis SA, Sharabi A, Tsokos GC. 2016. Empowering Regulatory T Cells in Autoimmunity. *Trends Mol Med* 22: 784-97
50. Grant CR, Liberal R, Mieli-Vergani G, Vergani D, Longhi MS. 2015. Regulatory T-cells in autoimmune diseases: challenges, controversies and--yet--unanswered questions. *Autoimmun Rev* 14: 105-16
51. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775-87
52. Heusschen R, Griffioen AW, Thijssen VL. 2013. Galectin-9 in tumor biology: a jack of multiple trades. *Biochim Biophys Acta* 1836: 177-85
53. Wang F, Wan L, Zhang C, Zheng X, Li J, Chen ZK. 2009. Tim-3-Galectin-9 pathway involves the suppression induced by CD4+CD25+ regulatory T cells. *Immunobiology* 214: 342-9
54. Julian MK. 2014. Autoimmune disease: cost-effective care. *Nurs Manage* 45: 24-9; quiz 30
55. Rosenblum MD, Gratz IK, Paw JS, Abbas AK. 2012. Treating human autoimmunity: current practice and future prospects. *Sci Transl Med* 4: 125sr1
56. Tang Q, Bluestone JA. 2013. Regulatory T-cell therapy in transplantation: moving to the clinic. *Cold Spring Harb Perspect Med* 3
57. Leslie M. 2011. Immunology. Regulatory T cells get their chance to shine. *Science* 332: 1020-1
58. Passerini L, Santoni de Sio FR, Porteus MH, Bacchetta R. 2014. Gene/cell therapy approaches for Immune Dysregulation Polyendocrinopathy Enteropathy X-linked syndrome. *Curr Gene Ther* 14: 422-8
59. Cheatem D, Ganesh BB, Gangi E, Vasu C, Prabhakar BS. 2009. Modulation of dendritic cells using granulocyte-macrophage colony-stimulating factor (GM-CSF) delays type 1 diabetes by enhancing CD4+CD25+ regulatory T cell function. *Clin Immunol* 131: 260-70
60. Vasu C, Dogan RN, Holterman MJ, Prabhakar BS. 2003. Selective induction of dendritic cells using granulocyte macrophage-colony stimulating factor, but not fms-like tyrosine kinase receptor 3-ligand, activates thyroglobulin-specific CD4+/CD25+ T cells and suppresses experimental autoimmune thyroiditis. *J Immunol* 170: 5511-22

61. Gangi E, Vasu C, Cheatem D, Prabhakar BS. 2005. IL-10-producing CD4+CD25+ regulatory T cells play a critical role in granulocyte-macrophage colony-stimulating factor-induced suppression of experimental autoimmune thyroiditis. *J Immunol* 174: 7006-13
62. Ganesh BB, Cheatem DM, Sheng JR, Vasu C, Prabhakar BS. 2009. GM-CSF-induced CD11c+CD8a--dendritic cells facilitate Foxp3+ and IL-10+ regulatory T cell expansion resulting in suppression of autoimmune thyroiditis. *Int Immunol* 21: 269-82
63. Sheng JR, Li L, Ganesh BB, Vasu C, Prabhakar BS, Meriggioli MN. 2006. Suppression of experimental autoimmune myasthenia gravis by granulocyte-macrophage colony-stimulating factor is associated with an expansion of FoxP3+ regulatory T cells. *J Immunol* 177: 5296-306
64. Sheng JR, Li LC, Ganesh BB, Prabhakar BS, Meriggioli MN. 2008. Regulatory T cells induced by GM-CSF suppress ongoing experimental myasthenia gravis. *Clin Immunol* 128: 172-80
65. Meriggioli MN, Sheng JR, Li L, Prabhakar BS. 2008. Strategies for treating autoimmunity: novel insights from experimental myasthenia gravis. *Ann N Y Acad Sci* 1132: 276-82
