Identification of a Novel OX40L^{+} Dendritic Cell Subset That Selectively Expands Regulatory T-Cells

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THESIS

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DEDICATION

I dedicate this doctoral dissertation to my mother, Elisa Gallego. Without her and her unwavering support, I would not have been able to complete the work I have today.

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AMM

CONTRIBUTION OF AUTHORS

Chapter 1 provides a review of literature that poses the significance of my research question in the broader context of the scientific field. I created all the illustrations within this chapter. Chapters 2, 3, and 4 include the results of my first author published manuscript (Marinelarena A, Bhattacharya P, Kumar P, Maker AV, Prabhakar BS. Identification of a Novel OX40L+ Dendritic Cell Subset That Selectively Expands Regulatory T cells. *Sci Rep*. 2018;8(1):14940) for which I was the primary author and major contributor of the published research and results. I performed the experiments, created all of the figures, and wrote the manuscript. Dr. Palash Bhattacharya, Dr. Prabhakaran Kumar, and Dr. Ajay Maker provided guidance, technical support, and reviewed the manuscript. My research mentor Dr. Bellur Prabhakar contributed to supervising the project and editing the manuscript. Chapter 5 is a summary of the research presented in this thesis, my conclusions, and the future directions of this research endeavor.

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Abbreviations

APC- Antigen-presenting cell

BLN - Brachial lymph node

BMDC - Bone-marrow derived dendritic cells

CLTA-4 - Cytotoxic T-lymphocyte-associated protein 4

DC - Dendritic cell

EAMG – Experimental Autoimmune Myasthenia Gravis

EAT – Experimental Autoimmune Thyroiditis

FLT3L - Fms like tyrosine kinase 3 ligand

GM-CSF - Granulocyte macrophage-colony stimulating factor

MHC - Major histocompatibility complex

PDL1 - Programmed death-ligand 1

PDL2 - Programmed death-ligand 2

T1D - Type 1 Diabetes

TCR - T-cell receptor

Teff - Effector T-cell

Treg - Regulatory T-cell

SUMMARY

Granulocyte macrophage-colony stimulating factor (GM-CSF) has been demonstrated to play a protective role in autoimmune disease through the dendritic cell-mediated expansion of regulatory T-cells (Tregs). We have previously shown that GM-CSF derived bone-marrow dendritic cells (G-BMDCs) can induce expansion of Tregs through the surface-bound molecule OX40L; however, the physiological relevance of this *ex vivo* derived DC subset has remained to be elucidated.

In the first part of this study, we determined $OX40L^+$ G-BMDCs, and not $OX40L^-$ G-BMDCs, were responsible for the selective expansion of Tregs expressing functionally suppressive markers in $CD4^+$ T-cell *ex vivo* co-cultures. Phenotypic characterization of $OX40L^+$ G-BMDCs revealed higher expression levels of surface marker Sirpα, co-stimulatory markers, CD80 and CD86, and co-inhibitory markers, PDL1 and PDL2.

In the second part of this study, we investigated whether an *in vivo* DC counterpart with similar phenotypic and functional properties to $OX40L^+$ G-BMDCs existed in physiology. Upon GM-CSF administration in mice, we observed an increased percentage of functionally suppressive Tregs in the spleen, brachial lymph nodes, and liver, of GM-CSF treated mice, but not the thymus. Concordantly, we identified $OX40L^+CD11c^+$ cells, phenotypically similar to $OX40L^+$ G-BMDCs, in the spleen, brachial lymph nodes and liver of GM-CSF treated mice but absent in the thymus, implying a role for $OX40L^+$ DCs in peripheral Treg expansion. Furthermore, isolated $CD11c^+$ DCs from GM-CSF treated

mice showed expansion of Tregs in $CD4^+$ T-cell co-cultures, which was abrogated upon addition of OX40L antagonist, suggesting *in vivo* derived $OX40L^+$ DCs share similar functional properties of $OX40L^+$ G-BMDCs in the expansion of suppressive Tregs.

Finally, to determine the role of $OX40L^+$ DCs in Treg homeostasis, we compared the transcriptome data from $OX40L^+$ G-BMDCs to that of all immune cell types from the ImmGen database which revealed $OX40L^+$ G-BMDCs to be distinct from steady-state immune cells, suggesting $OX40L^+$ DCs may be induced under inflammatory/infectious conditions. Furthermore, to confirm the tolerogenic properties of $OX40L^+$ DCs, a microarray analysis of $OX40L^+$ G-BMDCs and $OX40L^-$ G-BMDCs was performed and revealed significant differences in the expression of *PDL2*, *IL33*, *CCL17*, and *CCL22,* molecules which have been implicated in Treg function, expansion, or recruitment.

Overall, these findings suggest that $OX40L^+CD11c^+$ DCs represent a unique, tolerogenic, non-steady state DC subset which may play an essential role in maintaining Treg homeostasis and suppressing autoimmunity.

1.0 INTRODUCTION

Autoimmune disease is caused by the breakdown of the regulatory mechanisms of the immune system which causes an immune attack on self. The National Institutes of Health estimates autoimmune disease to approximately effect 23.5 million Americans. Incidence and prevalence of this disease have increased significantly over the last 30 years; however, treatment options have remained limited (Lerner and Matthias, 2015). Autoimmune disease has been shown to correlate with a decrease or an impairment in the function of regulatory T-cells (Tregs), a subpopulation of T-cells that suppress immune responses (Balandina et al., 2005). Therefore, much research has been devoted to exploring mechanisms to increase the number of functional Tregs as a potential therapeutic for autoimmune disease. Previously, in our lab, we had identified granulocyte-macrophage colony stimulating factor (GM-CSF) bone-marrow derived dendritic cells (G-BMDCs), capable of expanding Tregs *ex vivo* in a contact-dependent manner, independent of canonical TCR signaling, but dependent on the surface-bound molecule OX40L, a member of the tumor necrosis superfamily (Bhattacharya et al., 2011; Gopisetty et al., 2013). This suggested $OX40L^+$ G-BMDCs may play a regulatory role in the immune system. However, there has been much debate as to whether G-BMDCs have functional physiological equivalents *in vivo* or are they a byproduct of *ex vivo* manipulation with no relevance in physiology (Bhattacharya et al., 2015b; Helft et al., 2015). We hypothesize that $OX40L^+$ G-BMDCs may represent a critical *in vivo,* non-steady state, dendritic cell subset ($OX40L^+CD11c^+$) capable of expanding Tregs in an undiscovered mechanism of Treg homeostasis.

1.1 REGULATORY T-CELLS AS MEDIATORS OF IMMUNE TOLERANCE

1.1.1 T-cells and T-cell activation

The immune system was developed to protect hosts from pathogenic microorganisms, through a variety of effector cells such as T-cells and B-cells, however, when these protective mechanisms become dysregulated, deleterious effects to host tissues can occur and, as a consequence, lead to the pathogenesis of autoimmune diseases. T-cells play an integral role in adaptive cell-mediated immune responses. T-cells originate from hematopoietic stem cells in the bone marrow (BM) that then migrate to the thymus for maturation and selection. Upon completion of thymic development and selection, T-cells enter circulation, surveying the environment for infection. T-cells detect infection through their highly variable, surface expressed, T-cell receptor (TCR), however, the TCR cannot bind foreign antigens directly. Foreign antigens must be presented to the TCR by antigen presenting cells (APC) (Santana and Esquivel-Guadarrama, 2006). APCs can be dendritic cells, macrophages, or B-cells. APCs present foreign peptides bound to a surface molecule called major histocompatibility complex (MHC) (Santana and Esquivel-Guadarrama, 2006). There are two types of MHC classes: MHC class 1 and MHC class II. MHC class 1 presents to cytotoxic T-cells, also identified as CD8+ T-cells, while MHC class II presents to $CD4^+$ T-cells (Trombetta and Mellman, 2005).

For $CD4^+$ T-cells to participate in adaptive immune responses, the immune cells must be activated (Smith-Garvin et al., 2009). Activation requires three signals from an APC:

engagement of the T-cell receptor with the cognate foreign-peptide antigen bound self-MHC complex, co-stimulatory signals, and cytokine signals (Lenschow et al., 1996). Co-stimulation involves APC stimulation of co-stimulatory receptors on T-cells. The most researched co-stimulatory pathway involves the CD28 receptor, constitutively expressed on murine T-cells, and CD80/CD86, expressed mainly by APCs (Lenschow et al., 1996).

Upon proper activation, naïve T-cells differentiate into distinct T effector subsets depending on the signals that were provided at the time of activation, such as the co-stimulatory interaction and the cytokine present. $CD4^+$ T-cells can differentiate into several subsets of different effector cells, each with a distinct specific function to combat pathogens or regulate the immune system. These subsets can be classified as T_H1, T_H2, T_H17, $T_{FH,}$ and Tregs (Raphael et al., 2015). Once fully differentiated, T-cells can exert their effector responses. For example, T_H1 cells produce the cytokine IFN-γ to induce the microbiocidal activity of an infected macrophage to kill resident bacteria (Murphy and Reiner, 2002). Macrophage activation effectively destroys pathogens, however, due to the release of toxic mediators, this process is also associated with localized tissue destruction. Therefore, it is crucial that T-cell activation is a tightly regulated process in order to minimize tissue damage.

1.1.2 T-cell Tolerance Mechanisms

Autoimmunity is widely thought to be caused by a breakdown in the maintenance of T-cell tolerance. Tolerance is defined as the unresponsiveness of host immune cells to self-

antigens. Impaired tolerance is thought to be caused by the escape of self-reactive lymphocytes from tolerance mechanisms in the thymus, entering the periphery and causing the destruction of organs upon activation.

Uncontrolled T-cell activation is regulated by several mechanisms. The first mechanism is employed during T-cell development in the thymus and is termed central tolerance (Figure 1.1). During development, pre-T-cells undergo a process of elimination during which 95% of pre-T-cells are eliminated (Kyewski and Klein, 2006). In the thymus, during development and maturation, pre-T-cells are presented with self-antigens on endogenous MHC molecules to determine the level of self-reactivity. Pre-T-cells that are excessively activated through their T-cell receptors by self-antigens are directed to undergo apoptosis (Gregersen and Olsson, 2009). This process is considered negative selection and a component of central tolerance.

Unfortunately, thymic selection does not completely eliminate autoreactive T-cells. Therefore, the immune system developed a second major population of T-cells called regulatory T-cells (Tregs), capable of suppressing pathogenic, self-reactive T-cells (Figure 1.1). Tregs also develop in the thymus during thymic selection and are identified as natural Tregs (nTregs) (Sakaguchi et al., 2006). Tregs can also develop *de novo* in the periphery, outside of the thymus, from thymic precursors and these Tregs are termed induced Tregs (iTregs) (Chen et al., 2003). Once in the periphery, Tregs are the primary mediators of what is termed peripheral tolerance, where they can inhibit T-cell activation. Treg dysfunction has been linked to the pathogenesis of autoimmune disease highlighting

the importance of these cells in maintaining immune homeostasis and preventing autoimmune diseases (Sakaguchi and Sakaguchi, 2005).

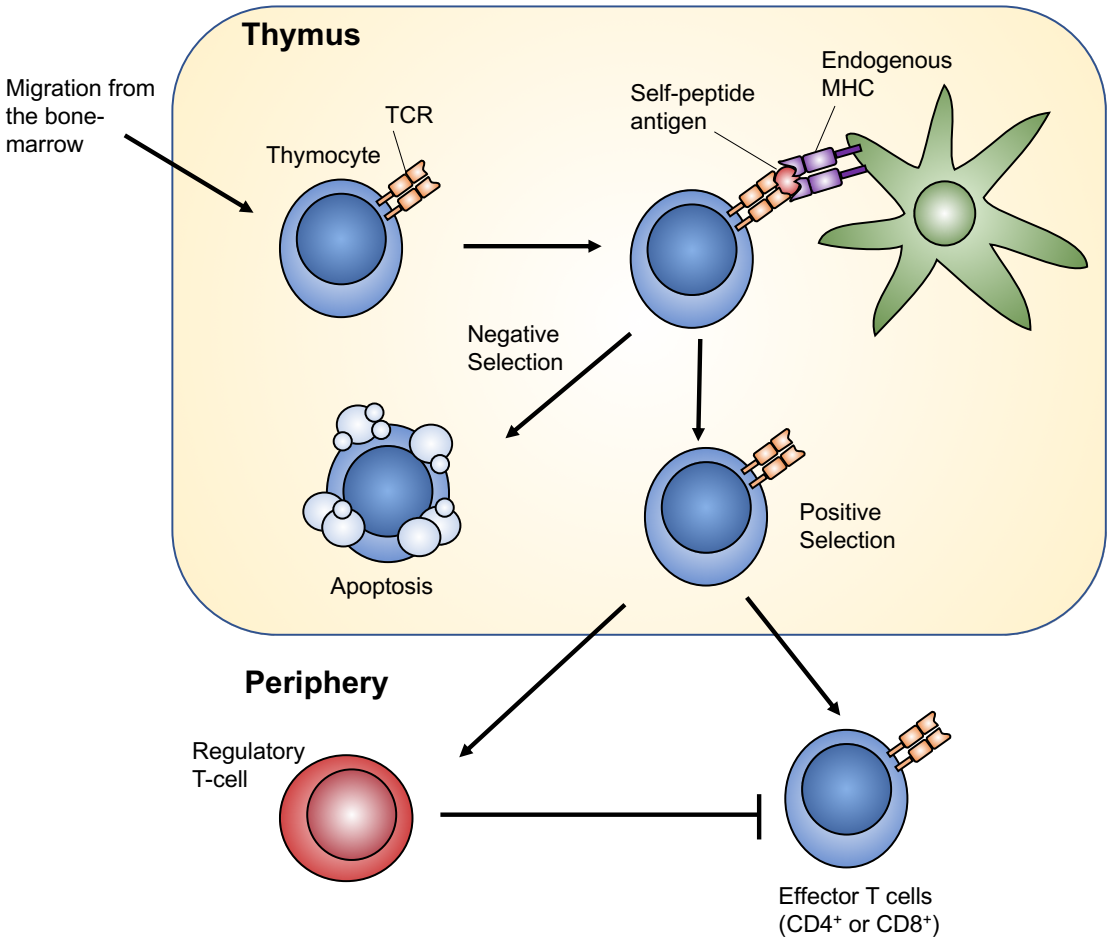

Figure 1.1. Central and Peripheral Tolerance. Central tolerance mechanisms occur in the thymus, which involves the maturation and selection process of thymocytes. Thymocytes strongly reactive to self-peptide antigen bound to MHC are deleted. Thymocytes not deleted, undergo positive selection and migrate to the periphery and develop into effector $CD8^+$ or $CD4^+$ T-cells which can then modulate humoral and cellular immune responses. Tregs also develop in the thymus. Upon migration to the periphery, Tregs participate in peripheral tolerance mechanisms by suppressing effector T-cell mechanisms.

1.1.3 Identification of Regulatory T-cells

The identity of Tregs was first revealed in 1969 through neonatal thymectomy (NTx) experiments of wild-type mice. Nishizuka and Sakakura showed that the NTx of wild-type mice, 2-4 days after birth, resulted in the autoimmune destruction of ovaries as well as tissue damage to other organs (Nishizuka and Sakakura, 1969). These results suggested the presence of a thymic cellular component critical for regulating self-reactive T-cell activation. Reconstitution of $CD4^+$ T-cells expressing high levels of CD25 or lower levels of CD45RB, markers later utilized to aid in the identification of Tregs, protected thymectomized mice from autoimmune pathologies (Morrissey et al., 1993; Sakaguchi et al., 1995; Suri-Payer et al., 1998). These studies led to the discovery that the normal immune system must contain naturally occurring thymocytes or T-cells with autoimmune-suppressive activity, that we now call Tregs.

Tregs are a subset of $CD4^+$ T-cells with the ability to suppress pathogenic self-reactive T-cells. As mentioned previously, Tregs can develop in the thymus (nTregs) or can be induced in the periphery from T-cell precursors (iTregs). Tregs were initially identified by the high expression of CD25, also known as the IL-2 receptor, or by specific CD45 isoforms. Activated $CD4^+$ T-cells (Teff cells), however, have also been shown to share expression of these molecules which led to much confusion (Kim and Leonard, 2002) and therefore, it was imperative to immunologists to identify a specific marker that distinguished Tregs from other $CD4^+$ T-cell subsets. It was not until the identification of the Treg-specific transcription factor forkhead box P3 (Foxp3) that led to the acceptance of Tregs as a distinct cell lineage (Benoist and Mathis, 2012). Mutations in Foxp3 were

found to be responsible for the spontaneous autoimmunity and fatal lymphoproliferation observed in Scurfy mice (Brunkow et al., 2001). Similarly, in humans, disruptions of Foxp3 was determined to cause the human disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked) which is characterized by severe multi-organ autoimmunity (Bennett et al., 2001; Wildin et al., 2001). Lethal autoimmune syndrome observed in Foxp3-null mice resulted from the depletion of $CD4^{+}CD25^{+}$ regulatory T-cells, implicating the specific role of Foxp3 in Treg development (Fontenot et al., 2003) and indicates the importance of Tregs in immunological tolerance.

