CBL-B DEFICIENT CD4⁺ T CELLS MEDIATE RESISTANCE AGAINST REGULATORY T CELLS THROUGH IL-2 OVERPRODUCTION

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Immunology
University of Toronto

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Master of Science
Department of Immunology
University of Toronto
2017

Abstract

Regulatory T cells play crucial roles in health and diseases through their immunosuppressive properties. In particular, Treg cells in the tumor microenvironment are known to restrict anti-tumor immune response and therefore understanding mechanisms of resistance to Treg-mediated immunosuppression serves as an attractive therapeutic avenue. E3 ubiquitin ligase Cbl-b is a regulator of multiple TCR signaling pathways, and has demonstrated its potential in rendering T cells impartial to the suppressive signals of Treg cells through regulating cytokines. This study specifically explored cellular mechanisms associated with Cbl-b KO CD4⁺Foxp3⁻ effector T cell resistance against Treg cells. Cbl-b KO CD4⁺Foxp3⁻ T cells hyper-produced IL-2 and together with IL-2Rα upregulation served as an important mechanism to escape suppression by Treg cells. Overall, the study offers insights into the potential development of CD4⁺ T cells resistant to suppression by regulatory T cells.
Acknowledgments

Every aspiring scientist matures personally and intellectually thanks to those around him/her. I would like to dedicate my Masters work to all of my family members, mentors, friends, and colleagues who provided me with the support and inspirations, enabling me to live through my childhood dream of becoming a scientist. I found sincere gratification during the 2.5 years I spent in the Ohashi laboratory, which have been the most rewarding years up to date. The following dossier highlights some of the works achieved through the mentorship, patience and support from everyone around me. Again, I am sincerely grateful for the opportunity provided thus far, as I look forward to the next step of my academic journey.

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<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ACT</td>
<td>adoptive cell transfer</td>
</tr>
<tr>
<td>Akt</td>
<td>Ak thymoma</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBA</td>
<td>cytometric bead array</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C motif ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte associated protein 4</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment crystalizable</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced TNFR-related protein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-2Ra</td>
<td>interleukin-2 receptor alpha</td>
</tr>
<tr>
<td>IPEX</td>
<td>immunodysregulation polyendocrinopathy enteropathy X-linked syndrome</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>Lck</td>
<td>lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>MFI</td>
<td>median fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>miR</td>
<td>micro-ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>mechanistic target of rapamycin</td>
</tr>
<tr>
<td>Nedd4</td>
<td>neuronal precursor cell-expressed developmentally downregulated 4</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cell</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>programmed cell death protein 1</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>phospholipase C gamma</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PTP</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RIP</td>
<td>rat insulin promoter</td>
</tr>
<tr>
<td>RORγt</td>
<td>retinoic acid receptor-related orphan receptor gamma</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>SHP</td>
<td>SH region 2 domain-containing phosphatase</td>
</tr>
<tr>
<td>SLP-76</td>
<td>SH2 domain-containing leukocyte phosphoprotein of 76kDa</td>
</tr>
<tr>
<td>SMAD</td>
<td>mothers against decapentaplegic homolog</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>T&lt;sub&gt;h&lt;/sub&gt;</td>
<td>helper T cells</td>
</tr>
<tr>
<td>TIL</td>
<td>tumor-infiltrating lymphocytes</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cells</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>UBA</td>
<td>ubiquitin associated</td>
</tr>
<tr>
<td>Vav1</td>
<td>vav guanine nucleotide exchange factor 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZAP70</td>
<td>zeta-chain-associated protein kinase 70</td>
</tr>
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CHAPTER I

1 INTRODUCTION

1.1 Introduction to Treg development and functions

1.1.1 Development of Treg cells

Regulatory T cells (Tregs) are integral components of the adaptive immune system responsible for maintaining tolerance to self-antigens and preventing autoimmune diseases (Sakaguchi et al. 2010). Tregs, defined as CD25^+CD127^-Foxp3^+ T cells within the CD4^+ T cell compartment in mice, can either be thymically or peripherally-derived (Figure 1.1), generating natural Tregs (nTreg) or induced Tregs (iTregs), respectively. While both display similar cellular phenotypes, some of the differences include the degree of Foxp3 plasticity and methylation profile (Floess et al. 2007 & Sakaguchi et al. 2008). Progenitor cells that eventually give rise to Treg cells originate from the bone marrow and undergo lineage commitment in the thymus. Beginning with the double negative stage of T cells (CD4^-CD8^-), α:β TCR gene re-arrangement occurs and the subsequently assembled TCR interacts with MHC on stromal cells of the thymic cortex to induce cell proliferation and co-expression of CD8 and CD4, leading the cell to become a double positive (DP) cell (Takahama 2006). TCR-expressing DP T cells interact with the cortical thymic epithelial cells (cTECs) through either MHC class I or II for lineage commitment toward either CD4^+CD8^- or CD4^+CD8^+ single positive (SP) populations. Alongside TCR
engagement with MHCI/II on cTECs for lineage commitment, positive selection occurs, eliminating cells that are incapable of recognizing self MHC molecules and ensuring a self-MHC-restricted T cell repertoire (Klein et al. 2009). Subsequently, SP thymocytes migrate to the medulla of thymus where the immature T cells engage with the medullary thymic epithelial cells (mTECs) for negative selection. During this stage, while strong affinity of TCR to self-antigen loaded MHCII induces apoptosis and low affinity induces naïve CD4\(^+\) T cell development, intermediate TCR-strength is crucial for the transcription of Foxp3, responsible for the immunosuppressive phenotype and lineage stability of nTreg cells (Jordan et al. 2001 & Zheng et al. 2007). The negative selection process plays an important role in generating self-tolerance. In addition to the roles of mTECs in negative selection of SP thymocytes, both mTECs and DCs present cellular signals for Treg maturation through either receptor mediated mechanisms or stromal cell-derived humoral factors. In conjunction with the appropriate interaction between TCR and MHC class II molecules, other accessory molecules and ligands such as CD28, CD40, LFA1 and CD80/86 also play integral role in generating mature Foxp3\(^+\) population in thymus (Sakaguchi 2005). Similarly, stromal cell-derived humoral factors, such as TSLP secreted by mTEC-derived Hassal’s corpuscles, target thymic DCs for Treg differentiation (Watanabe et al. 2005). Although not clearly understood, some reports have suggested the importance of IL-2 and TGF-β in nTreg differentiation (Liu et al. 2008 & Weist et al. 2015). At the end of thymic differentiation, Foxp3\(^+\) nTregs differ from other naïve lymphocytes derived from thymus, as they exit the organ as functionally mature and “antigen-primed” cells ready to counteract against autoimmune or pro-inflammatory conditions (Sakaguchi et al. 1982). Within the mature CD4\(^+\) thymic T cell compartment, Foxp3\(^+\) cells constitute approximately 5% of the total population, and this increase to 10-15% in the periphery as a result of the peripherally derived iTreg population which originates from naïve CD4\(^+\) T cell compartment (Fontenot et al. 2005).
Figure 1.1: Illustration of nTreg and iTreg development. Despite functional similarities between nTregs and iTregs, nTregs are derived directly from thymus whereas iTregs are differentiated from naïve CD4^+ T cells in the periphery.