66. Bhattacharya P, Gopisetty A, Ganesh BB, Sheng JR, Prabhakar BS. 2011. GM-CSF-induced, bone-marrow-derived dendritic cells can expand natural Tregs and induce adaptive Tregs by different mechanisms. *Journal of leukocyte biology* 89: 235-49
67. Gopisetty A, Bhattacharya P, Haddad C, Bruno JC, Jr., Vasu C, Miele L, Prabhakar BS. 2013. OX40L/Jagged1 cosignaling by GM-CSF-induced bone marrow-derived dendritic cells is required for the expansion of functional regulatory T cells. *J Immunol* 190: 5516-25
68. Takeda I, Ine S, Killeen N, Ndhlovu LC, Murata K, Satomi S, Sugamura K, Ishii N. 2004. Distinct roles for the OX40-OX40 ligand interaction in regulatory and nonregulatory T cells. *Journal of immunology* 172: 3580-9
69. Cahill EF, Tobin LM, Carty F, Mahon BP, English K. 2015. Jagged-1 is required for the expansion of CD4+ CD25+ FoxP3+ regulatory T cells and tolerogenic dendritic cells by murine mesenchymal stromal cells. *Stem Cell Res Ther* 6: 19
70. Kumar P, Alharshawi K, Bhattacharya P, Marinelarena A, Haddad C, Sun Z, Chiba S, Epstein AL, Prabhakar BS. 2017. Soluble OX40L and JAG1 Induce Selective Proliferation of Functional Regulatory T-Cells Independent of canonical TCR signaling. *Sci Rep* 7: 39751
71. Minami Y, Kono T, Miyazaki T, Taniguchi T. 1993. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol* 11: 245-68
72. Wuest TY, Willette-Brown J, Durum SK, Hurwitz AA. 2008. The influence of IL-2 family cytokines on activation and function of naturally occurring regulatory T cells. *J Leukoc Biol* 84: 973-80
73. Ellery JM, Nicholls PJ. 2002. Possible mechanism for the alpha subunit of the interleukin-2 receptor (CD25) to influence interleukin-2 receptor signal transduction. *Immunol Cell Biol* 80: 351-7
74. Papiernik M, Banz A. 2001. Natural regulatory CD4 T cells expressing CD25. *Microbes Infect* 3: 937-45
75. Coffer PJ, Burgering BM. 2004. Forkhead-box transcription factors and their role in the immune system. *Nat Rev Immunol* 4: 889-99
76. Ziegler SF. 2006. FOXP3: of mice and men. *Annu Rev Immunol* 24: 209-26
77. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27: 68-73
78. Sharpe AH, Freeman GJ. 2002. The B7-CD28 superfamily. *Nat Rev Immunol* 2: 116-26

79. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, Zheng XX, Strom TB, Bluestone JA. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 171: 3348-52
80. Guo F, Iclozan C, Suh WK, Anasetti C, Yu XZ. 2008. CD28 controls differentiation of regulatory T cells from naive CD4 T cells. *J Immunol* 181: 2285-91
81. Zheng J, Chan PL, Liu Y, Qin G, Xiang Z, Lam KT, Lewis DB, Lau YL, Tu W. 2013. ICOS regulates the generation and function of human CD4+ Treg in a CTLA-4 dependent manner. *PLoS One* 8: e82203
82. Redpath SA, van der Werf N, Cervera AM, MacDonald AS, Gray D, Maizels RM, Taylor MD. 2013. ICOS controls Foxp3(+) regulatory T-cell expansion, maintenance and IL-10 production during helminth infection. *Eur J Immunol* 43: 705-15
83. Walker LS. 2013. Treg and CTLA-4: two intertwining pathways to immune tolerance. *J Autoimmun* 45: 49-57
84. Kurtulus S, Sakuishi K, Ngiow SF, Joller N, Tan DJ, Teng MW, Smyth MJ, Kuchroo VK, Anderson AC. 2015. TIGIT predominantly regulates the immune response via regulatory T cells. *J Clin Invest* 125: 4053-62
85. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D, Grogan JL. 2009. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* 10: 48-57
86. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, Sharpe AH, Kuchroo VK. 2011. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* 186: 1338-42
87. Levin SD, Taft DW, Brandt CS, Bucher C, Howard ED, Chadwick EM, Johnston J, Hammond A, Bontadelli K, Ardourel D, Hebb L, Wolf A, Bukowski TR, Rixon MW, Kuijper JL, Ostrander CD, West JW, Bilsborough J, Fox B, Gao Z, Xu W, Ramsdell F, Blazar BR, Lewis KE. 2011. Vstm3 is a member of the CD28 family and an important modulator of T-cell function. *Eur J Immunol* 41: 902-15
88. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, Xia J, Tan TG, Sefik E, Yajnik V, Sharpe AH, Quintana FJ, Mathis D, Benoist C, Hafler DA, Kuchroo VK. 2014. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* 40: 569-81
89. Mahmud SA, Manlove LS, Schmitz HM, Xing Y, Wang Y, Owen DL, Schenkel JM, Boomer JS, Green JM, Yagita H, Chi H, Hogquist KA, Farrar MA. 2014. Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nat Immunol* 15: 473-81
90. Chen X, Nie Y, Xiao H, Bian Z, Scarzello AJ, Song NY, Anna TL, Yang D, Oppenheim JJ. 2016. TNFR2 expression by CD4 effector T cells is required to induce full-fledged experimental colitis. *Sci Rep* 6: 32834
91. Chen X, Baumel M, Mannel DN, Howard OM, Oppenheim JJ. 2007. Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells. *J Immunol* 179: 154-61
92. Chen X, Wu X, Zhou Q, Howard OM, Netea MG, Oppenheim JJ. 2013. TNFR2 is critical for the stabilization of the CD4+Foxp3+ regulatory T. cell phenotype in the inflammatory environment. *J Immunol* 190: 1076-84
93. Liao G, Nayak S, Regueiro JR, Berger SB, Detre C, Romero X, de Waal Malefyt R, Chatila TA, Herzog RW, Terhorst C. 2010. GITR engagement preferentially enhances proliferation of functionally competent CD4+CD25+FoxP3+ regulatory T cells. *Int Immunol* 22: 259-70
94. Croft M. 2010. Control of immunity by the TNFR-related molecule OX40 (CD134). *Annu Rev Immunol* 28: 57-78

95. Zheng G, Wang B, Chen A. 2004. The 4-1BB costimulation augments the proliferation of CD4+CD25+ regulatory T cells. *J Immunol* 173: 2428-34
96. Zhang P, Gao F, Wang Q, Wang X, Zhu F, Ma C, Sun W, Zhang L. 2007. Agonistic anti-4-1BB antibody promotes the expansion of natural regulatory T cells while maintaining Foxp3 expression. *Scand J Immunol* 66: 435-40
97. Smith SE, Hoelzinger DB, Dominguez AL, Van Snick J, Lustgarten J. 2011. Signals through 4-1BB inhibit T regulatory cells by blocking IL-9 production enhancing antitumor responses. *Cancer Immunol Immunother* 60: 1775-87
98. Fletcher JM, Lonergan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, Tubridy N, Mills KH. 2009. CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J Immunol* 183: 7602-10
99. Ponta H, Sherman L, Herrlich PA. 2003. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* 4: 33-45
100. Firan M, Dhillon S, Estess P, Siegelman MH. 2006. Suppressor activity and potency among regulatory T cells is discriminated by functionally active CD44. *Blood* 107: 619-27