1.1.4 Regulatory T-cell Mediated Suppression

The known mechanisms by which Tregs implement immunosuppression can be classified broadly into four categories: suppression by inhibitory cytokines, cytolysis, metabolic disruption, and modulation of DC function or maturation (Vignali et al., 2008). Inhibitory cytokines IL-10 and TGF-β have been identified as suppressive mediators released by Tregs (Figure 1.2A). IL-10 has been shown to be critical for the prevention of colitis in irritable bowel disease (IBD) mouse models (Asseman et al., 1999). However, Treg specific deletion of IL-10 does not induce spontaneous autoimmunity suggesting Treg-mediated IL-10 suppression may be context dependent and points to other mechanisms of Treg suppression (Rubtsov et al., 2008). Similar roles have been identified with the cytokines TGF-β and IL-35 (Collison et al., 2007; Green et al., 2003).

Cytolysis, a mechanism mediated by the secretion of granzymes, has been shown to play a role in the suppressive activity of Tregs (Figure 1.2B). Gene expression analysis identified the upregulation of granzyme B in mouse Treg cells (McHugh et al., 2002). Furthermore, deficiency of granzyme B in mouse Tregs reduced the *in vitro* suppressive capacity of Tregs (Gondek et al., 2005). Further evidence showing the ability of Tregs to kill B-cells in a granzyme B and perforin-dependent mechanism further supports the cytolytic suppressive activity of Tregs (Zhao et al., 2006).

Another mechanism by which regulatory T-cells exert their suppressive capacity can be described as the disruption of effector T-cell (Teff) metabolism (Figure 1.2C). Tregs have been shown to express CD39 and CD73, ectoenzymes capable of generating pericellular adenosine (Deaglio et al., 2007). The extracellular release of adenosine has been shown to suppress effector T-cell function through the activation of adenosine receptor 2A (A_{2A}), which reduces the pro-inflammatory properties of Teff cells (Borsellino et al., 2007; Kobie et al., 2006). Even further, activation of A_{2A} on Tregs has been shown to promote a self-reinforcing loop, which supports the expansion of Tregs by inhibiting the expression of IL-6, a proinflammatory cytokine, and promoting the secretion of TGF-β that induces FOXP3 expression and the differentiation of Tregs (Zarek et al., 2008).

Finally, in addition to the direct effect of Treg cells on Teff function, Tregs have been shown to modulate the maturation and/or function of DCs, and thus, disrupt the activation of Teffs (Figure 1.2D). While data is limited, intravital microscopy has demonstrated *in vivo* direct interactions between Tregs and DCs (Tadokoro et al., 2006; Tang et al., 2006).

Cytotoxic T-lymphocyte antigen 4 (CTLA-4), a molecule constitutively expressed on Tregs, is proposed to regulate DC function by competing with CD28 in the binding of co-stimulatory molecules CD80/CD86, and thus, downregulating the activation of Teff cells (Read et al., 2000). Additionally, it is thought the binding of CTLA-4 to CD80/CD86, downregulates the expression of these co-stimulatory molecules, consequently preventing the activation of Teff and maintaining tolerance (Oderup et al., 2006).

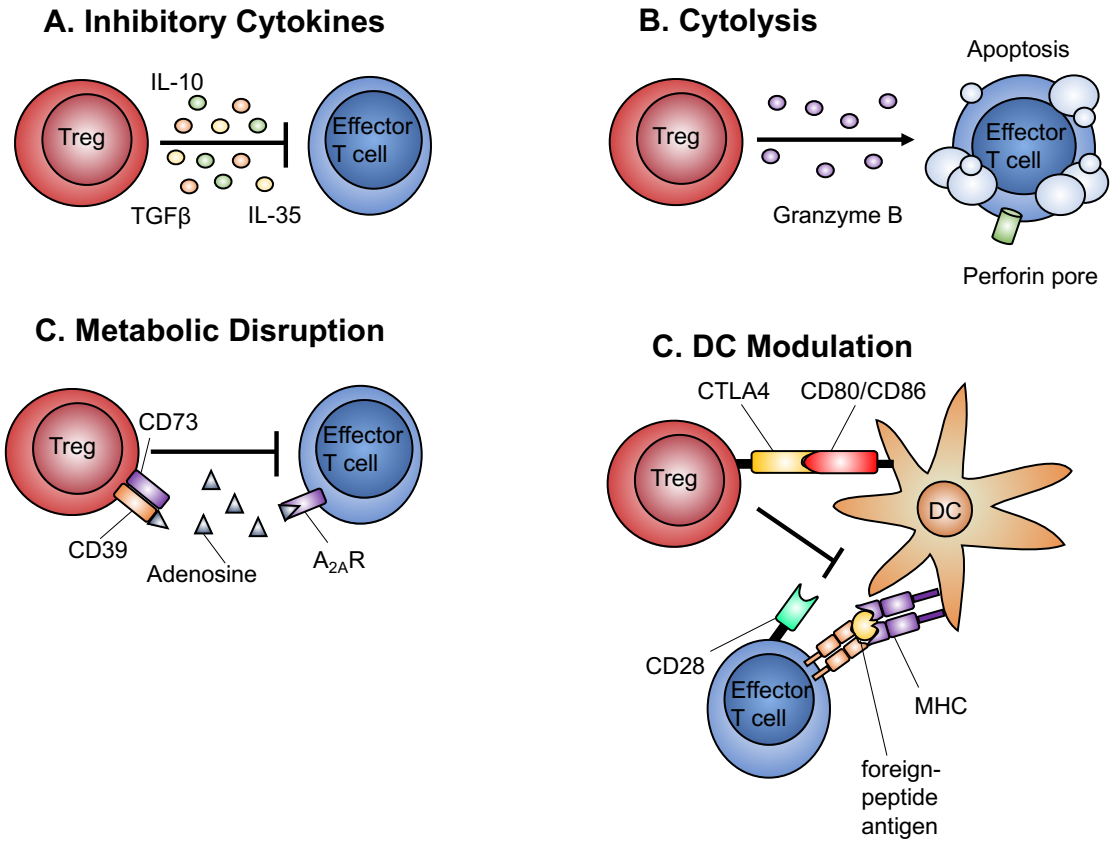

Figure 1.2. Mechanisms of Treg Suppression. The mechanisms by which Tregs exert their suppressive capacity in the periphery. **A.** Inhibitory cytokines IL-10, TGFβ, and IL-35. **B.** Cytolysis by granzyme B and perforin pore-dependent mechanisms**. C.** Metabolic disruption by CD39 and/or CD73 adenosine release. **D.** Dendritic cell modulation by CTLA-4-CD80/CD86 binding and downmodulation of T-cell activation.

1.1.5 Regulatory T-cell Transplantation for Autoimmune Disease Therapy

Tregs have been shown to be crucial for preventing the development of autoimmune disease by maintaining immunological self-tolerance (Sakaguchi et al., 2007). Type 1 Diabetes (T1DM) is an autoimmune disorder thought to be caused by the uncontrolled autoreactive T-cell attack on pancreatic β-cells (Eisenbarth, 1986; van Belle et al., 2011). Previous studies have suggested that this autoimmune destruction of the pancreas may be associated with a reduction in Treg numbers and/or function (Waid et al., 2008), and thus, much research has been dedicated to restoring Treg function/numbers as a treatment for autoimmune disease. Clinical adoptive Treg therapy has shown much promise as a successful treatment option for autoimmune disease. Studies in mice have shown the adoptive transfer of Tregs from wild-type mice to NOD mice, an experimental mouse model of diabetes, prevented the pathogenesis of this disease (Stumpf et al., 2013). Similarly, in humans, autologous expanded Tregs adoptively transferred to patients with T1DM patients, after one-year follow-up, have been shown to increase c-peptide levels, an indicator of endogenous insulin production, in the peripheral blood compared to non-treated patients, and 66% of TD1M patients treated with adoptive transfer remained in remission after one year, while non-treated patients were all dependent on insulin (Marek-Trzonkowska et al., 2014). These studies implicate the importance of maintaining Treg function/numbers in the prevention of autoimmune disease. It is, therefore, of much interest to identify regulators of Treg homeostasis in order to identify mechanisms to maintain tolerance.

1.2 DENDRITIC CELLS AS REGULATORS OF ADAPTIVE IMMUNE RESPONSE AND AUTOIMMUNITY

1.2.1 Dendritic Cell Classification and Role in Autoimmunity

Antigen-presenting cells (APCs) have been implicated as contributors to T1DM pathogenesis as these cells have been shown to exhibit a defect in the ability to stimulate functionally appearing Tregs in non-obese diabetic (NOD) mice and T1DM patients (Alard et al., 2006; Manirarora et al., 2008). Furthermore, phenotypic and functional abnormalities in dendritic cells (DCs), a highly specialized type of APC, have been reported in NOD mice as well as T1DM patients suggesting a compromised ability of DCs to stimulate immunoregulatory Tregs in an autoimmune state (Hotta-Iwamura and Tarbell, 2016; Turley et al., 2003; Unanue et al., 2016). As a result of these observations, it is thought that the restoration of DC and Treg function to homeostasis could potentially lead to an effective strategy to induce immune tolerance for clinical application in T1DM (Hotta-Iwamura and Tarbell, 2016).

Dendritic cells (DCs) comprise a heterogeneous population of antigen presenting cells which facilitate and regulate innate and adaptive immune response by initiating T-cell priming and differentiation. DCs are responsible for the capture, processing, and presentation of MHC Class-II bound antigenic peptides to T lymphocytes bearing cognate T cell receptors (Banchereau et al., 2000; Banchereau and Steinman, 1998; Coutant and Miossec, 2016; Merad et al., 2013). DCs can generally be identified by expression of

MHCII and CD11c, an integrin involved in DC function (Shortman and Liu, 2002). Depending on a combination of cell surface markers, anatomical location, function, and/or ability to influence T-cell fate, dendritic cells can be further divided into various distinct DC subpopulations. For the purpose of this thesis, DCs can broadly be divided into two categories in the steady-state, uninfected mouse: conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) (Belz and Nutt, 2012) (Figure 1.3). The function of cDCs is typically involved in antigen processing and presentation. cDCs can be divided into two main categories based on their tissue localization or migratory patterns: migratory DCs and lymphoid tissue-resident DCs (Naik, 2008). Migratory DCs, which travel from tissues to lymph nodes and act as sentinels for the immune system, can be divided into $CD11b^+$ DCs and $CD103^+$ $CD11b^-$ DCs. Migratory DCs are not found in the spleen. On the contrary, lymphoid tissue-resident DCs are found in major lymphoid organs, which include the spleen, thymus, and lymph nodes. Lymphoid tissue-resident DCs, for this purpose, can also be divided into two subpopulations on the basis of $CD8\alpha$ expression, $CD8\alpha^+$ and $CD8\alpha^-$. $CD8\alpha^+$ have been known to be involved in cross-presentation and the priming of CD8 T-cells, while $CD8\alpha^-$ DCs appear to be more efficient in the presentation of MHC-II bound antigens to $CD4^+$ T-cells (den Haan et al., 2000). The other major class of steady-state DCs are called plasmacytoid DCs (pDCs), which are quiescent cells found broadly distributed throughout murine tissues. While their exact contribution to the immune system are unclear, it is known that pDCs rapidly produce large amounts of type I interferons (IFN) upon viral infection and can be identified by the marker B220 (CD45RA) (Liu, 2005).

While cDCs and pDCs are present in the steady-state, uninfected mouse, circulating blood monocytes can be differentiated to possess prototypical features of DCs in the infected/inflammatory state (Shortman and Naik, 2007). Differentiated monocyte-derived DCs (moDCs) arise in the presence of inflammatory stimuli such as toll-like receptor ligands or bacteria. Similar to cDCs, moDCs also express similar DC molecules such as CD11c, MHC-II, and Sirpα, and function as professional antigen presenting cells (Dominguez and Ardavin, 2010). Other DC subsets can be induced in inflammatory environments. For example, it has been previously shown that after infection with *Listeria monocytogenes,* mice appear to generate a DC subset termed TipDCs, DCs that produce tumor-necrosis factor (TNF) and inducible nitric-oxide synthase (iNOS) (Serbina et al., 2003). TipDCs have been shown to exhibit potent microbiocidal activity and play an important role in adaptive immune responses during infection.

While DCs have predominately been shown to play a vital role in the initiation of immune responses to pathogens, studies have also suggested a critical role for DCs in the maintenance of immune tolerance (Hu and Wan, 2011). The specific deletion of CD11c, and at lower levels by the other cells of the innate immune system, has been shown to result in the induction of spontaneous autoimmunity, characterized by the infiltration of $CD4^+$ T-cells into peripheral tissues, autoantibody formation, and onset of inflammatory bowel disease (Ohnmacht et al., 2009) suggesting a role for $CD11c^+$ DCs in the maintenance of immune tolerance.

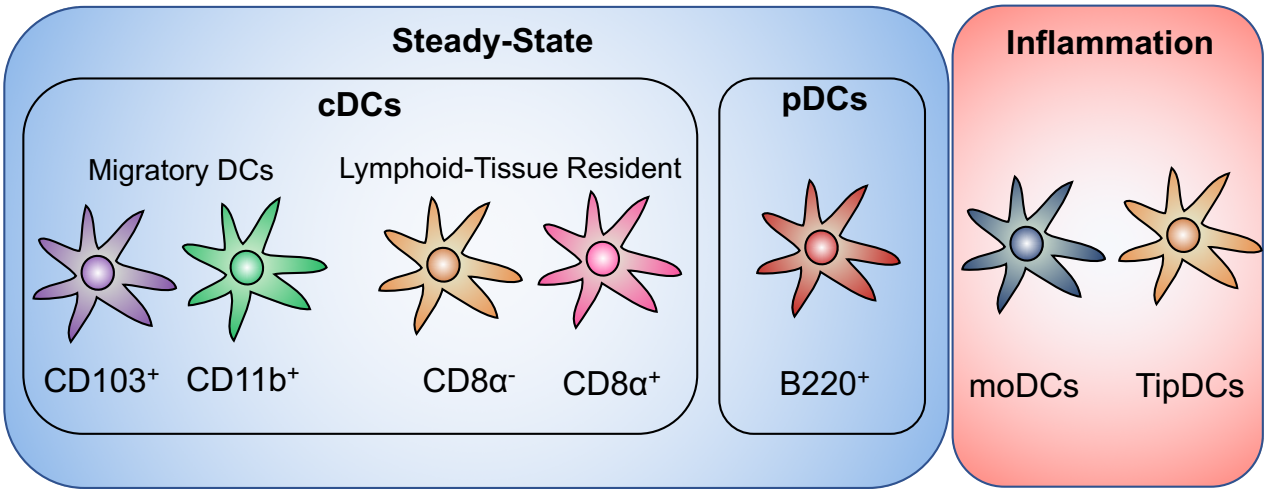

Figure 1.3. Dendritic Cell Classification. Dendritic cells can be divided in two general categories, DCs appearing in the steady-state and those generated in inflammatory processes. Steady state DCs can be separated into two categories, cDCs and pDCs. cDCs can be further divided into migratory DCs and lymphoid-tissue resident DCs. Inflammatory DCs can be generated from monocytes, termed moDCs or other DC subsets such as TipDCs.