Beyond nTregs, peripherally-derived iTregs also play important non-redundant immunosuppressive roles. While both class of regulatory T cells share closely matched transcriptional signatures and cellular functionality, several differences were previously explored including diversity in TCR repertoire (Haribhai et al. 2011). Distinct from nTregs, iTregs are derived from naïve CD4^+ T cells in the periphery. naïve CD4^+ T cells initially require TGF-β for transient Foxp3^+ expression (early differentiation) and IL-2 for sustaining the Treg functions as well as for inhibiting TGF-β/IL-6 mediated differentiation of Th17 cells through STAT5 signaling (Chen et al. 2011 & Zheng 2013). Throughout the differentiation process, Foxp3 cooperates and forms complexes with NFAT and AML1/Runx1 that repress IL-2, IL-4 and IFN-γ
production and enhances Treg-associated molecules including CD25 (IL-2Rα), and CTLA-4 (Wu et al. 2006 & Sakaguchi et al. 2008). Alongside direct regulation of genes encoding for cytokines and checkpoint molecules, Foxp3 regulates distinct metabolic program by restricting PI3K-Akt-mTOR signaling pathways, thus restricting glycolysis and enhancing oxidative metabolism required for maintaining cellular functions (Gerriets et al. 2016). Lineage stability between thymically and peripherally-derived Treg cells may differ, and pro-inflammatory cytokines may induce loss of suppressive phenotype and differentiation into other CD4+ T cell subsets for iTregs (Sakaguchi et al. 2013).

1.1.2 Cellular mechanisms of T cell suppression

Once fully differentiated, Tregs exploit diverse contact-dependent and cytokine-mediated mechanisms that limit T cell activities (Table 1). Under physiological conditions, the competition between Tregs and effector T cells for APCs, in particular dendritic cells, plays a crucial role in the regulation of the pro-inflammatory response (Sakaguchi et al. 2008). The expression of CD80 and CD86 on mature DCs result in the CD28-mediated co-stimulation necessary for T cell priming and cellular immunity, but also serve as the direct binding partner for CTLA-4 that is constitutively expressed on Tregs (Wing et al. 2011). While high CTLA-4 expression on Tregs may quantitatively compete for CD80/86 on DCs therefore restricting CD28-CD80/86 interaction, Tregs also promote downregulation of CD80/86 through CTLA-4-dependent mechanism (Wing et al. 2008). Additionally, Tregs highly express surface adhesion molecules such as LFA-1, subsequently enhancing the duration and strength of Treg stimulation.
with DCs over naïve CD4+ or CD8+ T cells (Onishi et al. 2008, Chen et al. 2017). Several other contact-dependent mechanisms are undergoing further investigation, including the ability of the Treg cells to directly induce perforin and granzyme-mediated lysis of effector T cells (Grossman et al. 2004, Gondek et al. 2005 & Cao et al. 2007). Lastly, CD39 and CD73 (ectonucleotidases used for hydrolysis of phosphate residues) expression by Tregs play catabolic role in the hydrolysis of extracellular ATP to adenosine, therefore shifting the metabolic milieu to directly inhibit T cells by triggering A2A receptor and subsequently elevating intra-cellular cAMP (Deaglio et al. 2007).