101. Bollyky PL, Lord JD, Masewicz SA, Evanko SP, Buckner JH, Wight TN, Nepom GT. 2007. Cutting edge: high molecular weight hyaluronan promotes the suppressive effects of CD4+CD25+ regulatory T cells. *J Immunol* 179: 744-7
102. Ermann J, Hoffmann P, Edinger M, Dutt S, Blankenberg FG, Higgins JP, Negrin RS, Fathman CG, Strober S. 2005. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. *Blood* 105: 2220-6
103. Fan Y, Lu D. 2016. The Ikaros family of zinc-finger proteins. *Acta Pharm Sin B* 6: 513-21
104. Shevach EM, Thornton AM. 2014. tTregs, pTregs, and iTregs: similarities and differences. *Immunol Rev* 259: 88-102
105. Singh K, Hjort M, Thorvaldson L, Sandler S. 2015. Concomitant analysis of Helios and Neuropilin-1 as a marker to detect thymic derived regulatory T cells in naive mice. *Sci Rep* 5: 7767
106. Szurek E, Cebula A, Wojciech L, Pietrzak M, Rempala G, Kisielow P, Ignatowicz L. 2015. Differences in Expression Level of Helios and Neuropilin-1 Do Not Distinguish Thymus-Derived from Extrathymically-Induced CD4+Foxp3+ Regulatory T Cells. *PLoS One* 10: e0141161
107. Ross EM, Bourges D, Hogan TV, Gleeson PA, van Driel IR. 2014. Helios defines T cells being driven to tolerance in the periphery and thymus. *Eur J Immunol* 44: 2048-58
108. Bettini ML, Pan F, Bettini M, Finkelstein D, Rehg JE, Floess S, Bell BD, Ziegler SF, Huehn J, Pardoll DM, Vignali DA. 2012. Loss of epigenetic modification driven by the Foxp3 transcription factor leads to regulatory T cell insufficiency. *Immunity* 36: 717-30
109. Sharma MD, Huang L, Choi JH, Lee EJ, Wilson JM, Lemos H, Pan F, Blazar BR, Pardoll DM, Mellor AL, Shi H, Munn DH. 2013. An inherently bifunctional subset of Foxp3+ T helper cells is controlled by the transcription factor eos. *Immunity* 38: 998-1012
110. Paterson DJ, Jefferies WA, Green JR, Brandon MR, Corthesy P, Puklavec M, Williams AF. 1987. Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. *Mol Immunol* 24: 1281-90
111. Tanaka Y, Inoi T, Tozawa H, Yamamoto N, Hinuma Y. 1985. A glycoprotein antigen detected with new monoclonal antibodies on the surface of human lymphocytes infected with human T-cell leukemia virus type-I (HTLV-I). *Int J Cancer* 36: 549-55
112. Baum PR, Gayle RB, 3rd, Ramsdell F, Srinivasan S, Sorensen RA, Watson ML, Seldin MF, Baker E, Sutherland GR, Clifford KN, et al. 1994. Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. *EMBO J* 13: 3992-4001
113. Gough MJ, Weinberg AD. 2009. OX40 (CD134) and OX40L. *Adv Exp Med Biol* 647: 94-107

114. Gramaglia I, Weinberg AD, Lemon M, Croft M. 1998. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol* 161: 6510-7
115. Rogers PR, Song J, Gramaglia I, Killeen N, Croft M. 2001. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity* 15: 445-55
116. Wajant H, Henkler F, Scheurich P. 2001. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell Signal* 13: 389-400
117. Mestas J, Crampton SP, Hori T, Hughes CC. 2005. Endothelial cell co-stimulation through OX40 augments and prolongs T cell cytokine synthesis by stabilization of cytokine mRNA. *Int Immunol* 17: 737-47
118. Song J, Salek-Ardakani S, Rogers PR, Cheng M, Van Parijs L, Croft M. 2004. The costimulation-regulated duration of PKB activation controls T cell longevity. *Nat Immunol* 5: 150-8