1.2.2 Granulocyte-Macrophage Colony Stimulating Factor and DC Development

Since the depletion of DCs can lead to autoimmune pathologies, it has been postulated that increasing DC populations could restore tolerance and prevent autoimmunity. Injection of Fms like tyrosine kinase 3 ligand (FLT3L), a hematopoietic cytokine required for DC development, increased the proportion of DCs and subsequently prevented diabetes onset in NOD mice (O'Keeffe et al., 2005). Similarly, we have previously reported the prevention and/or suppression of several experimental autoimmune diseases, such as type 1 diabetes (Cheatem et al., 2009), myasthenia gravis (Ganesh et al., 2009; Gangi et al., 2005; Vasu et al., 2003) and autoimmune thyroiditis (Meriggioli et al., 2008; Sheng et al., 2006; Sheng et al., 2008), upon treatment with granulocyte macrophage colony-stimulating factor (GM-CSF), another hematopoietic cytokine strongly linked to myeloproliferation as well as DC development. In each case, a significant increase in Tregs was observed in GM-CSF treated mice. Interestingly, the increase in Tregs in GM-CSF treated mice corresponded with an increase in $CD11c^+$ $CD8a^-$ DCs (Ganesh et al., 2009). We demonstrated that the therapeutic effect of GM-CSF was primarily mediated through the mobilization of DCs ($CD11c^+CD8\alpha^-$) that could stimulate the expansion of Tregs *in vivo* and suppress autoimmune disease through increased IL-10 production (Ganesh et al., 2009; Gangi et al., 2005).

Due to the scarcity of, and difficulty in, isolating DCs *in vivo* from tissues, most studies have long utilized bone-marrow progenitor culture systems with the use of hematopoietic

cytokines such as GM-CSF (Inaba et al., 1992) or FLT3L (Brasel et al., 2000) to generate bone-marrow derived DCs (BMDCs) to study DC biology. Therefore, to further investigate the phenomenon of GM-CSF induced generation of tolerogenic DCs capable of increasing Treg numbers and suppressing autoimmune disease, we utilized bone-marrow derived DCs generated from GM-CSF bone-marrow precursor cells (G-BMDCs). In these subsequent studies, we discovered that *ex vivo* derived G-BMDCs, generated from bone marrow (BM) precursor cells isolated from WT or MHC Class-II$^{-/-}$ mice, could selectively and robustly expand Foxp3^{+} Tregs when co-cultured with CD4^{+} T-cells (Bhattacharya et al., 2011; Gopisetty et al., 2013). We found this *ex vivo* G-BMDC mediated Treg proliferation to be cell-to-cell-contact dependent, independent of TCR-signaling and most importantly, dependent on the DC cell surface expression of OX40L (Bhattacharya et al., 2011).

1.2.3 OX40L Dendritic Cell Expression and Autoimmunity

OX40L is a member of the tumor necrosis factor superfamily and has been strongly implicated in the proliferation and survival of T cells (Croft, 2010) by playing a critical role as a co-stimulatory molecule in association with T-cell receptor engagement (Chen et al., 1999; Murata et al., 2000). OX40L expression has been detected on antigen presenting cells, such as dendritic cells (Jenkins et al., 2007), B-cells (Linton et al., 2003), and macrophages (Karulf et al., 2010), but can also be induced on various other immune cell types such as mast cells (Kashiwakura et al., 2004; Nakae et al., 2006), natural killer cells (Zingoni et al., 2004), and vascular endothelial cells (Imura et al., 1996). OX40L

expression has been observed to be induced by antigen presentation and corresponding co-stimulatory interactions (Croft, 2003) but can also be induced, in the absence of these interactions, by various inflammatory stimuli such as TSLP (Ito et al., 2005), IL-18 (Maxwell et al., 2006), and IFN-gamma (Kurche et al., 2012; Wang et al., 2008).

$OX40L^{+}CD11b^{+}CD11c^{+}$ DCs have been identified in various autoimmune contexts such as in the pancreatic lymph nodes of NOD mice around the time of diabetes onset (Pakala et al., 2004), and in the inflamed kidneys of Lupus patients (Jacquemin et al., 2015) which suggested a role for OX40L in the pathogenesis of autoimmune diseases. OX40L has also been found to have genetic associations with multiple autoimmune diseases including Systemic Lupus Erythematosus (SLE) (Cunninghame Graham et al., 2008), Systemic Sclerosis (Gourh et al., 2010), and Sjogren's syndrome (Nordmark et al., 2011).

On the contrary, OX40L/OX40 interactions have also been reported in the homeostatic regulation of Tregs. Mice genetically deficient in OX40 demonstrated a marked reduction in Treg numbers (Piconese et al., 2010; Takeda et al., 2004). Furthermore, overexpression of OX40L via an OX40L transgenic mouse model demonstrated an increase in Treg numbers (Xiao et al., 2012). Similarly, our findings have demonstrated a direct role for $OX40L^{+}$ G-BMDCs, derived either from WT or MHC Class-$II^{-/-}$ mice, in the selective expansion of Tregs, and not the Teff cells, in the absence of canonical antigen presentation (Bhattacharya et al., 2011).

1.3 STATEMENT OF THE PROBLEM

Recent reports have suggested that DCs generated from bone-marrow precursor cells differentiated by GM-CSF may lack physiological counterparts *in vivo* (Helft et al., 2015). While Flt3L deficiency severely affects the development of all types of DCs *in vivo,* no severe defects in DC development have been observed in GM-CSF or GM-CSFr deficient mice (Kingston et al., 2009; McKenna et al., 2000; Vremec et al., 1997) suggesting *ex vivo* generated G-BMDCs may not have physiological counterparts in the steady-state (Bhattacharya et al., 2015a; Helft et al., 2015). Thus, it has been proposed that GM-CSF may be required for the development and differentiation of inflammatory DCs, described as DCs that may not be present in the steady-state, but readily available during conditions of inflammation or infection (Shortman and Naik, 2007). Since we had previously generated tolerogenic G-BMDCs *ex vivo* that were capable of expanding Tregs and we had also observed profound beneficial effects of GM-CSF treatment in ameliorating experimental autoimmune diseases, we hypothesized that *in vivo,* $OX40L^+CD11c^+$ DCs may play a regulatory role through the expansion of Tregs. Thus, we sought to identify a biological equivalent to the GM-CSF derived $OX40L^+$ G-BMDC subset. To better understand the nature of tolerogenic $OX40L^+$ G-BMDCs and to identify a relevant biological functional counterpart, we characterized this *ex vivo* derived DC subset by analyzing cell surface marker and gene expression profiles. Collectively, our data suggest that $OX40L^+$ G-BMDCs may represent a non-steady-state myeloid cell subtype involved in Treg expansion *in vivo* and point to a hitherto undiscovered mechanism of peripheral Treg homeostasis.

2.0 OX40L^{+} G-BMDCS ARE RESPONSIBLE FOR THE SELECTIVE EXPANSION OF PHENOTYPICALLY FUNCTIONAL TREGS

Material from: Marinelarena et al., Identification of a Novel OX40L+ Dendritic Cell Subset That Selectively Expands Regulatory T cells. *Scientific Reports, 2018.*

2.1 INTRODUCTION

We have previously shown the *ex vivo* expansion of Tregs by G-BMDCs, DCs derived from bone-marrow precursor cells in culture with GM-CSF, was critically dependent on the surface-bound ligand OX40L in a contact-dependent manner (Bhattacharya et al., 2011). Furthermore, we have previously shown MHC$^{-/-}$ G-BMDCs, derived from MHC$^{-/-}$ bone-marrow precursor cells in culture with GM-CSF, sustained their capability of expanding Tregs independent of canonical TCR signaling (Bhattacharya et al., 2011). TCR-independence is of critical importance translationally in the expansion of Tregs since both Tregs and Teff express TCRs that interact with MHC, and thus, could both be activated upon TCR activation.

A current mechanism of Treg expansion stimulates proliferation through TCR activation by utilizing monoclonal antibodies that activate the TCR (anti-CD3) and provide a co-stimulatory signal through CD28 (anti-CD28) (Hoffmann et al., 2004; Putnam et al., 2009). While anti-CD3/anti-CD28 technology expands the Treg compartment, unfortunately, it also expands the Teff population as this mechanism is not Treg specific. To be used translationally, Tregs must be purified, expanded *ex vivo* and, then, subsequently,

transferred back into the host for therapeutic purposes (Tang and Bluestone, 2013). This arduous process comes with many complications as the stability of Treg function can be impaired upon Treg expansion after repeated stimulation (Hoffmann et al., 2009). As a result, it is crucial to assess the functional suppressive capacity of expanded Tregs.

Functional suppressive capacity can be assessed phenotypically with markers correlated with the Treg suppressive activity. Helios, an Ikaros family zinc-finger transcription factor, has been highly implicated in the stability of Treg suppressive function. Studies have shown Helios-deficient Tregs in mice exhibit impaired suppressive function, reduced Foxp3 expression, and increased pro-inflammatory cytokines (Kim et al., 2015). Other molecules implicated in the inhibitory activity of Tregs are CTLA-4 and CD39. The specific Treg deficiency of CTLA4 in mice results in systemic lymphoproliferation, fatal T-cell mediated disease, and impaired suppressive function of Tregs (Wing et al., 2008). CD39 is an ectonucleotidase that generates adenosine and has been found to be involved in the downregulation of pro-inflammatory cytokines of Teff (Borsellino et al., 2007; Deaglio et al., 2007). These suppressive Treg molecules can be used as markers to assess Treg stability and suppressive function. Here we established the tolerogenic nature of $OX40L^+$ G-BMDCs by evaluating their capacity of expanding functional Tregs. Furthermore, we phenotypically characterized $OX40L^+$ G-BMDCs to further define this unique tolerogenic DC subset.

2.2 MATERIALS AND METHODS

Animals. C57BL/6J female mice (6- to 12-week-old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the Biological Resources Laboratory Facility at the University of Illinois (Chicago, IL, USA) and provided food and water ad libitum. All animal experiments were approved by the University of Illinois at Chicago Animal Care and Use Committee.

Reagents. Recombinant mouse GM-CSF was purchased from Miltenyi Biotec (Auburn, CA). CellTrace Violet cell proliferation kit was purchased from ThermoFisher Scientific (Waltham, MA). Anti-FOXP3, anti-CD4, anti-OX40L, anti-CD11c, anti-Helios, anti-CTLA4, and anti-CD39, fluorescently conjugated antibodies were purchased from ThermoFisher Scientific (Waltham, MA). Foxp3/Transcription Factor Staining Buffer Kit was purchased from Tonbo Biosciences (San Diego, CA).

Flow Cytometry Analysis. For flow cytometry analysis, cells were washed with PBS containing 0.5% BSA. For surface staining, the cells were labeled with specified FITC-, PE-, EFluor® 450-, APC-, APC-Cy7- conjugated monoclonal antibodies for 30 min. For cell proliferation assays, the cells were labeled with CellTrace Violet, fixed, permeabilized, and incubated with fluorescent coupled antibodies for intracellular staining. Stained cells were washed and analyzed by CyAn ADP Analyzer (Beckman Coulter) and data analysis was performed using Summit v4.3 software (Beckman Coulter).

Isolation of DC and T-cell populations. Bone marrow cells were cultured in complete RPMI medium containing 10% heat-inactivated FBS in the presence of 20ng/ml GM-CSF. Fresh medium containing 20ng/mL GM-CSF was added on days 3 and 6. On the 8th day, non-adherent $CD11c^+$ DCs (G-BMDCs) or specific subpopulations of G-BMDCs (i.e. $OX40L^+$ or $OX40L^-$) were sorted using a MoFlo flow cytometer (Beckman/Coulter) following staining with appropriate antibodies. $CD4^+$ cells were isolated from the spleens by using the Mouse $CD4^+$ T Cell Isolation Kit II from Miltenyi Biotec (San Diego, CA).

In vitro DC and T-cell co-cultures. DC: T-cell co-culture experiments were conducted in triplicate with isolated T-cells and G-BMDCs. G-BMDCs (5×10^4) were cultured with $CD4^+$ T-cells (1×10^5) at a ratio of 1:2 for 5 days. For proliferation assays, $CD4^+$ T-cells were labeled with CellTrace Violet (ThermoFisher Scientific) according to manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using Prism GraphPad (V7.0). Data were expressed as Mean ± SEM of multiple experiments. A p-value < 0.05 was considered significant. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$.

2.3 RESULTS

2.3.1 $OX40L^+$ G-BMDCs are responsible for the selective expansion of phenotypically functional Tregs.

To elucidate the critical role of OX40L expression on $CD11c^+$ cells in the expansion of Tregs, we assessed the capacity of $OX40L^+$ G-BMDCs to expand $Foxp3^+$ Tregs upon co-culture with $CD4^+$ T-cells. $OX40L^+$ G-BMDCs and $OX40L^-$ G-BMDC populations were sorted, using fluorescence-activated cell sorting, from *ex vivo* expanded G-BMDCs. $CD4^+$ T-cells were stained with CellTrace Violet, a fluorescent marker which traces proliferation by dye dilution. CellTrace-labeled $CD4^+$ T-cells were then co-cultured with unsorted G-BMDCs, $OX40L^+$ G-BMDCs or $OX40L^-$ G-BMDC populations, and assessed for percent Treg proliferation. We compared this capacity to an alternative and common method of expanding Tregs *ex vivo* using anti-CD3/CD28 T-activator beads, which employs TCR stimulation and corresponding co-stimulation. Our results indicated co-cultures supplemented with anti-CD3/CD28 microbeads exhibited a modest Treg proliferative response (Figs 2.1A & 2.1B) with a prominent increase in the percentage of proliferating Teff cells ($CD4^+FoxP3^-$) (Figs 2.1A & 2.1C). In contrast, $CD4^+$ T-cells cultured with unsorted G-BMDCs exhibited a significant increase in the percentage of Tregs (Figs 2.1A & 2.1B) with minimal expansion of the Teff cells (Figs 2.1A & 2.1C) as we have observed in the past. Interestingly, we found a significant decrease in percent Treg proliferation in the presence of $OX40L^-$ G-BMDCs relative to bulk G-BMDCs. However, when compared to $OX40L^-$ G-BMDCs co-cultures and anti-CD3/CD28 microbeads, we see a significant increase in percent proliferation of $CD4^+$ T-cells co-cultured with $OX40L^+$G-BMDCs, thus implicating the significant role of OX40L expressed on $CD11c^+$ cells in selective Treg expansion (Figs 2.1A & 2.1B). Furthermore, since percent proliferating Tregs was similar in unsorted G-BMDC co-cultures and $OX40L^+$G-BMDCs co-cultures, it is suggested that

the $OX40L^+$ G-BMDC population is the *ex vivo* derived DC subset within the unsorted G-BMDC population that is responsible for Treg expansion.

OX40 has been previously shown to expand Tregs *in vivo,* as a potential therapeutic for autoimmune disease, however, Tregs expanded by this mechanism have been shown to exhibit impaired suppressive function due to exhaustion (Xiao et al., 2012). Therefore, we examined the expression of various suppressive Treg surface markers on proliferating Tregs expanded by G-BMDCs. Compared to proliferating Tregs stimulated by anti-CD3/CD28, Helios, a transcription factor that stabilizes the Treg suppressive phenotype (Kim et al., 2015), was expressed in a significantly higher percentage of proliferating Tregs in the G-BMDC co-cultures (Figs. 2.1D & 2.1E). Furthermore, we also assessed the expression of Treg suppressive markers CD39, an ectonucleotidase, and CTLA-4, a co-stimulatory molecule (Deaglio et al., 2007; Wing et al., 2008). Both these molecules, highly implicated in Treg suppression function, were expressed in similar high percentages of proliferating Tregs in both the G-BMDC and anti-CD3/anti-CD28 stimulated cultures (Figs. 2.1D & 2.1E), indicating that Treg proliferation did not impair the suppressive activity of expanded Tregs.