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Function</th>
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<th>Human</th>
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Table 1: Summary of key molecules associated with Treg suppression (Adapted from Sakaguchi et al. 2010) (Onishi et al. 2008, Patterson et al. 2016 & Chen et al. 2017). Tregs use a diverse repertoire of contact-dependent and cytokine-mediated mechanisms to restrict T cell activities. Suppressive mechanisms used by Treg cells are context-dependent and differs between murine models and humans.
Tregs also suppress other T cells via the production of inhibitory cytokines and chemokines. Tregs secrete TGF-β, IL-10 and IL-35, all of which dysregulate effector T cell function (Chen et al. 2003, Collison et al. 2007 & Belkaid 2007). More specifically, TGF-β signaling negatively regulates effector CD4+ or CD8+ T cell function, while working in tandem with either IL-2 or IL-6 to induce differentiation of naïve CD4+ T cells into Foxp3+ iTreg or RORγt+ T_h17 cells, respectively (Rubtsov & Rudensky 2007). Lastly, the constitutively high expression of CD25 on Tregs competes for IL-2, secreted by CD4+ T cells (Sakaguchi et al. 2008 & Chinen et al. 2016). Alongside CD25’s role in forming high-affinity complex with CD122 (IL-2Rβ) and CD132 (IL-2Rγ) to amplify its signaling pathways, including the STAT5 pathway, CD25 also serves to generate a reservoir of IL-2 to be recycled to support future T cell activation (Boyman & Sprent 2012 & Su et al. 2015). In the presence of Treg cells, T cells are deprived of IL-2 required for proliferation and survival, and subsequently undergo cytokine deprivation-mediated apoptosis, characterized by low Akt activation and induction of pro-apoptotic molecules including Bim (Pandiyan et al. 2007). Furthermore, IL-2 acquired by Tregs provides necessary STAT5 signaling to further enhance its immunosuppressive function (Chinen et al. 2016). In conjunction with previously described cytokine-driven suppressive mechanisms, it has been recently demonstrated in EAE and islet allograft models that chemokine secretion by Tregs, more specifically CCL3 and CCL4, play an important role in the recruitment of effector T cells for suppression (Patterson et al. 2016).

Adding to the complexity of suppressive mechanisms used by Tregs, each suppressive mechanism behaves in a context-dependent manner and more than one mechanism can be synergistically employed to restrict T cell function (Sakaguchi et al. 2008). In addition, the
phenotype of Tregs and the suppressive mechanisms differ between humans and mice (Table 1), further emphasizing its complexity. While suppressive mechanisms employed by human Tregs have been less extensively studied, reports have indicated their roles in the secretion of anti-inflammatory cytokines, dysregulation of DCs and direct cytolysis of effector T cells (Sakaguchi et al. 2010). Interestingly, human Tregs may mediate suppression of effector T cells independent of APCs, and only CD45RO⁺Foxp3ʰ Tregs highly express CTLA-4 (Miyara et al. 2009). Once activated through TCR signaling, the surface molecules expressed by human Tregs were sufficient in mediating suppression, independent of cell viability or function (Baecher-Allan et al. 2002). Lastly, while Foxp3 is often used as a marker for Treg cells in murine models, activated T cells in humans may gain transient expression of Foxp3 (Gavin et al. 2006). With inconsistency between human and mouse Treg cells, ongoing work is required to further clarify the suppressive mechanisms used by Treg cells.

1.1.3 The role of Treg cells in diseases

Regulatory T cells serve as one of the most potent inhibitors of hyperactive immune responses often observed in autoimmune diseases, alongside their distinct roles in non-immunological conditions (i.e. the poorly understood role of Tregs in muscle and tissue repair (Villalta et al. 2014)). While a robust anti-inflammatory response may be favored to counteract against certain pathologies, proper homeostatic regulation of pro-inflammatory CD4⁺ or CD8⁺ T cells and anti-inflammatory Tregs is prerequisite to preventing a diverse repertoire of immunological abnormalities. As exemplified by IPEX syndrome, caused by mutation of
transcription factor *Foxp3* required for Treg function, defective Treg function resulted in dysregulated activation of T and B cells, also leading to a severe, early onset multi-organ autoimmunity (Brunkow et al. 2001, Ochs et al. 2005 & Sakaguchi et al. 2008). Similar observations were made in allergic patients during hay fever season, displaying Treg impairment and dysregulation of T\(_h\)2 responses against allergens (Grindebacke et al. 2004 & Ling et al. 2004). Alternatively, hyperactive immunosuppression mediated by Tregs may also result in infectious diseases such as *Helicobacter pylori* infection (Lundgren et al. 2003). Overall, the complex interplay between Tregs and T cells has been demonstrated to play a pivotal role in the pathogenesis and treatment of an extensive number of human diseases such as multiple sclerosis, cancer, rheumatoid arthritis, and many more (Taams et al. 2006). Understanding the direct mechanisms which skew the homeostatic balance between the two cell types and the specific molecular targets are currently being explored for the development of therapeutics against many of these diseases.

In the context of cancer, prior to the early discovery of Foxp3 and its role in Treg cells in 2003 (Bennett et al. 2001, Hori et al. 2003 & Fontenot et al. 2003), Woo et al. provided early evidence for the ability of regulatory T cells (at that time identified as CD4\(^+\)CD25\(^+\) T cells) to mediate immunosuppression in patients with early-stage non-small cell lung cancer and late-stage ovarian cancer through mechanisms such as secretion of TGF-\(\beta\) and subsequent inhibition of CD8\(^+\) T cells (Woo et al. 2001). Since then, numerous studies have noted the presence of Treg cells in multiple types of cancer including melanoma, ovarian cancer and pancreatic cancer (Zou et al. 2006). Furthermore, evidence suggested that higher infiltration of Treg cells also correlated with the disease progression of cancer, and Treg infiltrate in each stages of cancer served as a good
metric for survival prediction (Curiel et al. 2004). However, several reports also suggest Treg infiltration to be a biomarker for good clinical response (Correale et al. 2010), highlighting the complexity associated with the use of Treg infiltrate as a biomarker. As effector T cell activities in cancer also increase Treg infiltration (Pages et al. 2005 & Galon et al. 2006), CD8⁺ T cell to Treg cell ratio was also recommended to address this challenge (Sato et al. 2005). Overall, the role of Treg cells in tumor progression has been demonstrated through the depletion of Treg cells in various tumor models and subsequent improvement in anti-tumor immune response (Shimizu et al. 1999). Alternatively, adoptive transfer of CD8⁺ T cells with Treg cells prevented effective adoptive cell therapy against B16 melanoma (Antony et al. 2005).