119. Kawamata S, Hori T, Imura A, Takaori-Kondo A, Uchiyama T. 1998. Activation of OX40 signal transduction pathways leads to tumor necrosis factor receptor-associated factor (TRAF) 2- and TRAF5-mediated NF-kappaB activation. *J Biol Chem* 273: 5808-14
120. Prell RA, Evans DE, Thalhofer C, Shi T, Funatake C, Weinberg AD. 2003. OX40-mediated memory T cell generation is TNF receptor-associated factor 2 dependent. *J Immunol* 171: 5997-6005
121. So T, Salek-Ardakani S, Nakano H, Ware CF, Croft M. 2004. TNF receptor-associated factor 5 limits the induction of Th2 immune responses. *J Immunol* 172: 4292-7
122. Hauer J, Puschner S, Ramakrishnan P, Simon U, Bongers M, Federle C, Engelmann H. 2005. TNF receptor (TNFR)-associated factor (TRAF) 3 serves as an inhibitor of TRAF2/5-mediated activation of the noncanonical NF-kappaB pathway by TRAF-binding TNFRs. *Proc Natl Acad Sci U S A* 102: 2874-9
123. Arch RH, Thompson CB. 1998. 4-1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor kappaB. *Mol Cell Biol* 18: 558-65
124. So T, Croft M. 2013. Regulation of PI-3-Kinase and Akt Signaling in T Lymphocytes and Other Cells by TNFR Family Molecules. *Front Immunol* 4: 139
125. So T, Soroosh P, Eun SY, Altman A, Croft M. 2011. Antigen-independent signalosome of CARMA1, PKCtheta, and TNF receptor-associated factor 2 (TRAF2) determines NF-kappaB signaling in T cells. *Proceedings of the National Academy of Sciences of the United States of America* 108: 2903-8
126. Ito T, Wang YH, Duramad O, Hanabuchi S, Perng OA, Gilliet M, Qin FX, Liu YJ. 2006. OX40 ligand shuts down IL-10-producing regulatory T cells. *Proc Natl Acad Sci U S A* 103: 13138-43
127. So T, Croft M. 2007. Cutting edge: OX40 inhibits TGF-beta- and antigen-driven conversion of naive CD4 T cells into CD25+Foxp3+ T cells. *J Immunol* 179: 1427-30
128. Vu MD, Xiao X, Gao W, Degauque N, Chen M, Kroemer A, Killeen N, Ishii N, Li XC. 2007. OX40 costimulation turns off Foxp3+ Tregs. *Blood* 110: 2501-10
129. Pakala SV, Bansal-Pakala P, Halteman BS, Croft M. 2004. Prevention of diabetes in NOD mice at a late stage by targeting OX40/OX40 ligand interactions. *Eur J Immunol* 34: 3039-46
130. Weinberg AD, Bourdette DN, Sullivan TJ, Lemon M, Wallin JJ, Maziarz R, Davey M, Palida F, Godfrey W, Engleman E, Fulton RJ, Offner H, Vandenbark AA. 1996. Selective depletion of myelin-reactive T cells with the anti-OX-40 antibody ameliorates autoimmune encephalomyelitis. *Nat Med* 2: 183-9
131. Higgins LM, McDonald SA, Whittle N, Crockett N, Shields JG, MacDonald TT. 1999. Regulation of T cell activation in vitro and in vivo by targeting the OX40-OX40 ligand

interaction: amelioration of ongoing inflammatory bowel disease with an OX40-IgG fusion protein, but not with an OX40 ligand-IgG fusion protein. *J Immunol* 162: 486-93
132. Ruby CE, Yates MA, Hirschhorn-Cymerman D, Chlebeck P, Wolchok JD, Houghton AN, Offner H, Weinberg AD. 2009. Cutting Edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right. *Journal of immunology* 183: 4853-7
133. Griseri T, Asquith M, Thompson C, Powrie F. 2010. OX40 is required for regulatory T cell-mediated control of colitis. *The Journal of experimental medicine* 207: 699-709