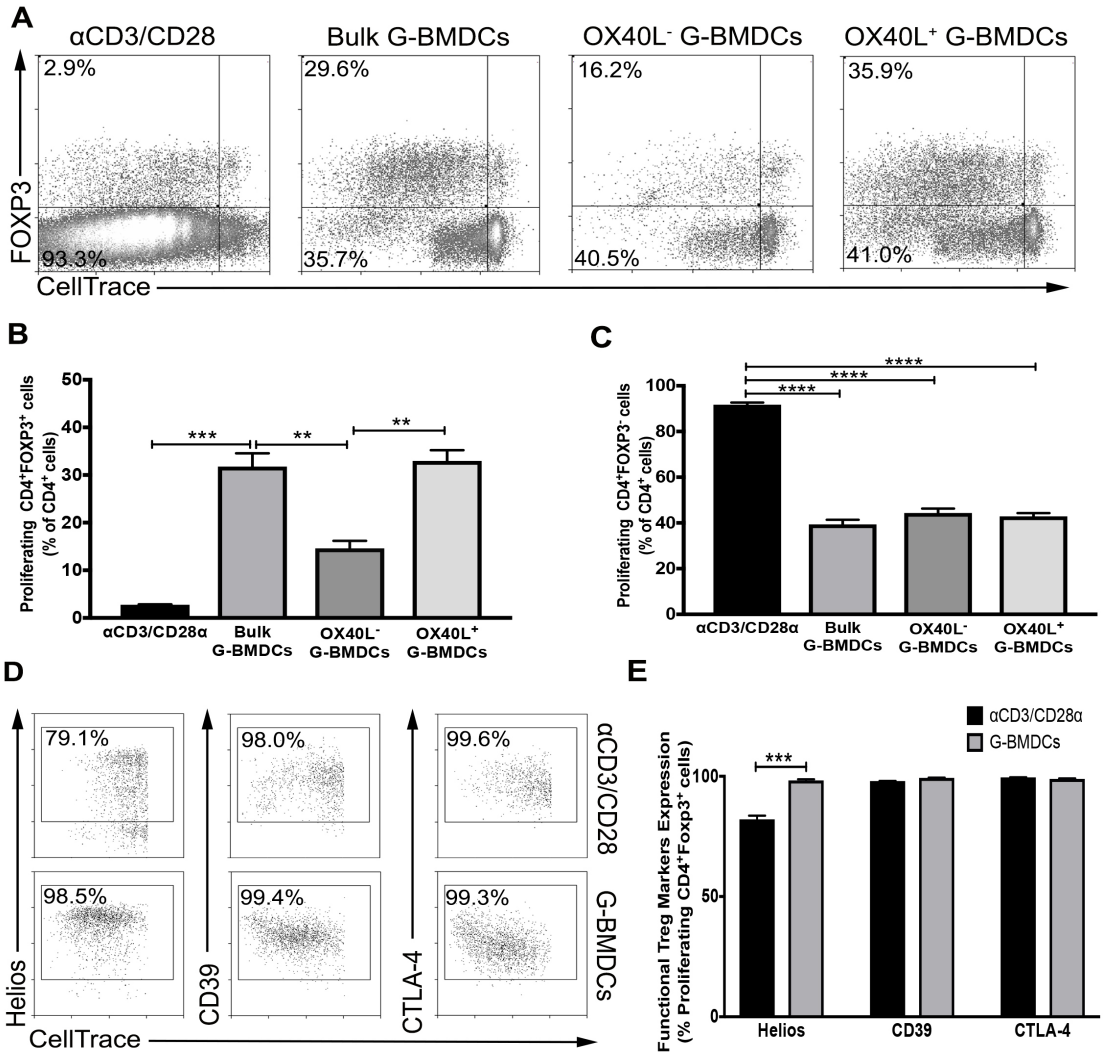

Figure 2.1. OX40L$^+$CD11c$^+$ G-BMDCs are responsible for the expansion of functional Tregs. Splenic C57BL/6 CD4$^+$ T-cells were stained with CellTrace and separately co-cultured with anti-CD3/CD28 microbeads, bulk G-BMDCs (pre-sort) population, sorted OX40L$^-$ CD11c$^+$ G-BMDCS, and sorted OX40L$^+$CD11c$^+$ G-BMDCS. **A)** Representative dot plots of percent proliferating Tregs using CD4$^+$FOXP3$^+$ as a marker for Tregs. All flow cytometry plots were gated on CD4$^+$ cells. **B&C)** Summary bar graphs for data shown in A (n=3). **D)** Representative dot plots of Treg suppressive markers on proliferating Tregs. **E)** Summary bar graphs for data shown in D (n=3)

2.3.2 OX40L$^+$ G-BMDCs highly express co-stimulatory and co-inhibitory molecules.

To assess the potential functional differences between OX40L$^+$ and OX40L$^-$ G-BMDCs and to more thoroughly characterize the tolerogenic OX40L$^+$ G-BMDC population, we analyzed the expression of various co-stimulatory/co-inhibitory markers as these molecules have been implicated in the regulation of Tregs. Cells were first identified by their expression of OX40L or lack thereof on CD11c expressing cells (Fig. 2.2A). We then identified increased expression of MHC-II, CD11b, and Sirpα on OX40L$^+$ G-BMDCs (Fig. 2.2B and 2.2D). Sirpα expression has been shown to be inversely correlated with CD8α expression suggesting G-BMDCs are of CD11c$^+$CD8α^- origin (Naik et al., 2005). We found OX40L$^+$ G-BMDCs expressed increased levels of the co-stimulatory markers CD80 and CD86 compared to OX40L$^-$ DCs. DC-restricted deficiency of CD80 and CD86 has been shown to lead to a reduction in peripheral Treg frequencies implicating a role for CD80/CD86 in peripheral Treg homeostasis (Bar-On et al., 2011). Evidence has also shown the co-inhibitory molecules PD-L1 and PD-L2 to play a role in the regulation of Tregs and immune responses. PD-L1 on liver DCs has been shown to induce tolerance mice through the induction of Tregs (Liu et al., 2013). PD-L2 has been implicated to play a critical role in immune tolerance by negatively regulating T-cell immune responses (Zhang et al., 2006). Our assessment revealed OX40L$^+$ G-BMDCs to express significantly higher levels of PDL1 and PDL2 when compared to OX40L$^-$ counterparts (Fig 2.2C & 2.2D). These results suggested that OX40L$^+$ G-BMDCs highly express co-stimulatory molecules involved in DC Treg

homeostasis and can be identified by MHCII, Sirpα, CD11b, CD80, CD86, PDL1, and PDL2 expression.

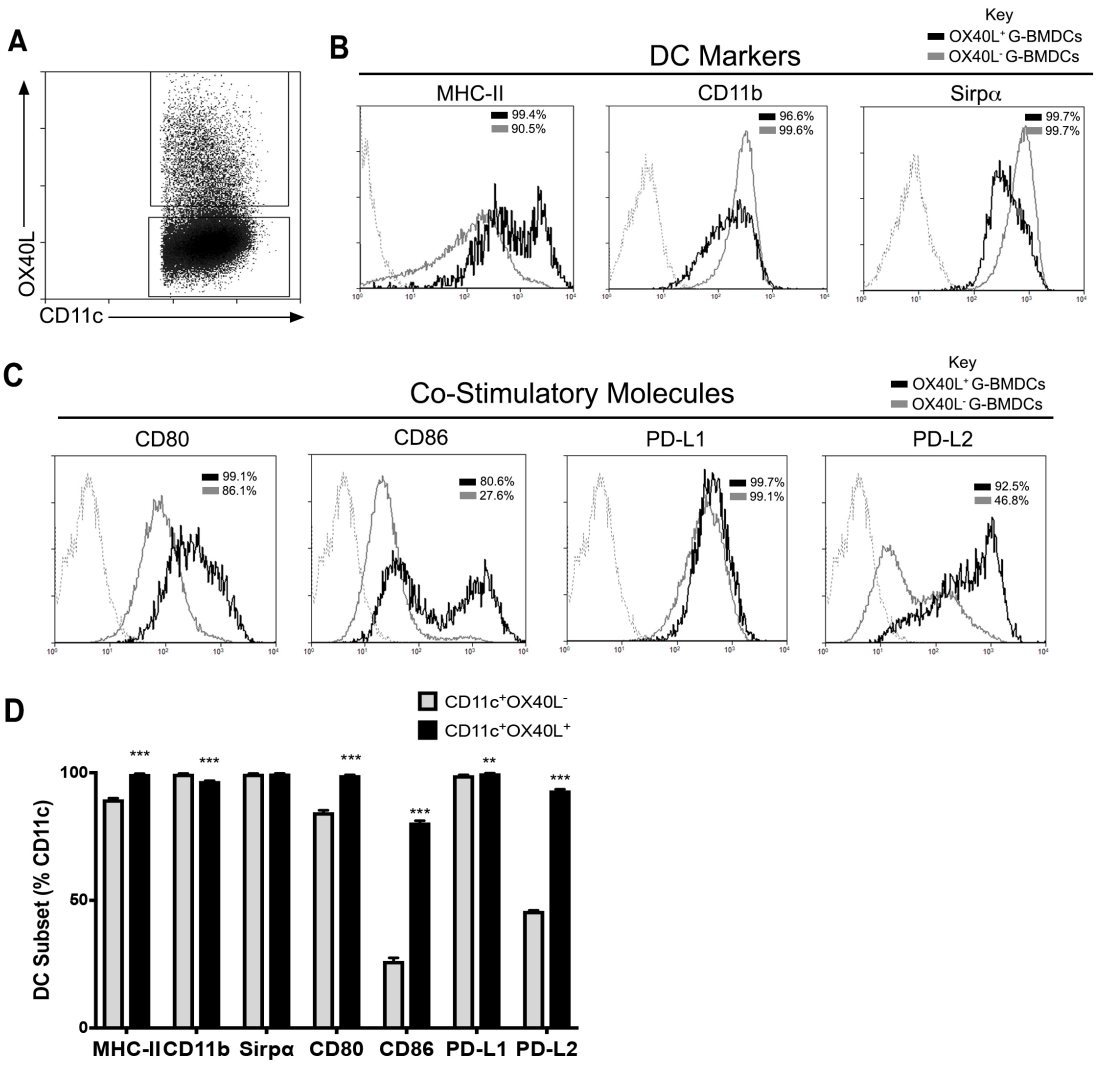

Figure 2.2 Costimulatory and co-inhibitory molecules are highly expressed on OX40L$^+$ G-BMDCs. G-BMDCs were generated from GM-CSF BM cultures after 7 days. **A)** Representative dot plot indicating the subdivided populations OX40L$^+$ BMDCs (top quadrant) and CD11c$^+$OX40L$^-$ (bottom quadrant). **B-C)** Representative histograms showing surface expression of the indicated markers by OX40L$^+$ G-BMDCs (black) and OX40L$^-$ G-BMDCs (grey) subsets. Dotted line represent stained controls. **D)** Summary bar graphs for data shown in B&C (n=3).

2.4 Discussion

In these studies, we have determined OX40L$^+$ G-BMDCs are responsible for the selective expansion of Tregs in co-cultures with CD4$^+$ T-cells. OX40L$^+$ G-BMDC mediated Treg expansion was accompanied by only a moderate expansion in the Teff (CD4$^+$Foxp3$^-$) compartment. Translationally, the selective nature of OX40L$^+$ G-BMDCs to expand Tregs is important as OX40L$^+$ G-BMDCs can increase the percentage of Tregs while the Teff population remains relatively unchanged, generating a tolerogenic environment. Even further, these studies determined the Tregs expanded by G-BMDCs highly express Treg suppressive markers Helios, CTLA-4, and CD39, suggesting the functionally suppressive activity of Tregs remains unimpaired by Treg expansion.

Overall, these results suggest OX40L$^+$ G-BMDCs may represent a tolerogenic DC subset; however, there has been much debate as to whether G-BMDCs have physiological equivalents in physiology (Helft et al., 2015). Furthermore, we have previously shown upon treatment with GM-CSF in mice a significant increase in Tregs with a corresponding increase in the CD11c$^+$CD8α^- DC subset (Ganesh et al., 2009; Gao et al., 2012). We suggested that the CD11c$^+$CD8α^- DC subset may play a role in the amelioration of autoimmune diseases through Treg expansion, however, based on our current findings, we suggest that a subset of the CD8α^- DCs may express OX40L and they may be the critical “tolerogenic” DC subtype capable of selectively expanding Tregs and thus suppressing autoimmunity. CD8α^- DCs in *ex vivo* derived co-cultures have previously been identified by high levels of expression of the molecule Sirpα (Naik et al., 2005). In

this regard, we found OX40L$^+$ G-BMDCs to express higher levels of Sirpα, thus identifying OX40L$^+$ G-BMDCs as a subset of CD8α^- DCs. In addition to Sirpα expression, we determined OX40L$^+$ G-BMDCs expressed significantly increased levels of co-stimulatory molecules such as CD80 and CD86, as well as the co-inhibitory molecules PDL1 and PDL2. Selective reduction of CD80 and CD86 on CD11c$^+$ DCs in mice has been shown to significantly reduce the frequencies of peripheral Tregs suggesting a role for CD80/CD86 in Treg homeostasis (Bar-On et al., 2011). Furthermore, PDL2 has been implicated in negatively regulating T-cell immune responses and thus, promoting tolerance (Zhang et al., 2006). In conclusions, these results suggest OX40L$^+$ G-BMDCs mediate the expansion of phenotypically functionally suppressive and stable Tregs and may represent a novel tolerogenic DC subset identified by the markers CD11c, CD11b, Sirpα, CD80, CD86, PDL1, and PDL2

3.0 GM-CSF ADMINISTRATION INDUCES OX40L^{+}CD11c^{+} DENDRITIC CELLS CAPABLE OF EXPANDING REGULATORY T-CELLS IN THE PERIPHERY

Material from: Marinelarena et al., Identification of a Novel OX40L+ Dendritic Cell Subset That Selectively Expands Regulatory T cells. *Scientific Reports, 2018.*

3.1 INTRODUCTION

Previously, we had observed the suppression of various autoimmune diseases upon administration of GM-CSF (Cheatem et al., 2009; Ganesh et al., 2009; Gangi et al., 2005; Meriggioli et al., 2008; Sheng et al., 2006; Sheng et al., 2008; Vasu et al., 2003). We determined this disease suppression was caused by the mobilization of a tolerogenic DC subset (CD11c^{+}CD8α^{-}) capable of expanding Tregs and, thus, preventing autoimmune disease (Ganesh et al., 2009). However, utilizing *ex vivo* G-BMDCs in culture with CD4^{+} T-cells, we found that the surface-bound, costimulatory molecule OX40L played a significant role in the *ex vivo* selective proliferation of Tregs (Ganesh et al., 2009; Gopisetty et al., 2013). Although these studies determined OX40L^{+} G-BMDCs to be significant in the *ex vivo* expansion of Tregs, we had never assessed in GM-CSF treated mice whether *in vivo* derived CD11c^{+}CD8α^{-} DCs capable of Treg expansion and autoimmune disease suppression, expressed the OX40L molecule. Furthermore, reports have suggested *ex vivo derived* BMDCs, generated from GM-CSF bone-marrow cultures, may not in fact yield *in vivo* physiological equivalents, and may only represent an *ex vivo* phenomenon (Helft et al., 2015). Thus, we hypothesize that in GM-CSF treated mice, the OX40L molecule could have been expressed on a subset of CD11c^{+}CD8^{-} DCs and this OX40L expressing DC subset may have been the main mediator of Treg expansion and

suppression of autoimmune disease. Therefore, we treated mice with GM-CSF to assess for Treg expansion and the potential generation of $OX40L^+$ DCs.

3.2 MATERIALS AND METHODS

Animals. C57BL/6J female mice (6- to 12-week-old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the Biological Resources Laboratory Facility at the University of Illinois (Chicago, IL, USA) and provided food and water ad libitum. All animal experiments were approved by the University of Illinois at Chicago Animal Care and Use Committee.

Reagents. Recombinant mouse GM-CSF was purchased from Miltenyi Biotec (Auburn, CA). CellTrace Violet cell proliferation kit was purchased from ThermoFisher Scientific (Waltham, MA). Anti-FOXP3, anti-CD4, anti-OX40L, anti-CD11c, anti-CD11b, anti-Sirpα, anti-CD80, anti-CD86, anti-CD274, anti-CD273, anti-MHCII, anti-Ki67, and anti-MGL2 fluorescently coupled antibodies were purchased from ThermoFisher Scientific (Waltham, MA). Foxp3/Transcription Factor Staining Buffer Kit was purchased from Tonbo Biosciences (San Diego, CA). Purified anti-OX40L (RML134L) was purchased from Biolegend (San Diego, CA) and purified anti-OX40 agonist (OX-86) was purchased from ThermoFisher Scientific (Waltham, MA).

Flow Cytometry Analysis. For flow cytometry analysis, cells were washed with PBS containing 0.5% BSA. For surface staining, the cells were labeled with specified FITC-,

PE-, EFluor® 450-, APC-, APC-Cy7- conjugated monoclonal antibodies for 30 min. For cell proliferation assays, the cells were labeled with CellTrace Violet, fixed, permeabilized, and incubated with fluorescent coupled antibodies for intracellular staining. Stained cells were washed and analyzed by CyAn ADP Analyzer (Beckman Coulter). Data analysis was performed using Summit v4.3 software (Beckman Coulter).

GM-CSF treatment. Amine-directed PEGylation of the recombinant mouse GM-CSF was performed following the protocol as detailed previously (Sainathan et al., 2005). Age and sex-matched C57BL/6J mice were treated daily for 4 days with 5μg/mouse of pegylated GM-CSF intraperitoneally or with PBS as a vehicle control. 24 hours after the last treatment, mice were sacrificed, and organs were harvested for flow cytometric analysis.