In alignment with previously studied immunosuppressive mechanisms, several different Treg cell-based mechanisms were noted in tumor progression. In the context of head and neck squamous cell carcinoma, TGF-β and IL-10 secretion by CD4⁺CD25hiFoxp3⁺ T cells in the tumor played a key role in regulating pro-inflammatory T cell responses (Strauss et al. 2007). Furthermore, Cao et al. provided evidence suggesting that the tumor-resident Treg cells induced NK and CD8⁺ T cell death in a granzyme B and perforin-dependent manner (Cao et al. 2007). Interestingly, the role of Treg cells in cancer has been studied beyond their immunosuppressive functions. Treg cells may secrete VEGF and are directly associated with angiogenic re-programming, further illuminating the multi-faceted functions of Treg cells (Giatromanolaki et al. 2008 & Facciabene et al. 2011). Reports suggest that the tumor microenvironment enhances such Treg activities and promotes the immunosuppressive state through increased recruitment of Treg cells rather than augmenting their functional state (Curiel et al. 2004). Treg cells are recruited to the tumor microenvironment by CCL22 (secreted by tumors or tumor-resident macrophages) or
CCL28 (secreted by ovarian cancer cells in response to hypoxia) via ligation of the cognate receptors CCR4 or CCR10 on Treg cells (Ishida et al. 2006 & Facciabene et al. 2011). The tumor microenvironment is not only capable of recruiting Treg cells and subsequently expanding the population, but is also capable of generating iTregs through IL-10 and TGF-β (Seo et al. 2001 & Ghiringhelli et al. 2005). Overall, these specific mechanisms employed by tumor and Treg cells to restrict the anti-tumor immune response highlight the significance and complexity of Treg cells in the clinical outcome of cancer patients.

1.1.4 Current therapeutic strategies targeting Treg cells

Acknowledging the significance of Treg cells and its role in the restriction of anti-tumor immune responses, multiple therapeutic strategies have been considered and developed. First, several non-specific anti-cancer drugs have been shown to regulate Treg activities. Taking advantage of Treg cells’ proliferative advantage over effector T cell proliferation in the tumor microenvironment, cyclophosphamide (CTX), a common chemotherapeutic agent known to target rapidly dividing cells, restricted intra-tumoral Treg expansion while maintaining effector T cell function (Galluzzi et al. 2012). Thus, while the overuse of chemotherapeutic agents were traditionally known to have immunosuppressive effects as a result of systemic lymphocyte depletion, an appropriate dosage and treatment strategy may foster an immunostimulatory effect by targeting rapidly dividing intra-tumoral Treg cells. Such a treatment method was further improved in its selectivity and efficacy through combination therapy with OX40 agonist, demonstrating increased intra-tumoral Teff/Treg cell ratio and subsequent regression of B16

Despite the ability of chemotherapeutic agents to target Treg cells, a Treg-specific therapeutic strategy is currently in demand. While specific targeting of tumor infiltrating Treg cells are limited due to lack of a Treg-specific biomarker, several agents including basiliximab/daclizumab (CD25 blocking antibody), denileukin diftitox (Ontak, IL-2-diphtheria toxin conjugate protein), and CCR4 neutralizing antibody were proposed to specifically target effector Treg population and enhance anti-tumor immunity (Waldmann 2006, Litzinger et al. 2007 & Sugiyama et al. 2013). Similar to CD25 neutralization, the use of denileukin diftitox for Treg targeting and eliciting an anti-tumor immune response remains controversial with varying clinical responses. While the FDA approved agent effectively relieved inhibition to promote anti-tumor immunity in patients with renal cell carcinoma, the opposite trend was observed in patients with metastatic melanoma (Dannull et al. 2005 & Attia et al. 2005). With patient heterogeneity and CD25 as a non-specific biomarker, several limitations in the therapeutic strategies exist despite several instances of success. An alternative strategy devised by the Sakaguchi group involves targeting CCR4 expressed by terminally differentiated effector Tregs but not naïve Tregs, which would selectively deplete suppressive Tregs. Anti-CCR4 selectively depleted the effector Treg population (CD45RA Foxp3^{hi}CD4^{+} Tregs), which are predominant among tumor-infiltrating Tregs but not the naïve Treg population (CD45RA^{+}Foxp3^{hi}CD4^{+} Tregs). This enabled a more effective anti-tumor T cell response in cancer patients (Sugiyama et al. 2013). Thus, the
therapeutic strategy offered the advantage of avoiding systemic Treg depletion while simultaneously enabling an anti-tumor T cell response. More recently, CD25-targeted near-infrared photoimmunotherapy (NIR-PIT) was developed using murine models to address some of the limitations with previous Treg depletion strategies. Using a CD25-targeting approach conjugated to a light-sensitive dye, NIR-PIT enabled localized Treg depletion in tumors to avoid systemic depletion (Sato et al. 2016). Lastly, CTLA-4 checkpoint inhibition therapy, originally designed to restrict inhibitory signals on effector T cells, may also play a role in regulating Treg responses. With Treg cells constitutively expressing a high level of CTLA-4, the addition of anti-CTLA-4 mAb resulted in the depletion of intra-tumoral CTLA-4\textsuperscript{+}Foxp3\textsuperscript{+} Treg cells which was dependent on Fcγ receptor-expressing macrophages in the tumor microenvironment (Simpson et al. 2013). This was consistent with the correlation of decreased tumor-infiltrating Tregs with the usage of ipilimumab in patients with bladder cancer (Liakou et al. 2008). In summary, despite significant improvements in Treg targeting strategies, the research area still faces several limitations, including lack of Treg-specific biomarker in humans.