134. Bluestone JA. 2005. Regulatory T-cell therapy: is it ready for the clinic? *Nat Rev Immunol* 5: 343-9
135. Putnam AL, Brusko TM, Lee MR, Liu W, Szot GL, Ghosh T, Atkinson MA, Bluestone JA. 2009. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 58: 652-62
136. Webb GJ, Hirschfield GM, Lane PJ. 2016. OX40, OX40L and Autoimmunity: a Comprehensive Review. *Clin Rev Allergy Immunol* 50: 312-32
137. Ruby CE, Yates MA, Hirschhorn-Cymerman D, Chlebeck P, Wolchok JD, Houghton AN, Offner H, Weinberg AD. 2009. Cutting Edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right. *J Immunol* 183: 4853-7
138. Gaudreau S, Guindi C, Menard M, Besin G, Dupuis G, Amrani A. 2007. Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of CD4+CD25+ regulatory T cells. *J Immunol* 179: 3638-47
139. Bernasconi E, Favre L, Maillard MH, Bachmann D, Pythoud C, Bouzourene H, Croze E, Velichko S, Parkinson J, Michetti P, Velin D. 2010. Granulocyte-macrophage colony-stimulating factor elicits bone marrow-derived cells that promote efficient colonic mucosal healing. *Inflamm Bowel Dis* 16: 428-41
140. Ephrem A, Epstein AL, Stephens GL, Thornton AM, Glass D, Shevach EM. 2013. Modulation of Treg cells/T effector function by GITR signaling is context-dependent. *Eur J Immunol* 43: 2421-9
141. Delgoffe GM, Woo SR, Turnis ME, Gravano DM, Guy C, Overacre AE, Bettini ML, Vogel P, Finkelstein D, Bonnevier J, Workman CJ, Vignali DA. 2013. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature* 501: 252-6
142. Mandapathil M, Lang S, Gorelik E, Whiteside TL. 2009. Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression. *J Immunol Methods* 346: 55-63
143. Wu C, Thalhamer T, Franca RF, Xiao S, Wang C, Hotta C, Zhu C, Hirashima M, Anderson AC, Kuchroo VK. 2014. Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. *Immunity* 41: 270-82
144. Ishii N, Takahashi T, Soroosh P, Sugamura K. 2010. OX40-OX40 ligand interaction in T-cell-mediated immunity and immunopathology. *Adv Immunol* 105: 63-98
145. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 201: 723-35
146. Baier G, Telford D, Giampa L, Coggeshall KM, Baier-Bitterlich G, Isakov N, Altman A. 1993. Molecular cloning and characterization of PKC theta, a novel member of the protein kinase C (PKC) gene family expressed predominantly in hematopoietic cells. *J Biol Chem* 268: 4997-5004
147. Zhang EY, Kong KF, Altman A. 2013. The yin and yang of protein kinase C-theta (PKCtheta): a novel drug target for selective immunosuppression. *Adv Pharmacol* 66: 267-312
148. Meller N, Altman A, Isakov N. 1998. New perspectives on PKCtheta, a member of the novel subfamily of protein kinase C. *Stem Cells* 16: 178-92

149. Meller N, Elitzur Y, Isakov N. 1999. Protein kinase C-theta (PKCtheta) distribution analysis in hematopoietic cells: proliferating T cells exhibit high proportions of PKCtheta in the particulate fraction. *Cell Immunol* 193: 185-93
150. Liu Y, Graham C, Parravicini V, Brown MJ, Rivera J, Shaw S. 2001. Protein kinase C theta is expressed in mast cells and is functionally involved in Fcepsilon receptor I signaling. *J Leukoc Biol* 69: 831-40