Treg Suppression Assay. $CD4^+CD25^+$ splenic Tregs were sorted from control and GM-CSF treated mice and were co-cultured with CellTrace Violet labeled, anti-CD3/anti-CD28 microbead stimulated $CD4^+CD25^-$ Teff cells at 1:8, 1:4, 1:2, 1:1 ratios. Proliferation was measured by the dilution of CellTrace Violet, and the percentage of Teff cell proliferation suppression was calculated.

In vitro DC and T-cell co-cultures. DC: T-cell co-culture experiments were conducted in triplicate with isolated T-cells and isolated DC populations from WT or GM-CSF-treated mice, pooled from three mice. $OX40L^+CD11c^+$ DCs (5×10^4) were cultured with $CD4^+$ T-cells (1×10^5) at a ratio of 1:2 for 5 days. For proliferation assays, $CD4^+$ T-cells were labeled with CellTrace Violet according to manufacturer's protocol. Certain cultures were

supplemented with anti-OX40L (10 μg/ml) and/or OX40 agonist (OX86; 10 μg/ml) when indicated.

Statistical analysis. Statistical analyses were performed using Prism GraphPad (V7.0). Data were expressed as Mean ± SEM of multiple experiments. A p-value < 0.05 was considered significant. * indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001

3.3 RESULTS

3.3.1 GM-CSF administration expands Tregs in the periphery.

Since we had previously observed Treg expansion in *ex vivo* co-cultures of $CD4^+$ T-cells with $OX40L^+$ G-BMDCs, we attempted to replicate *ex vivo* conditions by administering short-term treatment of GM-CSF to age- and sex-matched C57/BL6 mice. Due to the bioavailability and the rapid clearance of GM-CSF from mouse circulatory systems, recombinant mouse GM-CSF was modified by lysine direct polyethylene glycol conjugation (PEGylation) following the protocol described in Sainathan et al. 2005 (Sainathan et al., 2005). Subsequently, pegylated GM-CSF (pegGM-CSF) was administered daily (5 μg/mouse/day) for four days to age-matched and sex-matched mice. Mice were sacrificed 24 hours after last pegGM-CSF injection and organs were harvested and collected. Initial analysis revealed hepatomegaly, splenomegaly, and lymphadenopathy (unpublished). We first analyzed Treg frequencies within various tissues of GM-CSF treated mice. Treg analysis of thymic tissue revealed no differences

in Treg frequencies between WT and GM-CSF treated mice (Fig 3.1A & 3.1B). However, upon further investigation, we observed a significant increase in the percentage of Tregs in the spleen, brachial lymph node (BLN), and the liver of GM-CSF treated mice compared to untreated mice (Fig 3.1A & 3.1B).

To determine whether the increased Treg frequencies was due to enhanced proliferation or recruitment of Tregs to peripheral organs, we assessed the expression of the cell cycle proliferation marker Ki67 by intracellular staining. After 4 days of GM-CSF treatment, the proportion of proliferating Tregs ($Ki67^+$ Tregs) nearly doubled in the spleen of GM-CSF treated mice compared to WT controls (Fig 3.1C & 3.1D). Thus, the increased proportions of Tregs in GM-CSF treated mice appears to be due to the proliferation of Treg cells rather than mobilization or recruitment. These results suggest GM-CSF induced the proliferation of peripheral Tregs.

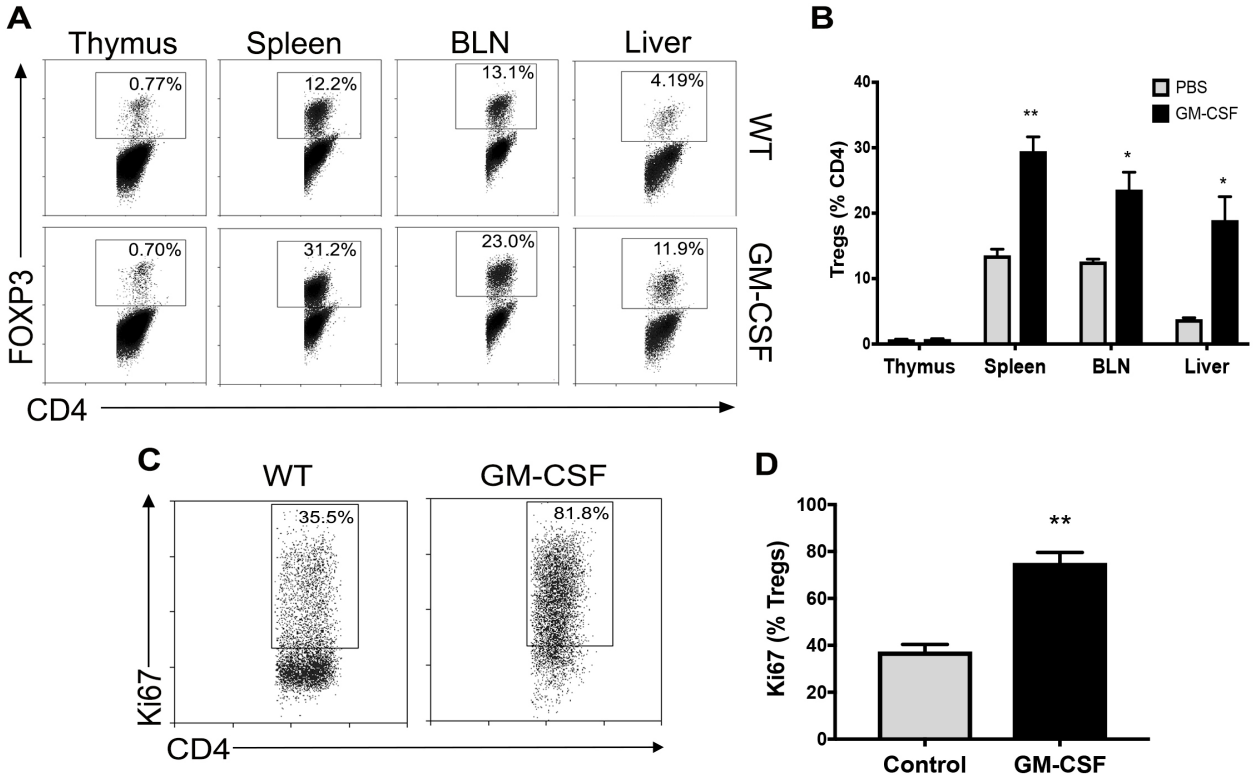

Figure 3.1. GM-CSF administration induces the proliferation of Tregs in peripheral lymphoid organs. pegGM-CSF was administered to C57/BL6 mice daily for 4 days. **A)** Representative dot plots of percent Tregs within various tissues of untreated and GM-CSF treated mice. **B)** Summary bar graphs for data shown in A (n=3). **C)** Representative dot plot of percent Tregs with Ki67 expression from the spleen of untreated and GM-CSF treated mice. **D)** Summary bar graphs for data shown in C (n=3).

3.3.2 GM-CSF expanded Tregs are functionally suppressive.

To determine whether the suppressive function of Tregs was diminished due to their expansion, we assessed the suppressive capacity of the Tregs isolated from control and GM-CSF treated mice. Tregs ($CD4^+CD25^+$) were magnetically isolated and purified from the spleen of WT and treated mice. Concurrently, Teff ($CD4^+CD25^-$) cells were also purified from the spleen of WT mice and stained with the dilutional proliferation marker, CellTrace Violet. Subsequently, CellTrace labeled-Teff cells were stimulated to proliferate by anti-CD3/CD28 microbeads and were cultured with Tregs from WT or GM-CSF treated mice at differing dilutions. Assessment of Treg activity is investigated by assessing the amount of Teff proliferation that is suppressed, which is indicated by increased CellTrace Violet retention. Tregs isolated from GM-CSF treated mice suppressed the proliferation of stimulated T-effector cells at similar levels as WT Tregs (Fig 3.2A). Our suppressive assay revealed the percent Teff suppression was not significantly different at any dilution of Tregs to T-effectors between WT Tregs and Tregs from GM-CSF treated mice (Fig 3.2B), suggesting Treg suppression was not impaired or altered with GM-CSF treatment or upon undergoing proliferation. Collectively, these results suggest GM-CSF induced the proliferation of functionally suppressive Tregs in the periphery.

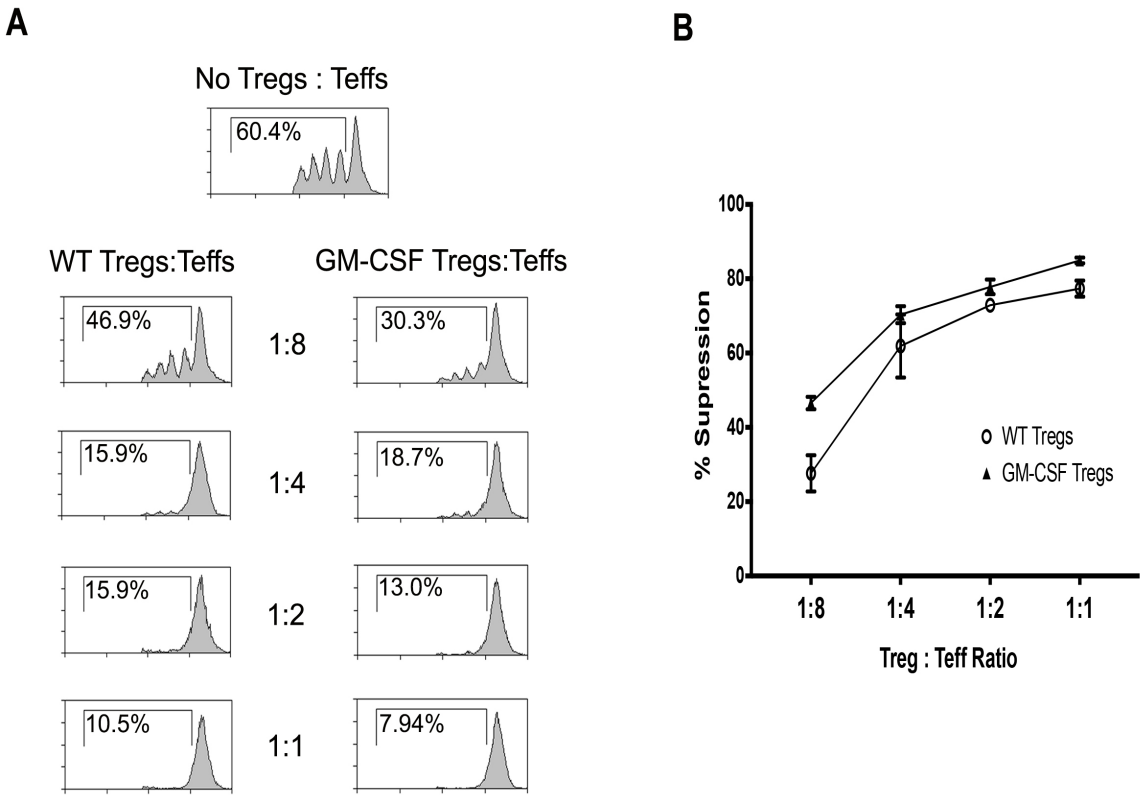

Figure 3.2. Tregs expanded by GM-CSF administration are functionally suppressive.

Tregs were isolated from WT and GM-CSF treated mice, and subsequently were cultured with WT Teff cells that were stimulated with anti-CD3-CD28. Treg suppression analysis was performed on Tregs isolated from the spleens of untreated and GM-CSF mice. **A)** Representative histograms are shown. **B)** Percent suppression was determined (n=3).

3.3.3 GM-CSF induces OX40L$^+$CD11c$^+$ DCs in the periphery.

Since we observed a pronounced increase in Tregs upon GM-CSF administration, we investigated whether GM-CSF induced the generation of OX40L$^+$CD11c$^+$ DCs. In WT mice, we did not find an observable amount of OX40L$^+$CD11c$^+$ DCs in the indicated tissues (Fig 3.3A and 3.3B). Upon GM-CSF treatment, we also did not see a remarkable amount of OX40L$^+$CD11c$^+$ DCs in the thymus, a tissue that also did not observe an increase in Treg frequency (Fig 3.3A and 3.3B). We did, however, observe significant increases of OX40L$^+$CD11c$^+$ DCs in the spleen, brachial lymph nodes, and the liver of GM-CSF treated mice (Fig 3.3A and 3.3B); tissues where we had previously observed significant increases in the percent Treg population upon GM-CSF treatment. These results suggested that OX40L$^+$CD11c$^+$ DCs may have been involved in the proliferation of peripheral Tregs found upon GM-CSF administration.

Furthermore, since we had identified a set of phenotypic markers that identified *ex vivo* derived OX40L$^+$ G-BMDCs, we investigated the expression of these markers on *in vivo* derived OX40L$^+$CD11c$^+$ DCs isolated from the spleen of GM-CSF treated mice. CD11b and Sirpα were highly expressed on *in vivo* derived OX40L$^+$CD11c$^+$ (Fig 3.3C and 3.3D) which we had been seen previously on OX40L$^+$ G-BMDCs. Furthermore, CD8α expression was found to be significantly reduced on OX40L$^+$CD11c$^+$ which we predicted as Sirpα expression is inversely correlated with this molecule (Naik et al., 2005). Additionally, PDL2, a surface molecule we found to be significantly upregulated on OX40L$^+$ G-BMDCs compared to OX40L$^-$ G-BMDC controls, was also found to be

upregulated on *in vivo* derived OX40L$^+$ CD11c$^+$ DCs compared to OX40L$^-$ CD11c$^+$ (Fig 3.3C and 3.3D). Furthermore, we investigated the expression of another molecule MGL2 on OX40L$^+$ CD11c$^+$ DCs. The molecules PDL2 and MGL2 have been recently found on a DC subset identified in a tumor-induced GM-CSF microenvironment with the capability of suppressing CD8$^+$ T-cells and expanding Tregs, respectively (Kenkel et al., 2017). Similarly, on our splenic OX40L$^+$ DCs isolated from GM-CSF treated mice, expression of MGL2 was observed (Fig 3.3C and 3.3D). These results suggested that *in vivo* derived OX40L$^+$CD11c$^+$ DCs share phenotypic similarities with *ex vivo* derived OX40L$^+$ G-BMDCs and may be physiological equivalents.

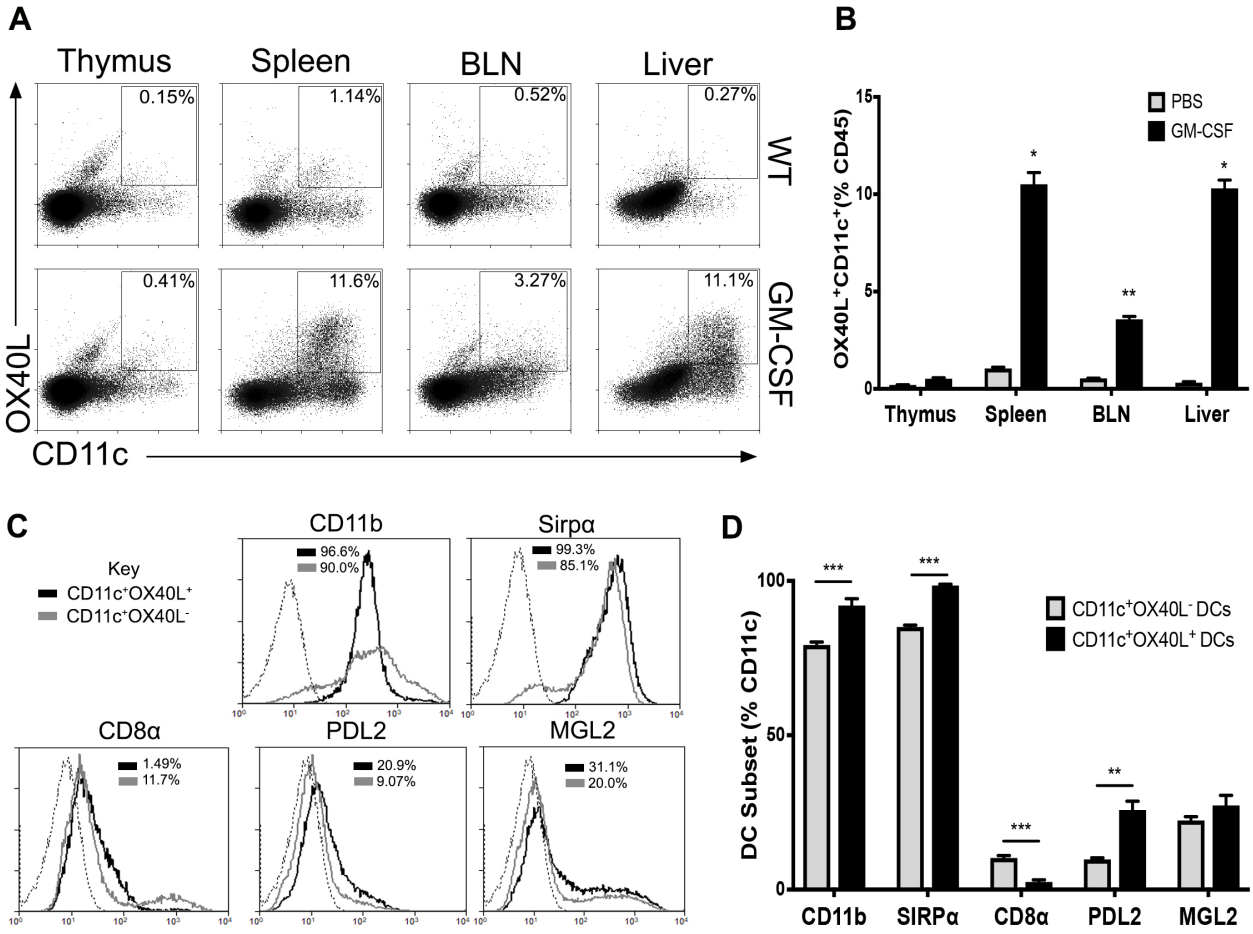

Figure 3.3. GM-CSF induces the generation of OX40L$^+$CD11c$^+$ DCs. Mice were treated daily with GM-CSF for 4 consecutive days. **A)** Representative dot plots of OX40L$^+$CD11c$^+$ expression within various tissues of untreated and GM-CSF treated mice. **B)** Summary bar graphs for data shown in A (n=3). **C)** Representative histograms of indicated markers on OX40L$^+$CD11c$^+$ (black) compared to OX40L$^+$CD11c$^-$ (grey) isolated cells. Dotted lines represent stained controls. **D)** Summary bar graphs for data shown in C (n=3).