1.2 T cell activation & Treg resistance

1.2.1 T cell development and activation

Consistent with the origin of Treg cells, T cells are initially derived from progenitor cells in the bone marrow prior to migration to thymus for T cell maturation. After DN and DP stage of T cell development, CD4\textsuperscript{+}CD8\textsuperscript{+}TCR\textsuperscript{+} thymocytes engage with either class I or II MHC of cTECs
for positive selection and lineage commitment to either immature CD8+ or CD4+ T cells (Klein et al. 2009). Subsequently, the single positive thymocytes migrate from the thymic cortex to medulla, where negative selection occurs based on TCR affinity to self-antigen loaded mTECs. Thus, the thymocytes exiting thymus as naïve CD4+ or CD8+ T cells have a self-MHC-restricted repertoire and display low-reactivity against self-antigens (Palmer et al. 2003 & Starr et al. 2003).

Once in the periphery, T cells interact with APCs for robust activation (Smith-Garvin et al. 2009). In the context of naïve CD4+ T cells, coordinated CD3/TCR engagement with MHCII, in conjunction with the ligation of CD28 with CD80/86, is important for T cell activation. Co-stimulatory signals from CD28 are also important for avoiding T cell anergy (Harding & Gross 1992). Upon TCR signaling, ITAM motifs on the CD3ζ are phosphorylated by Lck and this enables the binding of ZAP70. ZAP70 is then activated by Lck (brought close through CD4 co-receptor ligation to the MHCII complex), which is important for LAT phosphorylation. Subsequently, LAT phosphorylation and binding of SLP-76 enables the recruitment of important TCR signaling molecules including PLC-γ and Vav1 (Wang & Samelson 1996 & Kane et al. 2000). PLC-γ activation is required for PKCθ and calcium flux-dependent NFAT activation (Figure 1.2). PKCθ is important for NFκB and AP-1 activation (Brownlie & Zamoyska 2013).

Together, NFκB, AP-1 and NFAT transcriptionally regulate a diverse repertoire of genes responsible for cell proliferation, pro-inflammatory cytokine secretion (e.g. IL-2), cytoskeletal rearrangements and more (Brownlie & Zamoyska 2013). IL-2 secretion followed by T cell activation plays an important role in maintaining T cell function and proliferation (Malek & Bayer 2004). While TCR and CD28 signaling is sufficient for initial T cell activation, cytokine signaling is required for further differentiation of T cells into different compartments specialized to generate context-dependent immune response. Dependent on the cytokines available, naïve
CD4⁺ T cells differentiate into T₇₁, T₇₂, T₇₉, T₇₁₇, T₇₂₂, T₇₀ and iTreg, each with distinct transcriptional signatures and cellular functions (Murphy & Reiner 2002 & Caza & Landas 2015).

Figure 1.2: Overview of TCR signaling pathways (Adapted from Brownlie & Zamoyska 2013). TCR stimulation involves propagation of intracellular signaling cascade important for cell survival, proliferation, differentiation and cytokine production. Along with several other key intracellular signaling molecules, PLC-γ1 plays an important role in the activation of NFκB, NFAT and AP-1 signaling pathways.

1.2.2 Immuno-stimulatory/inhibitory surface molecules

While TCR and CD28 signal serve as pre-requisite to T cell activation, the overall response can be further amplified or reduced depending on the presence of immuno-stimulatory or inhibitory molecules. There exists a diverse repertoire of stimulatory surface molecules including the TNF receptor superfamily (GITR, CD40L, 4-1BB, HVEM, and OX40), specific
immunoglobulin superfamily such as ICOS and CD28, along with several other molecules (Croft 2003 & Chen & Flies 2013). Together with TCR signaling, many of these receptors enhance cellular proliferation, cytokine production, cell differentiation, survival, and cytotoxic phenotype. In contrast, inhibitory molecules such as PD-1, CTLA-4, LAG-3, BTLA-4, and TIGIT utilize diverse mechanisms to negatively regulate effector T cell function or cell differentiation (Chen & Flies 2013). In particular, CTLA-4 competitively excludes CD28 out of the central supra-molecular activation complexes (cSMAC) of the immune synapse through direct ligation to CD80/86 (Tseng et al. 2005 & Wing et al. 2011). Upon CTLA-4 signaling, activation of SHP2 and PPA2 results in inhibition of PLC-γ and Akt signaling pathways, indirectly inhibiting proliferation, IL-2 secretion and cellular survival. While PD-1 also exploits PTPs similar to CTLA-4, their molecular targets are different (Chen & Flies 2013).