151. Monks CR, Kupfer H, Tamir I, Barlow A, Kupfer A. 1997. Selective modulation of protein kinase C-theta during T-cell activation. *Nature* 385: 83-6
152. Yokosuka T, Kobayashi W, Sakata-Sogawa K, Takamatsu M, Hashimoto-Tane A, Dustin ML, Tokunaga M, Saito T. 2008. Spatiotemporal regulation of T cell costimulation by TCR-CD28 microclusters and protein kinase C theta translocation. *Immunity* 29: 589-601
153. Gupta S, Manicassamy S, Vasu C, Kumar A, Shang W, Sun Z. 2008. Differential requirement of PKC-theta in the development and function of natural regulatory T cells. *Mol Immunol* 46: 213-24
154. Baier-Bitterlich G, Uberall F, Bauer B, Fresser F, Wachter H, Grunicke H, Utermann G, Altman A, Baier G. 1996. Protein kinase C-theta isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Mol Cell Biol* 16: 1842-50
155. Werlen G, Jacinto E, Xia Y, Karin M. 1998. Calcineurin preferentially synergizes with PKC-theta to activate JNK and IL-2 promoter in T lymphocytes. *EMBO J* 17: 3101-11
156. Coudronniere N, Villalba M, Englund N, Altman A. 2000. NF-kappa B activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-theta. *Proc Natl Acad Sci U S A* 97: 3394-9
157. Wang XD, Gong Y, Chen ZL, Gong BN, Xie JJ, Zhong CQ, Wang QL, Diao LH, Xu A, Han J, Altman A, Li Y. 2015. TCR-induced sumoylation of the kinase PKC-theta controls T cell synapse organization and T cell activation. *Nat Immunol* 16: 1195-203
158. Shevach EM, Tran DQ, Davidson TS, Andersson J. 2008. The critical contribution of TGF-beta to the induction of Foxp3 expression and regulatory T cell function. *Eur J Immunol* 38: 915-7
159. Scholzen A, Mittag D, Rogerson SJ, Cooke BM, Plebanski M. 2009. Plasmodium falciparum-mediated induction of human CD25Foxp3 CD4 T cells is independent of direct TCR stimulation and requires IL-2, IL-10 and TGFbeta. *PLoS Pathog* 5: e1000543
160. Raab M, Pfister S, Rudd CE. 2001. CD28 signaling via VAV/SLP-76 adaptors: regulation of cytokine transcription independent of TCR ligation. *Immunity* 15: 921-33
161. Marinari B, Costanzo A, Marzano V, Piccolella E, Tuosto L. 2004. CD28 delivers a unique signal leading to the selective recruitment of RelA and p52 NF-kappaB subunits on IL-8 and Bcl-xL gene promoters. *Proc Natl Acad Sci U S A* 101: 6098-103
162. Tuosto L. 2011. NF-kappaB family of transcription factors: biochemical players of CD28 co-stimulation. *Immunol Lett* 135: 1-9
163. Porciello N, Tuosto L. 2016. CD28 costimulatory signals in T lymphocyte activation: Emerging functions beyond a qualitative and quantitative support to TCR signalling. *Cytokine Growth Factor Rev* 28: 11-9
164. Song J, So T, Croft M. 2008. Activation of NF-kappaB1 by OX40 contributes to antigen-driven T cell expansion and survival. *J Immunol* 180: 7240-8
165. Burchill MA, Yang J, Vang KB, Moon JJ, Chu HH, Lio CW, Vegoe AL, Hsieh CS, Jenkins MK, Farrar MA. 2008. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. *Immunity* 28: 112-21
166. Liston A, Nutsch KM, Farr AG, Lund JM, Rasmussen JP, Koni PA, Rudensky AY. 2008. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. *Proc Natl Acad Sci U S A* 105: 11903-8