3.3.4 CD11c$^+$ DCs isolated from GM-CSF treated mice expand Tregs in OX40L dependent manner.

While *in vivo* derived OX40L$^+$ DCs may share phenotypic similarities to OX40L$^+$ G-BMDCs, we wanted to determine whether these cells also shared the similar functional property of expanding Tregs. Therefore, in order to assess whether *in vivo* derived OX40L$^+$ DCs could also expand Tregs, we magnetically isolated CD11c$^+$ cells from the spleens of GM-CSF treated mice and of WT mice. We then cultured isolated CD11c$^+$ cells with CellTrace labeled-CD4$^+$ T-cells isolated from the spleen of WT mice. After 5 days of culture, we assessed through flow cytometry the percentage of proliferating Tregs. In comparison to cultures with WT CD11c$^+$ DCs, CD4$^+$ T-cell cultures with CD11c$^+$ DCs from GM-CSF treated mice showed a significant increase in the percentage of proliferating Tregs (Fig 3.4A and 3.4B). Furthermore, in order to determine the role of OX40L on CD11c$^+$ DCs in this Treg expansion, we utilized an OX40L antagonist in these T-cell: CD11c$^+$ DC co-cultures. Upon addition of the OX40L antagonist to the CD4$^+$ T-cell co-culture with CD11c$^+$ DCs isolated from GM-CSF treated mice, we observed a significant reduction in the percentage of proliferating Tregs in the co-cultures (Fig 3.4A and 3.4B). However, upon reconstitution of the co-cultures with an OX40 agonist, we found the percent Treg expansion to be restored to previous levels (Fig 3.4A and 3.4B). These results showed that *in vivo* generated OX40L$^+$CD11c$^+$ DCs share similar function with OX40L$^+$G-BMDCs and were likely the DC involved in the expansion of peripheral Tregs in GM-CSF treated mice.

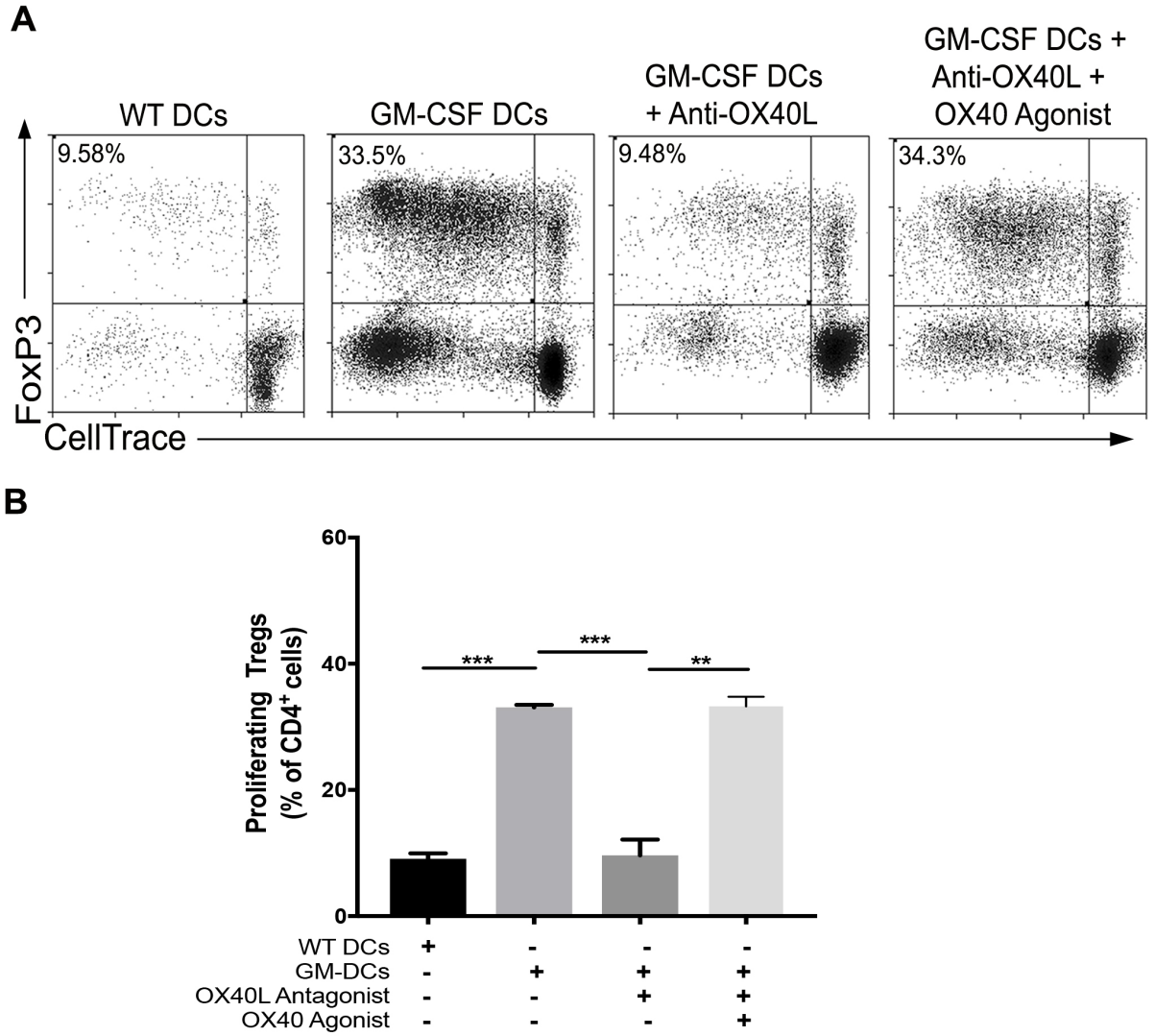

Figure 3.4. CD11c$^+$ DCs isolated from GM-CSF treated mice expand Tregs in OX40L dependent manner. A) CD11c$^+$ DCs were isolated from the spleens of GM-CSF treated or WT mice and co-cultured with CD4 T-cells with or without OX40L blockade and/or supplemented with OX40 agonist. Representative dot plots are shown. **B)** Summary bar graphs for data shown in A (n=3).

3.4 DISCUSSION

Our current studies show that treatment with GM-CSF increased Treg populations in the spleen, brachial lymph nodes, and the liver of treated mice. Furthermore, we determined that GM-CSF treatment in mice expanded Tregs without causing loss of their suppressive function as Tregs from both WT and GM-CSF treated mice efficiently suppressed proliferation of Teffs to similar levels. Furthermore, investigation of OX40L expression on $CD11c^+$ DCs in GM-CSF treated mice, tissues that had exhibited increased frequencies in Treg percentages, revealed a significant increase of $OX40L^+$ $CD11c^+$ DCs in the spleen, BLN, and the liver of treated mice. OX40L remained to be negligibly expressed in WT mice and in the thymus of GM-CSF treated mice, where Treg-specific increases were also not observed. Since tissue-specific increases in $OX40L^+CD11c^+$ DCs correlated with increases in Treg populations in the same tissues upon GM-CSF treatment, these results suggest GM-CSF may induce $OX40L^+CD11c^+$ DCs in peripheral tissues and expand Tregs.

In our previous studies where we ameliorated autoimmune diseases with GM-CSF administration in mouse models, we identified $CD11c^+CD8\alpha^-$ DCs as the critical mediators of Treg expansion, and thus suppression of disease (Ganesh et al., 2009). However, in *ex vivo* co-cultures, using G-BMDCs, we identified the surface-bound ligand $OX40L^+$ as the primary mediator of Treg expansion (Bhattacharya et al., 2011). Therefore, we hypothesized that a subset of $CD11c^+CD8\alpha^-$ DCs in GM-CSF treated mice may express

OX40L and represent a unique DC subset that may play a role in Treg homeostasis. Phenotypic characterization of *in vivo* derived OX40L$^+$ DCs provided support for this theory as OX40L$^+$CD11c$^+$ DCs expressed Sirpα, a marker inversely correlated with CD8α, but also lacked expression of CD8α.

Furthermore, *in vivo* derived OX40L$^+$CD11c$^+$ DCs also expressed, CD11b, CD80, CD86, PD-L1 and PD-L2, markers similarly expressed on OX40L$^+$ G-BMDCs, suggesting *in vivo* derived OX40L$^+$CD11c$^+$ DCs are physiological equivalents to *ex vivo* derived OX40L$^+$ G-BMDCs. This was further supported by evidence of functional similarity since CD11c$^+$ DCs isolated from the spleens of GM-CSF treated mice were capable of expanding Tregs in CD4$^+$ T-cell co-cultures in an OX40L dependent manner. Taken all together, these results overall suggest a role for OX40L$^+$CD11c$^+$ DCs in peripheral Treg expansion and immune homeostasis.

4.0 OX40L^{+} DENDRITIC CELLS REPRESENT TOLEROGENIC NON-STEADY STATE IMMUNE CELLS

Material from: Marinelarena et al., Identification of a Novel OX40L+ Dendritic Cell Subset That Selectively Expands Regulatory T cells. *Scientific Reports, 2018.*

4.1 INTRODUCTION

There has been much debate over the role of GM-CSF in DC development and DC homeostasis. GM-CSF is a known hematopoietic cytokine that stimulates the production of DCs, macrophages, monocytes, neutrophils, eosinophils, and microglia (Hamilton, 2008). This cytokine can be produced by many cells of the immune system such as myeloid cells, DCs, T-cells, B-cells, and even nonhematopoetic cells such as endothelial cells or tumor cells (Hamilton, 2008; Shiomi and Usui, 2015; Stosser et al., 2011). It is thought to promote the survival of myeloid cells and therefore is suggested to play a role in innate immune homeostasis (Hamilton and Achuthan, 2013).

However, reports have indicated that GM-CSF has pro-inflammatory properties and may play a role in the development of autoimmune disease. Early findings originally found elevated GM-CSF in the synovial fluid from patients with inflammatory disorders such as chronic arthritis (Williamson et al., 1988; Xu et al., 1989). Furthermore, a rheumatoid arthritis patient treated with GM-CSF observed arthritis flares upon administration of this cytokine (Hazenberg et al., 1989). These observations suggested to researchers that GM-CSF antagonism could be useful in the prevention of autoimmune disease. In some

instances, GM-CSF deficiency protected mice from autoimmune disease. For example, GM-CSF deficient mice were protected from the development of collagen-induced arthritis, even though antibodies to collagen were still observed (Campbell et al., 1998). Further confirming this suspicion, GM-CSF blocking agents administered post-onset of collagen-induced arthritis into mice ameliorated the disease (Cook et al., 2001). Moreover, in mouse models of EAE, GM-CSF deficiency was seen to be beneficial in the prevention of this disease as GM-CSF (-/-) mice were resistant to the induction of EAE (McQualter et al., 2001). These studies supported the hypothesis that GM-CSF may play a pro-inflammatory role in the pathogenesis of autoimmune disease.

On the contrary, we, and others have shown, GM-CSF administration to protect mice from autoimmune disease (Cheatem et al., 2009; Ganesh et al., 2009; Gangi et al., 2005; Gaudreau et al., 2007; Meriggioli et al., 2008; Sheng et al., 2006; Sheng et al., 2008; Vasu et al., 2003). GM-CSF has proved to cause disease remission in patients with Crohn's disease, an inflammatory disease affecting the gut (Dieckgraefe and Korzenik, 2002). Furthermore, studies have shown GM-CSF administered to prediabetic NOD mice protected mice from developing diabetes, which correlated with an expansion of Tregs (Gaudreau et al., 2007). Similarly, our lab has also shown the GM-CSF mediated protection from experimental autoimmune diseases, such as Type 1 diabetes, EAE, and EAMG (Cheatem et al., 2009; Ganesh et al., 2009; Gangi et al., 2005; Meriggioli et al., 2008; Sheng et al., 2006; Sheng et al., 2008; Vasu et al., 2003). Therefore, these studies suggest GM-CSF may play dual roles in pro-inflammatory and tolerogenic processes of the immune system.

Since there has been controversial debate as to whether GM-CSF plays an essential role in inflammatory DC or steady-state DC homeostasis, we performed a principal component analysis comparing the transcriptomes of all immunological steady-state cell types to that of OX40L$^+$ G-BMDCs to elucidate the physiological role of OX40L$^+$ DCs in immune homeostasis (Greter et al., 2012; Steinman et al., 2003; Xu et al., 2007). The dual nature of GM-CSF in pro-inflammatory and anti-inflammatory properties suggested that we further characterize the OX40L$^+$ G-BMDC population to confirm and identify further mechanisms that may be involved in tolerance induction.

4.2 MATERIALS AND METHODS

Animals. C57BL/6J female mice (6- to 12-week-old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the Biological Resources Laboratory Facility at the University of Illinois (Chicago, IL, USA) and provided food and water ad libitum. All animal experiments were approved by the University of Illinois at Chicago Animal Care and Use Committee.

Isolation of DC and T-cell populations. Bone marrow cells were cultured in complete RPMI medium containing 10% heat-inactivated FBS in the presence of 20ng/ml GM-CSF. Fresh medium containing 20ng/mL GM-CSF was added on days 3 and 6. On the 8th day, non-adherent CD11c$^+$ DCs (G-BMDCs) or specific subpopulations of G-BMDCs (i.e.,

OX40L$^+$ or OX40L$^-$) were sorted using a MoFlo flow cytometer (Beckman/Coulter) following staining with appropriate antibodies.

Microarray and gene expression analysis. OX40L$^+$ and OX40L$^-$ G-BMDCs were sorted by fluorescence-activated cell sorting and total RNA was extracted using RNAeasy Mini isolated kit (Qiagen). For each population, mRNAs were profiled on Affymetrix MoGene-1_0-st-v1 expression microarrays. Labeling, hybridization, and staining of microarrays were performed according to the manufacturer's protocol. Data was analyzed using Bioconductor 3.4 (http://ww.bioconductor.org) running on R 3.3.2 (http://www.R-project.org). Raw data was preprocessed by RMA normalization using the R Bioconductor "oligo" package (Carvalho and Irizarry, 2010). Top differentially expressed genes between two OX40L positive and two OX40L negative samples were obtained using the Bioconductor "limma" package (Smyth, 2004) using a false-discovery rate (FDR) of less than 0.05 and absolute fold change greater than 2.0 as thresholds. Heat maps were created by calculating row Z-score. A principal component analysis (PCA) was performed on two microarray datasets with the same Affymetrix platform from the Immunological Genome Project (ImmGen) (Heng et al., 2008): GSE15907 and GSE37448, along with our samples. Raw datasets were preprocessed by RMA normalization. DC and macrophage gene signatures were acquired from the following sources: Gautier et al., 2012; Miller et al., 2012.

RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) following manufacturer's instructions. cDNA synthesized from total RNA was used for RT-qPCR

analysis with Fast SYBR green master mix (Applied Biosystems) and gene-specific primers (listed in supplementary table-1) by using AB ViiA7 RT-qPCR instrument (Applied Biosystems). Gene expression values were calculated by comparative ΔCt method after normalization to 18s internal control and expressed as log fold change over respective controls. The following primer sets were used to amplify the indicated products: OX40L-F: AATCTGGAAAACGGATCAAGGC; OX40L-R: CAGGCAGACATAGATGAAGCAC; PDL1-F: GCTCCAAAGGACTTGTACGTG; PDL1-R: TGATCTGAAGGGCAGCATTTC; CCR7-F: TGTACGAGTCGGTGTGCTTC, CCR7-R: GGTAGGTATCCGTCATGGTCTTG; IL33-F: TCCAACTCCAAGATTTCCCCG; IL33-R: CATGCAGTAGACATGGCAGAA, CCL22-F: AGGTCCCTATGGTGCCAATGT; CCL22-R: CGGCAGGATTTTGAGGTCCA, 18s rRNA-F: GATCCATTGGAGGGCAAGTCT; 18s rRNA-R: CCAAGATCCAACTACGAGCTTTTT.

Statistical analysis. Statistical analyses were performed using Prism GraphPad (V7.0). Data were expressed as Mean ± SEM of multiple experiments. A p-value < 0.05 was considered significant. * indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001.

4.3 RESULTS

4.3.1 OX40L$^+$CD11c$^+$ DCs may represent a non-steady state DC subset.

To determine whether the induction of OX40L on CD11c$^+$ is a specific property of GM-CSF, we assessed the functional capacity of GM-CSF and FLT3L, two cytokines known

to be strongly linked to DC development, to differentiate OX40L$^+$ BMDCs from BM precursor cells. BM precursor cells cultured with FLT3L failed to differentiate OX40L$^+$ BMDCs (Fig. 4.1A & 4.1B). In contrast, OX40L$^+$ G-BMDCs are abundant in GM-CSF BM cultures suggesting the induction of OX40L$^+$ BMDCs is a unique property of the GM-CSF cytokine (Fig. 4.1A & 4.1B).

Since we have identified OX40L$^+$CD11c$^+$ cells as functional and physiological equivalents to OX40L$^+$ G-BMDCs, it was important to understand the purpose this DC subset plays in physiology if it is to be used therapeutically in the treatment of autoimmune disease. The role of GM-CSF in DC development has previously been suggested to be involved in inflammatory conditions (Bhattacharya et al., 2015b). To determine whether OX40L$^+$ G-BMDCs represented a non-steady state immune cell, we performed a principal component analysis (PCA) comparing the transcriptome of OX40L$^+$ G-BMDCs to that of all myeloid and lymphoid lineages in the Immgen database. The ImmGen Project is a collaboration between a group of immunologists and computational biologists who seek to collect and generate comprehensive gene expression data to molecularly characterize the immune system of the mouse (Heng et al., 2008). The principal-component analysis (PCA) of the expression of all genes revealed OX40L$^+$ G-BMDCs clustered closely with dendritic cell and macrophage populations (Fig. 4.1C). When this PCA was restricted to only the transcriptomes of macrophages and dendritic cells, OX40L$^+$ G-BMDCs more closely clustered with the macrophage population with the closest relatives being alveolar macrophages, peritoneal macrophages and liver macrophages (Fig. 4.1D). Consistent with these data, OX40L$^+$ G-BMDCs expressed some of the core signature genes of

alveolar and peritoneal macrophages, suggesting that while OX40L$^+$ G-BMDCs cells may have a similar transcriptome to those of alveolar and peritoneal macrophages, they are distinct from the steady-state cells of the immune system (Fig. 4.1E & 4.1F). Taken together, these results suggest that OX40L$^+$ G-BMDCs can be induced *in vivo* upon GM-CSF administration and may represent a non-steady state DC subtype involved in Treg homeostasis.

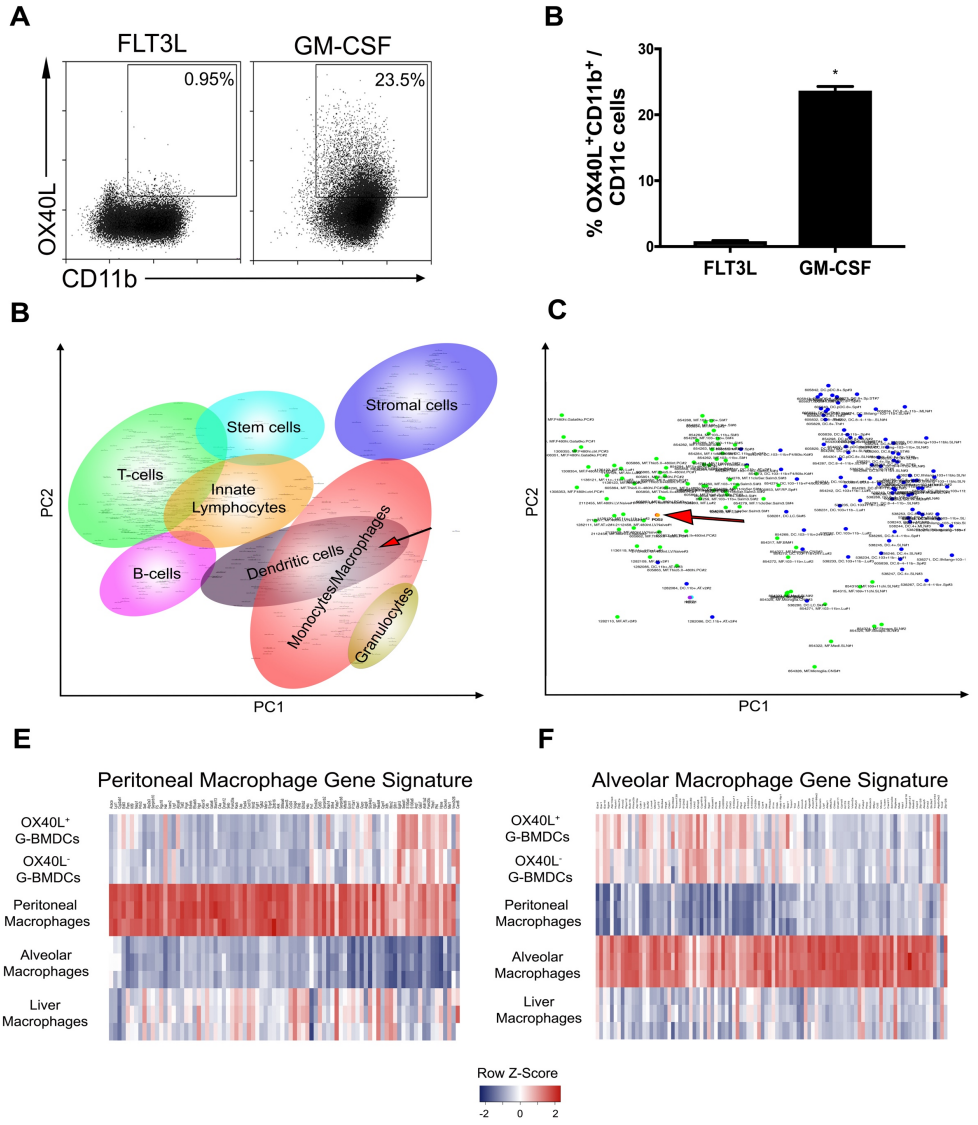

Figure 4.1. OX40L⁺ CD11c⁺ DCs represent non-steady state identity. A-B) Bone marrow precursor cells cultured in indicated cytokines and assessed for the differentiation of OX40L⁺ G-BMDCs. **C)** PCA of all ImmGen immune cell types compared to OX40L⁺ G-BMDCs. Red arrow represents OX40L⁺ G-BMDCs. Each dot represents cell type sample. **D)** Subsequent PCA of ImmGem macrophage and dendritic cell subtypes compared to OX40L⁺ G-BMDCs. Green dots are macrophages subtypes and blue dots are dendritic cell subtypes. **E-F)** Heatmap of alveolar and peritoneal gene signatures in OX40L⁺ G-BMDCs compared to indicated cell subsets from the ImmGen database.

4.3.2 OX40L G-BMDCS express a unique transcriptional tolerogenic profile.

To better understand the molecular signature of OX40L$^+$ G-BMDCs, and thus the tolerogenic mechanism implemented by CD11c$^+$OX40L$^+$ G-BMDCs, we conducted a genome-wide microarray analysis of mRNA expression between OX40L$^+$ and OX40L$^-$ G-BMDCs and compared the expression profiles between OX40L$^+$ G-BMDCs and OX40L$^-$ G-BMDCs. Microarray analysis revealed vast differences in transcriptome phenotype of OX40L$^+$ G-BMDCs compared to OX40L$^-$ G-BMDCs. Confirming the phenotype of these cells, *OX40L* (*Tnfsf4)* was the highest differentially expressed costimulatory gene with a 6-fold increase in expression in OX40L$^+$ G-BMDCs (Fig. 4.2A). As we saw previously from the flow cytometry results, the OX40L$^+$ G-BMDC population presented a stronger co-stimulatory phenotype with significantly increased expression of *CD80*, *CD86*, as well as co-inhibitory molecules *PDL1* and *PDL2* (Fig. 4.2A). Additionally, CCR7, a chemokine receptor known to be involved in the trafficking of Tregs to lymphoid sites and implicated in the maturation and mobilization of dendritic cells, was found to be the most significantly upregulated in the OX40L$^+$ G-BMDCs (Fig. 4.2B). The lack of CCR7 has been implicated in the manifestation of spontaneous autoimmunity (Winter et al., 2011). Furthermore, among the cytokines, IL-33, a cytokine highly implicated in the generation of Foxp3$^+$ Tregs (Matta et al., 2014; Turnquist et al., 2011) was the most differentially expressed cytokine with higher expression levels in OX40L$^+$ G-BMDCs compared to OX40L$^-$ G-BMDCs (Fig. 4.2B). *IL7R* was the most differentially expressed among the interleukin receptor family (Fig. 4.2B). *IL7R* deficiencies have been shown to contribute to autoimmune diseases such as SCID (Puel et al., 1998). Furthermore, *CCL17* and *CCL22*, chemokine ligands

shown to be involved in the trafficking of Tregs to inflammatory sites were also among the chemokine ligands significantly upregulated (Mizukami et al., 2008) (Fig. 4.2B). RT-qPCR confirmed the differential expression *OX40L*, *CCL22*, *CCR7*, and *PDL1* with increased transcriptional expression compared to $OX40L^-$ G-BMDCs (Fig. 4.2C). Taken together, these results demonstrate $OX40L^+CD11c^+$ DCs to differentially express molecules that may contribute significantly to the tolerogenic function of the novel $OX40L^+$ DC subset.

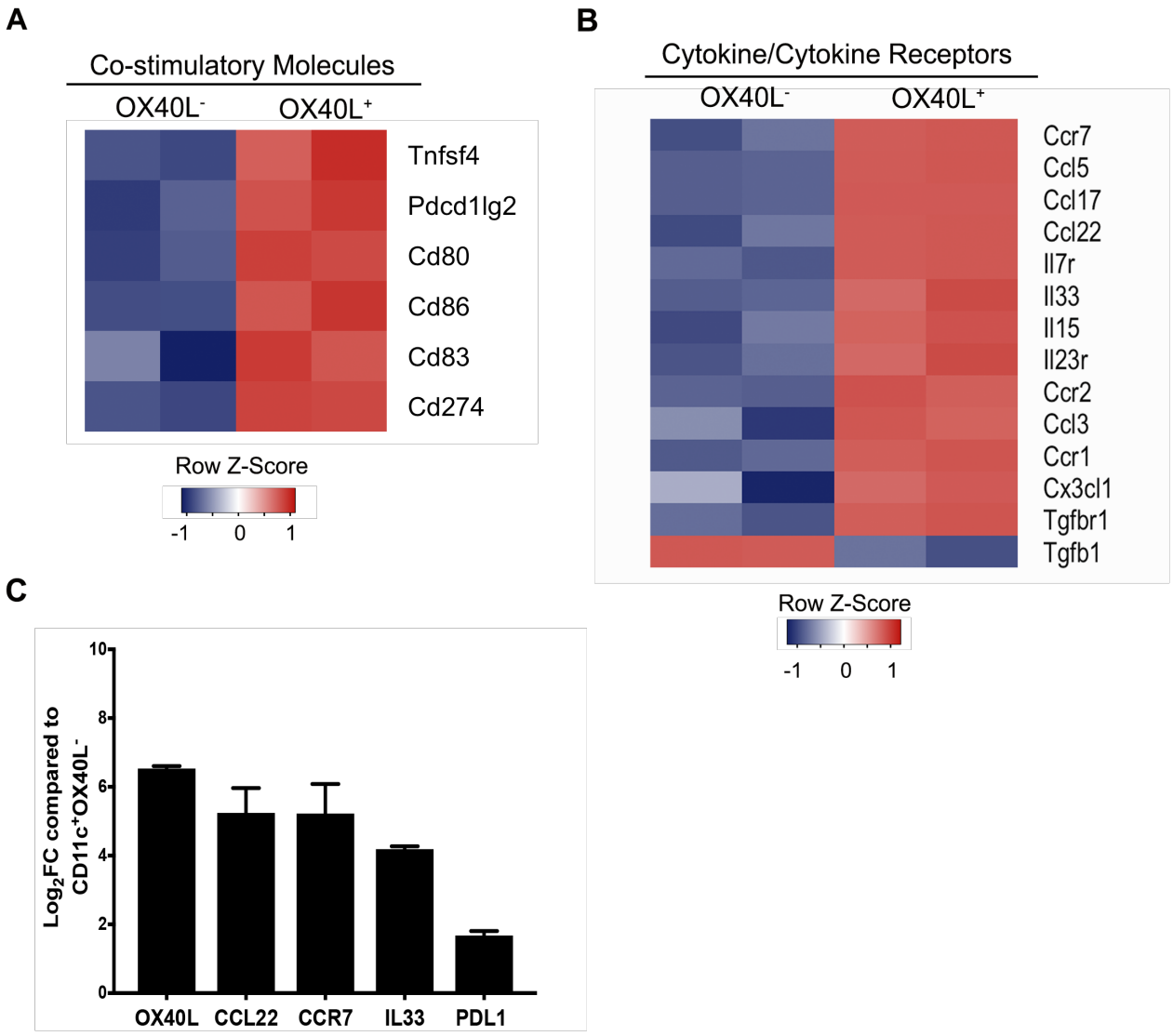

Figure 4.2. Microarray analysis reveals tolerogenic profile of OX40L$^+$ G-BMDCs. A-B) Heatmap of gene expression for select indicated categories of genes comparing sorted OX40L$^+$ G-BMDCs to OX40L$^-$ G-BMDCs. Individual replicates are shown. **C)** RT-qPCR of selected genes. Fold changes are shown relative to OX40L$^-$ G-BMDCs (n=4).

4.4 DISCUSSION

There has been much debate over the role of GM-CSF in DC development. GM-CSF has been postulated to play a role in inflammatory processes rather than under steady-state conditions due to the lack of alterations of hematopoietic populations in GM-CSF receptor-deficient mice (Paine et al., 2001). Only minor alterations in alveolar macrophages have been observed in GM-CSF receptor-deficient mice (Paine et al., 2001). These results suggested GM-CSF may play important roles in stress responses rather than in immune cell development. This is further supported by evidence of GM-CSF levels being typically very low or absent in steady-state conditions; however, upon exposure to strong inflammatory stimuli GM-CSF production is rapidly increased (Cheers et al., 1988).

Our results suggest $OX40L^+$ DCs to be a DC subset uniquely differentiated by GM-CSF, as FLT3L cultures failed to differentiate these cells in bone-marrow precursor cultures. In order to identify a steady-state equivalent DC, we performed a principal component analysis using gene expression data from $OX40L^+$ G-BMDCS and immune cell subsets from the ImmGen database. This analysis revealed $OX40L^+$ G-BMDCS to closely cluster with alveolar, peritoneal, and liver macrophages, however, although $OX40L^+$ G-BMDCs expressed some of the gene signatures of alveolar and peritoneal macrophages, the $OX40L^+$ G-BMDC gene signature remained distinct from those of the other cells. A limitation of the ImmGen database is that all the immune cell transcriptional data acquired are from steady-state non-infected mice. Therefore, our data suggests that $OX40L^+$ G-

BMDCs could represent a non-steady state dendritic cell subtype induced under inflammatory conditions.