1.2.3 Cytokine signaling

In conjunction with direct cell-to-cell contact and subsequent activation of co-stimulatory or inhibitory molecules serving an important role in T cell activation or regulation, soluble mediators such as cytokines also modulate powerful receptor-mediated T cell signaling required for cell proliferation, survival, differentiation and activation. Anti-inflammatory cytokines such as TGF-β serve as potent negative regulators of T cell responses and inducers of iTreg or Th17 differentiation (Li & Flavell 2008). In contrast, pro-inflammatory cytokines including interferons (IFNγ and IFNα), those binding to receptors that include the common γ-chain (IL-2, IL-4, IL-7, IL-15, TSLP and others), gp130 (IL-6) and IL-1 (IL-1β, and IL-18) families employ diverse
combinations of intracellular JAK/STAT signaling pathways to mediate robust T cell response or differentiation (Hirano et al. 1997, Schluns & Lefrancois 2003, Schindler et al. 2007 & Sims & Smith 2010). IL-2, one of the most rapidly secreted cytokines upon TCR stimulation (Sojka et al. 2004), serves multi-faceted functions in driving T cell expansion and differentiation. IL-2 has a 15kDa 4-bundle α-helical structure and binds to CD25, (IL-2Ra), CD122 (IL-2Rβ) and CD132 (IL-2Rγ) (Wang et al. 2005 & Stauber et al. 2006). In particular, IL-2Ra is crucial for generating an initial IL-2Ra-IL-2 binary complex that is required for subsequent association with IL-2Rβ and IL-2Rγ, generating the maximal binding affinity of $K_d = 10^{-11}$M (Malek & Castro 2010). Upon high-affinity quaternary IL-2-IL2R complex formation, tyrosine kinases JAK1 and JAK3 also initiate a STAT1, STAT3, and STAT5-dependent response, along with the induction of the PI3K signaling pathway (Nelson & Willerford 1998 & Gaffen 2001). Subsequent to IL-2 signaling, while IL-2Rβ and IL-2Rγ are internalized for degradation, IL-2Ra is recycled back to the cell surface (Yu & Malek 2001 & Su et al. 2015). While IL-2 secretion and signaling induces positive feedback of T cell expansion and differentiation to its effector state, exposure to prolonged high levels of IL-2 may restrict T cell survival and activation status (Lenardo 1991). Beyond TCR signaling and the NFκB, AP-1 and NFAT requirement, IL-2 along with many other pro-inflammatory cytokines are regulated by the family of suppressor of cytokine signaling proteins (SOCS) established for their role in JAK/STAT pathway inhibition (Yoshimura et al. 2007). In particular, Cish, SOCS1 and SOCS3 have shown a direct effect in regulating IL-2 and STAT5 signaling pathways (Matsumoto et al. 1999, Cohney et al. 1999 & Sporri et al. 2001).

Other γ-chain family proteins including IL-7 and IL-15 utilize some of the commonly shared pathways for inducing T cell activation, survival, proliferation and cytolytic functions (Schluns &
Lefrancois 2003). In many cases, lack of certain cytokines may be compensated through the use of other similar cytokines with overlapping signaling pathways. Furthermore, despite tendencies to categorize cytokines and their functions as either pro- or anti-inflammatory, many of the proteins generate context-dependent responses and therefore must be explored in a context dependent manner. In particular, IL-10, an anti-inflammatory cytokine secreted by Treg cells along with several other immunoregulatory cells (von Boehmer 2005), has an important role in the induction of a memory T cell population (Foulds et al. 2006). Thus, while cytokines serve as potent autocrine or paracrine molecules to amplify or restrict T cell functions, much work is required to understand their overlapping and context-dependent functions.

1.2.4 Resistance to regulatory T cells

Studies have demonstrated that molecular pathways and cellular mechanisms exist that render T cells resistant to Treg-mediated suppression. First, the expression of several co-stimulatory molecules including GITR or OX40 by conventional T cells have shown potential in mediating Treg resistance (Shimizu et al. 2002, Ji et al. 2004 & Piconese et al. 2008). Intracellular immunoregulatory molecules such as Casitas B-Lineage Lymphoma-b (Cbl-b), p105 (NFkB precursor protein), miR-155, Akt1 and MyD88 have also been reported to render effector T cells resistant against Treg cell mediated suppression in both in vivo and in vitro models (Wohlfert et al. 2006, Stahl et al. 2009, Chang et al. 2009, Schenten et al. 2014 & Mercadante & Lorenz 2016). In addition, TLR signaling pathways in T cells and DCs along with their roles in Treg resistance have been further characterized in the context of cytokine signal
transduction pathways (Pasare & Medzhitov 2003). Overall, multiple studies have highlighted the role of pro-inflammatory cytokines as the central driver of Treg-resistance; in particular IL-1β, IL-2, IL-4, IL-6, IL-7, IL-15 and IL-21 were noted for their potential role in mediating Treg-resistance through induction of PI3K or other related pathways (O’Sullivan et al. 2006, Pace et al. 2006, Wan et al. 2007, van Amelsfort et al. 2007, Peluso et al. 2007, Ahmed et al. 2009 & Mercadante & Lorenz 2016). The precise molecular and cellular mechanisms behind Treg resistance remain relatively unexplored, but previous literature offers insights into the potential development of therapeutic strategies designed to overcome Treg-mediated immunosuppression.