167. Xiao X, Balasubramanian S, Liu W, Chu X, Wang H, Taparowsky EJ, Fu YX, Choi Y, Walsh MC, Li XC. 2012. OX40 signaling favors the induction of T(H)9 cells and airway inflammation. *Nat Immunol* 13: 981-90
168. Iwanaga R, Ozono E, Fujisawa J, Ikeda MA, Okamura N, Huang Y, Ohtani K. 2008. Activation of the cyclin D2 and cdk6 genes through NF-kappaB is critical for cell-cycle progression induced by HTLV-I Tax. *Oncogene* 27: 5635-42
169. Santamaria D, Barriere C, Cerqueira A, Hunt S, Tardy C, Newton K, Caceres JF, Dubus P, Malumbres M, Barbacid M. 2007. Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* 448: 811-5
170. Pfoertner S, Jeron A, Probst-Kepper M, Guzman CA, Hansen W, Westendorf AM, Toepfer T, Schrader AJ, Franzke A, Buer J, Geffers R. 2006. Signatures of human regulatory T cells: an encounter with old friends and new players. *Genome Biol* 7: R54
171. Pacholczyk R, Kern J. 2008. The T-cell receptor repertoire of regulatory T cells. *Immunology* 125: 450-8
172. Birkeland ML, Copeland NG, Gilbert DJ, Jenkins NA, Barclay AN. 1995. Gene structure and chromosomal localization of the mouse homologue of rat OX40 protein. *Eur J Immunol* 25: 926-30
173. Nocentini G, Bartoli A, Ronchetti S, Giunchi L, Cupelli A, Delfino D, Migliorati G, Riccardi C. 2000. Gene structure and chromosomal assignment of mouse GITR, a member of the tumor necrosis factor/nerve growth factor receptor family. *DNA Cell Biol* 19: 205-17
174. Hamano R, Huang J, Yoshimura T, Oppenheim JJ, Chen X. 2011. TNF optimally activatives regulatory T cells by inducing TNF receptor superfamily members TNFR2, 4-1BB and OX40. *Eur J Immunol* 41: 2010-20
175. Cabal-Hierro L, Rodriguez M, Artime N, Iglesias J, Ugarte L, Prado MA, Lazo PS. 2014. TRAF-mediated modulation of NF-kB AND JNK activation by TNFR2. *Cell Signal* 26: 2658-66

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PUBLICATIONS: 1. **Alharshawi, K.**, A. Marinelarena, P. Kumar, O. El-Sayed, P. Bhattacharya, Z. Sun, A.L. Epstein, A.V. Maker, and B. S. Prabhakar. 2017. PKC-Ɵ is dispensable for OX40L-induced TCR-independent

Treg proliferation but contributes by enabling IL-2 production from effector T-cells. Sci Rep (**In Press**).

2. Kumar, P., **K. Alharshawi**, P. Bhattacharya, A. Marinelarena, C. Haddad, Z. Sun, S. Chiba, A. L. Epstein, and B. S. Prabhakar. 2017. Soluble OX40L and JAG1 Induce Selective Proliferation of Functional Regulatory T-Cells Independent of canonical TCR signaling. Sci Rep 7: 39751.

3. Haddad, C. S., P. Bhattacharya, **K. Alharshawi**, A. Marinelarena, P. Kumar, O. El-Sayed, H. A. Elshabrawy, A. L. Epstein, and B. S. Prabhakar. 2016. Age-dependent divergent effects of OX40L treatment on the development of diabetes in NOD mice. Autoimmunity 49: 298-311.

4. Bhattacharya, P., M. Thiruppathi, H. A. Elshabrawy, **K. Alharshawi**, P. Kumar, and B. S. Prabhakar. 2015. GM-CSF: An immune modulatory cytokine that can suppress autoimmunity. Cytokine 75: 261-271.

5. Bhattacharya, P., I. Budnick, M. Singh, M. Thiruppathi, **K. Alharshawi**, H. Elshabrawy, M. J. Holterman, and B. S. Prabhakar. 2015. Dual Role of GM-CSF as a Pro-Inflammatory and a Regulatory Cytokine: Implications for Immune Therapy. Journal of interferon & cytokine research. 2015. 35(8):585-599.