We further evaluated the transcriptional profile of $OX40L^+$ G-BMDCs compared to $OX40L^-$ G-BMDCs to reveal molecules that may play a role in the functional differences between the two subsets. $OX40L^+$ G-BMDCs expressed significantly increased levels of various co-stimulatory molecules such as CD80 and CD86, as well as the co-inhibitory molecules PDL1 and PDL2, which we had previously observed through FACs analysis. Selective reduction of CD80 and CD86 on $CD11c^+$ DCs in mice has been shown to significantly reduce the frequencies of peripheral Tregs suggesting a role for CD80/CD86 in Treg homeostasis (Bar-On et al., 2011). Furthermore, PDL2 has been implicated in negatively regulating T-cell immune responses, and thus, promoting tolerance (Zhang et al., 2006). Further supporting this theory, studies have shown PDL2-deficient APCs from mice were found to activate T-cells more potently than those of WT mice (Zhang et al., 2006). Additionally, of the most upregulated genes, $OX40L^+$ G-BMDCs exhibited increased expression of IL-33, a cytokine shown to be involved in Treg expansion (Matta et al., 2014; Turnquist et al., 2011). IL-33 has also been shown to promote the development of $CD11c^+$ DCs *in vitro* that fail to activate immunologically naïve T-cells in a GM-CSF dependent manner, suggesting IL-33 may play a role in GM-CSF derived tolerogenic DC development (Mayuzumi et al., 2009). Furthermore, CCL22 and CCL17 were also found to be upregulated in $OX40L^+$ G-BMDCs. CCL22 and CCL17 have been shown to induce *in vitro* Treg migration, suggesting $OX40L^+$ G-BMDCs utilize these chemokines as a mechanism to attract Tregs to inflammatory sites (Mizukami et al., 2008). These results

further confirm the tolerogenic immune phenotype of OX40L$^+$ G-BMDCs and may point to potential mechanisms of Treg homeostasis that could be explored further. Overall, these results suggest OX40L$^+$ DCs may be induced under inflammatory conditions through the increased production of GM-CSF and could play a regulatory feedback role to temper inflammation through the induction of Tregs.

5.0 SUMMARY AND FUTURE DIRECTIONS

In this study, we determined *ex vivo* derived OX40L$^+$ G-BMDCs are phenotypically and functionally equivalent to *in vivo* derived OX40L$^+$CD11c$^+$ DCs induced upon GM-CSF treatment. We have previously shown, upon treatment with GM-CSF in mice, a significant increase in Tregs with a corresponding increase in the CD11c$^+$CD8a$^-$ DC subset and suppression of autoimmune disease (Ganesh et al., 2009). Subsequent studies from our laboratory showed that BMDCs, derived from the bone marrow of either WT or MHC Class-II$^{-/-}$ mice differentiated *ex vivo* in the presence of GM-CSF (G-BMDCs), could selectively expand Tregs independent of TCR signaling but critically dependent on the expression of OX40L on the DC surface (Bhattacharya et al., 2011; Gopisetty et al., 2013). Supporting this data, in this study we have demonstrated that sorted OX40L$^+$ G-BMDCs were responsible for the selective proliferation of Tregs expressing suppressive Treg phenotypic markers indicative of functional Treg suppressive capacity. Furthermore, our current studies show that treatment with GM-CSF induced the proliferation of suppressive Treg populations in the spleen, brachial lymph nodes, and the liver of treated mice, which correlated with tissue-specific increases in OX40L$^+$ on CD11c$^+$ DCs suggesting a role for OX40L$^+$CD11c$^+$ DCs in peripheral Treg expansion. These OX40L$^+$CD11c$^+$ DCs not only shared expression of similar surface markers observed on OX40L$^+$ G-BMDCs but also showed the similar function of expanding Tregs in an OX40L-dependent manner. Moreover, we determined OX40L$^+$ DCs are of non-steady state identity, and we discovered potential mechanisms by which OX40L$^+$ DCs exert tolerance,

suggesting OX40L$^+$ DCs may be induced by GM-CSF during inflammatory conditions as a mechanism of immune homeostasis.

Historically, physiological OX40L$^+$ expression on CD11c$^+$ DCs has appeared to be restricted to inflammatory sites and relatively absent in wild-type, uninfected mice (Webb et al., 2016). For example, OX40L$^+$ DCs have been described in the pancreas and secondary lymphoid organs of 11-13 week-old pre-diabetic NOD mice (Pakala et al., 2004), in the inflamed kidneys of Lupus patients (Jacquemin et al., 2015), and in EAE brain tissues, which correlated with disease severity (Hong et al., 2013). Furthermore, based on the outcome of studies using agonistic OX40 signaling or OX40L blockade, it was postulated that OX40L$^+$ DCs were the cause of inflammation or autoimmune diseases. However, OX40L has been shown to be induced on various immune cells such as B-cells (Linton et al., 2003), macrophages (Karulf et al., 2010), mast cells (Kashiwakura et al., 2004; Nakae et al., 2006) and natural killer cells (Zingoni et al., 2004), and therefore, systemic administration of OX40L and/or blockade of OX40 may have broader effects, and thus differ mechanistically from the way in which OX40L$^+$ DCs specifically exert their tolerogenic effect through Treg expansion. Thus, the tolerogenic effect of OX40L may be context dependent.

Our studies are of significant importance as GM-CSF derived DCs have long been utilized in therapeutic vaccines for cancer. The efficacy, however, of these DC tumor vaccines has been called into question (Mantia-Smaldone and Chu, 2013). While many DC vaccines have exhibited antigen-specific immune responses, clinical responses have

been low (Mantia-Smaldone and Chu, 2013). Based on our findings, it could be hypothesized that OX40L$^+$ G-BMDCs, when used for DC-based vaccination, may suppress anti-tumor effector immune responses through the expansion of Tregs, and therefore, lead to suboptimal immune response and/or treatment failure. In fact, in a melanoma DC vaccine clinical trial with low-dose cytokines (IL-2 and IFN-gamma), Tregs were significantly increased by the fourth dose of the DC vaccine and were correlated with disease progression (Bjoern et al., 2011). These findings combined with our current results strongly suggest that it is prudent to consider the possibility of inducing tolerogenic DCs, such as OX40L$^+$ G-BMDCs, by GM-CSF, while developing/optimizing therapeutic tumor DC vaccinations.

The tolerogenic properties of OX40L$^+$ DCs are also of critical significance in metastatic tumor microenvironments. PDL2 and MGL2 have been identified on CD11c$^+$CD11b$^+$ cells in a metastatic tumor-released GM-CSF microenvironment and implicated in the suppression of CD8 T-cells and the expansion of Tregs, respectively (Kenkel et al., 2017). In many cancers, poor outcomes are associated with increased Tregs frequencies and low CD8 T-cell infiltration (Fridman et al., 2012; Gooden et al., 2011). Depletion of this DC subset was found to enhance tumor immunity and inhibit tumor metastasis (Kenkel et al., 2017). In our studies, we detected expression of MGL2 and CD11b, along with the upregulation of the co-inhibitory molecule PDL2 on *in vivo* derived OX40L$^+$ DCs from GM-CSF treated mice. It is possible that OX40L$^+$ DCs may play another not yet discovered role in the suppression of CD8 T-cells through PDL2 signaling. Furthermore, it is possible that OX40L$^+$ DCs may play a significant role in tumor-released GM-CSF

microenvironments in the process of tumor metastasis. Therefore, further investigation into the development and function of $OX40L^+$ DCs could yield tumor-specific mechanisms in the treatment of metastatic cancers.

In conclusion, our results suggest $OX40L^+$ G-BMDCs are equivalent to $OX40L^+$ DCs induced *in vivo* by GM-CSF and may play a role in the expansion of functional Tregs as a mechanism of maintaining immune homeostasis during inflammation or infection. The purpose of $OX40L^+$ DCs should be further explored as they can be manipulated for optimal therapeutic use in the treatment of autoimmune disease, cancers, or DC-based vaccine development.

6.0 CITED LITERATURE

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7.0 APPENDICES

APPENDIX A:

Statement from Nature Research in regards to author reuse of publication in thesis.

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APPENDIX B:

Animal Care Committee (ACC) approval:

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

February 2, 2018

Bellur S. Prabhakar
Microbiology & Immunology
M/C 790

Dear Dr. Prabhakar:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **1/16/2018**. *The protocol was not initiated until final clarifications were reviewed and approved on* **2/2/2018**. *The protocol is approved for a period of* ***3*** years with annual continuation.

Title of Application: GM-CSF Directed Tregs Induction in Non-Obese Diabetic Mice

ACC Number: 17-227

Initial Approval Period: 2/2/2018 to 1/16/2019

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 3

Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*G-BMDC-Induced Treg Mediated Suppression of Type 1 Diabetes in NOD Mice (Institutioanl #0029120)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 AI 07516 (A1 version years 1-5)	*Funded*	*201400352*	*UIC*	*Bellur S Prabhakar*
Funding Agency	**Funding Title**			**Portion of Proposal Matched**
JDRF- Juvenile Diabetes Research Foundation	*TCR- Independent Selective Induction of Regulatory T Cells to Treat Type-1 Diabetes (Institutioanl #0026229)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
2-SRA-2016-245-S-B	*Funded*	*201602947*	*UIC*	
Funding Agency	**Funding Title**			**Portion of Proposal Matched**

APPENDIX B (continued):

NIH	*Expansion of regulatory T cells as a means of tolerance induction in treatment of type 1 diabetes (Institutional # 00308443)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
F31 DK111183- (years 1-3 A1 version)	*Pending*		*UIC*	*Alejandra Marienelarena*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Timothy J. Koh, PhD
Chair, Animal Care Committee
TJK /*mbb*
cc: BRL, ACC File, Prabhakaran Kumar, Palash Bhattacharya, Alejandra Marinelarena

8.0 VITA

EDUCATION

University of Illinois, College of Medicine — Chicago, IL
M.D./Ph.D., Ph.D. expected in Microbiology and Immunology — Expected May 2020

Harvard College — Cambridge, MA
B. A., Molecular and Cellular Biology — May 2011

TRAINING AWARDS

Ruth L. Kirschstein Pre-Doctoral National Research Service Award — April 2017 – April 2020
National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health

American Heart Association Predoctoral Fellowship — January 2017 – April 2017
American Heart Association Midwest Affiliate

Postbaccalaureate Intramural Research Training Award — August 2011 – May 2013
National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health

Short-term Education Program for Underrepresented Persons — Summers 2008, 2009, 2010.
National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health

PROFESSIONAL EXPERIENCE

University of Illinois, College of Medicine at Chicago — Chicago, IL
MSTP Trainee with Dr. Prabhakar — July 2015 – June 2018
Investigating the TCR-independent expansion of regulatory T cells as a clinical application for the treatment of type 1 diabetes.

Phoenix Epidemiology and Clinical Research Branch, NIH — Phoenix, AZ
Research intern with Dr. Leslie Baier — August 2011 – May 2013
Identified genetic variants that may play a role in the development of type 2 diabetes and obesity in the Pima Indians of Arizona.

Joslin Diabetes Center, Harvard Medical School — Boston, MA
Thesis student with Dr. Susan Bonner-Weir — January 2010 – June 2011

Developed an *in vitro* mouse model using islets extracted from neonatal rats to study the role of glucocorticoids in the maturation of neonatal β-cells to glucose-responsive β-cells.

Feinberg School of Medicine, Northwestern University Chicago, IL
Research intern with Dr. Brian Layden June 2009 – August 2009
Confirmed the differential expression of novel molecules potentially involved in β-cell mass regulation during pregnancy.

University of California, Los Angeles Los Angeles, CA
Research intern with Dr. Stephen Smale June 2008 – August 2008
Created expression plasmids to investigate the role of Ikaros, a zinc finger transcription factor, in the pathogenesis of BCR-ABL1 acute lymphoblastic leukemia.

PUBLICATIONS

Marinelarena A, Bhattacharya P, Kumar P, Maker AV, and Prabhakar BS. Identification of a Novel OX40L+ Dendritic Cell Subset That Selectively Expands Regulatory T cells. *Sci Rep* 2018;8(1): 14940.

Kumar, P., **Marinelarena, A**., Raghunathan, D., Ragothaman, V.K., Saini, S., Bhattacharya, P., Fan, J., Epstein, A.L., Maker, A.V., and Prabhakar, B.S. (2018). Critical role of OX40 signaling in the TCR-independent phase of human and murine thymic Treg generation. Cell Mol Immunol.

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

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

Traurig, M., Hanson, R.L., **Marinelarena, A**., Kobes, S., Piaggi, P., Cole, S., Curran, J.E., Blangero, J., Goring, H., Kumar, S., et al. (2016). Analysis of SLC16A11 Variants in 12,811 American Indians: Genotype-Obesity Interaction for Type 2 Diabetes and an Association With RNASEK Expression. Diabetes 65, 510-519.

Bian, L., Traurig, M., Hanson, R.L., **Marinelarena, A**., Kobes, S., Muller, Y.L., Malhotra, A., Huang, K., Perez, J., Gale, A., et al. (2013). MAP2K3 is associated with body mass index in American Indians and Caucasians and may mediate hypothalamic inflammation. Hum Mol Genet 22, 4438-4449.

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Ma, L., Murea, M., Snipes, J.A., **Marinelarena, A**., Kruger, J., Hicks, P.J., Langberg, K.A., Bostrom, M.A., Cooke, J.N., Suzuki, D., et al. (2013). An ACACB variant implicated in diabetic nephropathy associates with body mass index and gene expression in obese subjects. PLoS One 8, e56193.

Aguayo-Mazzucato, C., Zavacki, A.M., **Marinelarena, A**., Hollister-Lock, J., El Khattabi, I., Marsili, A., Weir, G.C., Sharma, A., Larsen, P.R., and Bonner-Weir, S. (2013). Thyroid hormone promotes postnatal rat pancreatic beta-cell development and glucose-responsive insulin secretion through MAFA. Diabetes 62, 1569-1580.

Layden, B.T., Durai, V., Newman, M.V., **Marinelarena, A.M.**, Ahn, C.W., Feng, G., Lin, S., Zhang, X., Kaufman, D.B., Jafari, N., et al. (2010). Regulation of pancreatic islet gene expression in mouse islets by pregnancy. J Endocrinol 207, 265-279.

CONFERENCE PRESENTATIONS

Marinelarena AM, Bhattacharya P, Alharshawi K, El-Sayed O, Kumar P, Prabhakar BS. Identification of a novel tolerogenic cell subset in the treatment of autoimmune diseases. Poster. Immunology 2017; Washington D.C., P127.16.

Marinelarena AM, Bhattacharya P, Alharshawi K, Osama El-Sayed, Kumar P, Prabhakar BS. Identification of a novel tolerogenic dendritic cell subset in the treatment of type 1 diabetes. Oral and Poster. *Autumn Immunology Conference.* 2016 November 18-22. Chicago, Illinois.

Marinelarena AM, Bhattacharya P, Alharshawi K, Kumar P, Prabhakar, BS. GM-CSF induced bone-marrow derived dendritic cells from NOD mice selectively expand T regulatory cells independent of TCR activation. Poster. *AAP-ASCI-APSA Joint Meeting.* 2016 April 13-17; Chicago, Illinois.

Marinelarena AM, Aguayo-Mazzucato C, Bonner-Weir S. Dexamethasone as a regulator of β-cell maturation. Poster. *ENDO 2011: The 93rd Annual Meeting and Expo;* 2011 June 4-7; Boston, Massachusetts. *Endocrine Reviews*; 2011. P2-501.

CONFERENCE PRESENTATIONS (continued)

Marinelarena AM, Aguayo-Mazzucato C, Bonner-Weir S. The development of an in vitro model investigating the role of glucocorticoids in the functional maturation of neonatal β-cells. Oral Presentation and Poster. *The Short-Term Education Program for Underrepresented Persons (STEP-UP) Research Symposium*; Atlanta, GA. August 2010.

Marinelarena AM, Layden BT, Lowe WL Jr. Identification of novel genes involved in β-cell mass regulation. Oral Presentation and Poster. *STEP-UP Research Symposium*; Los Angeles, CA. August 2009.

Marinelarena AM, Schjerven H, Smale ST. Ikaros: Potential contributor to BCR-ABL1 lymphoblastic leukemia. Oral Presentation and Poster. *STEP-UP Research Symposium*; Washington D.C. August 2008.