1.3 The role of E3 ubiquitin ligase Cbl-b in T cell biology

1.3.1 Molecular function

Within the list of immunoregulatory molecules studied thus far in the context of Treg cell-resistance, the E3 ubiquitin ligase Cbl-b has shown promising results and its molecular functions have been previously explored. Cbl-b, structurally and functionally distinct from isoforms c-Cbl and Cbl-3, is 982 amino-acids long and contains an N-terminal tyrosine kinase binding (TKB) domain (consisting of a four-helical bundle, calcium-binding EF-hand and Src homology 2 sub-domains), the RING domain, proline-rich domain and UBA domain, along with several serine and tyrosine phosphorylation sites at Y360, Y363, S630, S634, Y665, Y709 and Y802 (Figure 1.3) (Schmidt & Dikic 2005).
Despite structural similarities between Cbl-b and c-Cbl, Cbl-b targets unique set of substrates important for TCR signaling. Cbl-b is preferentially expressed in peripheral lymphoid organs, and was demonstrated to play crucial role in T cells, B cells, as well as NK cells (Lutz-Nicoladoni et al. 2015). Through direct ubiquitination (and in many cases, subsequent ubiquitin-mediated degradation) of target TCR signaling proteins, Cbl-b serves as a negative regulator of antigen-induced T cell activation, and its deficiency results in a hyper-inflammatory T cell status (Paolino & Penninger 2010). Several direct molecular targets have been identified, including PKCθ, Nedd4, PLC-γ1, Vav1, LAT and p85, along with several other TCR signaling molecules that control the pro-inflammatory T cell response (Lin & Mak 2007, Qingjun et al. 2014 & Matalon et al. 2016). Consequently, through the regulation of these molecules, Cbl-b controls a diverse repertoire of intracellular mechanisms associated with the pro-inflammatory response, such as calcium influx, cytoskeletal rearrangement, immune synapse formation, cytokine secretion as well as proliferation (Krawczyk et al. 2000 & Lin & Mak 2007). Furthermore, Cbl-b plays an important role in regulating
activation-induced apoptosis in Th1 cells, consistent with previous literature suggesting prevention of apoptosis with Cbl-b deficiency (Hanlon et al. 2005). Alternatively, Cbl-b expression may be negatively regulated through the induction of TCR signaling pathways through PKCθ and Nedd4 (Yang et al. 2008 & Gruber et al. 2009). Upon TCR and CD28 signaling, PKCθ phosphorylates Cbl-b (Figure 1.4), changing its structural conformation, and Nedd4, an E3 ligase and negative regulator of PTEN, is capable of poly-ubiquitination of Cbl-b for subsequent proteosomal degradation (Yang et al. 2008). Together, Cbl-b and TCR signaling molecules negatively regulate each other, playing a crucial role in the activation or anergic state of the T cell.

**Figure 1.4:** The role of Cbl-b in T cell anergy and activation (Adapted from Schmitz 2009). Cbl-b, serving as a negative regulator of p85, PLC-γ and PKCθ activities through poly-ubiquitination and
subsequent proteosomal degradation, plays an important role in T cell anergy upon TCR signaling. Co-stimulatory signals by CD28 trigger PKCθ activation for negative regulation of Cbl-b and subsequent T cell activation.

As demonstrated in anergic T cells, enhanced Cbl-b expression restricts multiple TCR signaling pathways, therefore serving as an efficient molecular target intertwined with many inhibitory signaling pathways. Recent reports suggest the importance of Cbl-b as the downstream target of PD-1 and CTLA-4 signaling pathways (Li et al. 2004 & Fujiwara et al. 2017). Li et al. highlight Cbl-b as the central molecule controlled by CD28 and CTLA-4 to tune the T cell activation threshold, where CTLA-4 signaling induced higher expression of Cbl-b (Li et al. 2004). Furthermore, while induction of PD-1 and CTLA-4 are known to restrict T cell activation, Cbl-b deficient T cells demonstrated resistance to both inhibitory signaling pathways (Li et al. 2004 & Fujiwara et al. 2017). Alongside the role of inhibitory checkpoint molecules in upregulating Cbl-b expression, Cbl-b itself promotes other inhibitory signaling pathways such as TGF-βR signaling. Cbl-b is a negative regulator of SMAD7, a protein known to abrogate TGF-β signaling, thus indirectly enabling TGF-β signaling (Gruber et al. 2013). Other potential regulatory mechanisms of TGF-β signaling include SMAD2 phosphorylation (Wohlfert et al. 2006). Consistent with this finding, Cbl-b KO CD4⁺ T cells did not respond to TGF-β signaling (Adams et al. 2010). Lastly, Cbl-b’s regulation of Foxo3a/Foxo1 activities plays an important role in the induction of Foxp3 and iTreg differentiation (Harada et al. 2010). In summary, with Cbl-b’s multi-faceted role in negatively regulating multiple TCR signaling pathways and interacting with multiple inhibitory signaling pathways, T cells deficient in Cbl-b demonstrate TCR sensitivity, increased cytokine production, CD28 independent stimulation and context-dependent TGF-β
insensitivity (Chiang et al. 2000 & Wohlfert et al. 2006). Such qualities make it an attractive therapeutic target to generate a robust pro-inflammatory response.

1.3.2 Physiological & Therapeutic implications

With emerging evidence highlighting the importance of Cbl-b in T cell activation, multiple studies have investigated its role at a physiological level and in disease settings. In humans, several groups discovered links between cbl-b genetic variants and susceptibility to various autoimmune diseases such as multiple sclerosis, asthma, type I diabetes and lupus erythematosus, with single nucleotide polymorphisms located at the promoter region (Bergholdt et al. 2005, Sanna et al. 2010, Doniz-Padilla et al. 2011 & DeWan et al. 2012). More specifically, reduced expression of Cbl-b in human CD4\(^+\) T cells was commonly discovered in multiple autoimmune diseases including systemic lupus erythematosus, most likely contributing to massive T cell proliferation and IL-2 secretion (Gómez-Martín et al. 2013 & Banica et al. 2014). In both humans and mice, while the precise mechanisms employed by Cbl-b-low T cells remain yet to be elucidated, other disease models in mice were studied to further our understanding of Cbl-b deficient T cell response in a physiological setting. Gronski et al. demonstrated Cbl-b’s role in controlling T cell activation threshold, as 100% of Cbl-b deficient P14/RIP-Gp transgenic mice developed diabetes when introduced with LCMV expressing the low-affinity ligand LF6 (in contrast to <50% achieving diabetes in P14/RIP-Gp mice) (Gronski et al. 2004). Additionally, Adams et al. demonstrated the role of Cbl-b in CD4\(^+\) T cells’ resistance to Treg cells through a graft-versus-host disease model, where Treg cells failed to suppress Cbl-b KO CD4\(^+\) T cell
activities in vivo (Adams et al. 2010). To date, the precise identity and phenotype of Cbl-b KO helper T cells remain controversial. Qiao et al. reported Cbl-b deficiency in the context of allergic airway inflammation, highlighting loss of Cbl-b facilitating T\(_h\)2 and T\(_h\)9 differentiation. More specifically, Cbl-b induced ubiquitination and subsequent proteosomal degradation of STAT6 through its TKB domain. Consequently, Cbl-b deficiency in CD4\(^+\) T cells promoted the development of T\(_h\)2 cells upon in vitro stimulation (Oh et al. 2011 & Qiao et al. 2014). Such findings were inconsistent with other research demonstrating a T\(_h\)1 response in Cbl-b-deficient T cells in vitro and in vivo. With no consensus achieved, further work is required to understand Cbl-b KO CD4\(^+\) T cell identity and mechanisms in healthy and pathological states.

Beyond the role of Cbl-b in autoimmune diseases, Cbl-b deficient T cells have been studied in the context of eliciting tumor immune surveillance and anti-tumor immunity. Cbl-b deficiency promoted effective immune surveillance in both genetically engineered and transplanted tumor models (Chiang et al. 2007 & Loeser et al. 2007). Using Cbl-b KO mice, Loeser et al. and Chiang et al. demonstrated increased CD8\(^+\) T cell infiltration in transplanted TC-1 and EL4/EG7 tumors. In both cases, CD4\(^+\) effector T cell infiltration did not increase. Interestingly, despite the increased infiltration of Tregs in the tumor of Cbl-b KO mice, T cells were able to either reject or attenuate tumor growth (Chiang et al. 2007 & Loeser et al. 2007). A similar observation was made when Cbl-b KO mice were crossed with ataxia telangiectasia mutated (ATM) KO mice, demonstrating a robust tumor rejection in vivo (Chiang et al 2007). Some of the proposed mechanisms include enhanced secretion of IFN-\(\gamma\) and insensitivity to TGF-\(\beta\). While Cbl-b deficiency promoted spontaneous rejection of TC-1 tumor, Cbl-b KO mice crossed with CD4Cre-SMAD\(7^{\text{fl/fl}}\) KO mice abrogated the overall anti-tumor immune response, thus highlighting the
importance of Cbl-b KO CD4⁺ T cells in anti-tumor immunity and the ability of Cbl-b deficient T cells to potentially overcome immunosuppressive signals generated in the tumor microenvironment (Gruber et al. 2013). To date, adoptive transfer of Cbl-b deficient T cells in tumor-bearing mice yielded inconsistent results across literature. While some demonstrated robust anti-tumor immune response using adoptive cell transfer of Cbl-b KO CD8⁺ T cells (Chiang et al. 2007), some were unsuccessful in generating anti-tumor T cell response against EG7 and B16-Ova tumors upon adoptive transfer of Cbl-b KO CD8⁺ T cells, and therefore resorted to a DC co-vaccination strategy to boost Cbl-b KO CD8⁺ T cell functions (Lutz-Nicoladoni et al. 2012).

While Cbl-b has demonstrated its potential as a promising therapeutic target in cancer immunotherapy, further understanding of Cbl-b biology is required for translational purposes. To date, Cbl-b targeting agents have been developed but clinical studies are yet to be published. Apeiron Biologics Inc.’s APN401 (siRNA against Cbl-b) has been developed for ex vivo usage in a phase I clinical trial and Progenra Inc. recently identified a selective molecular inhibitor against Cbl-b that targets substrate ubiquitination, identifying Cbl-b as an intracellular checkpoint inhibitor (Triozzi et al. 2015 & Agarwal et al. 2016). While Cbl-b inhibitors are currently under development and validation, existing therapeutic strategies may already target Cbl-b. For instance, regulation of Cbl-b by PD-1 and CTLA-4 checkpoint inhibition potentially contributes to the clinical benefits observed in patients undergoing the checkpoint inhibitor therapy.
1.4 Research objective & hypothesis

Despite the emergence of multiple studies characterizing the role of immunoregulatory molecules and their roles in mediating CD4\(^+\) T cell resistance against Tregs, no consensus was achieved in identifying the specific cellular mechanism. This study investigated the role of the E3 ubiquitin ligase Cbl-b, miR-155 and NFkB1 in CD4\(^+\) T cells’ resistance against Treg cells, to address the challenge. With multiple studies reporting enhanced pro-inflammatory cytokine secretion (IL-2, IFN-\(\gamma\), IL-4 and TNF-\(\alpha\)) by Cbl-b deficient CD4\(^+\) T cells and the role of pro-inflammatory cytokines in inducing Treg cell resistance, we hypothesized pro-inflammatory cytokines produced by Cbl-b deficient T cells, more specifically IL-2, renders T cells resistant to suppression by Treg cells.
CHAPTER II

2 MATERIALS AND METHODS

2.1 Mice

C57BL/6 Wild-type (WT), NFκB1 KO, and CD25 KO mice were purchased from The Jackson Laboratory. miR-155 KO mice were a kind gift from Klaus Rajewsky and Cbl-b KO mice in C57BL/6 background were a kind gift from Josef Penninger. All mice were maintained and bred under the guideline and policy set by UHN Animal Resource Centre. Mice used for experiments were 2 to 4 months old.

2.2 Cell Isolation

CD4+ T or CD8+ T cells were negatively selected from spleens and lymph nodes of mice using magnetic purification kit (CAT# 130-104-454 & 130-104-075, Miltenyi Biotec). BD Fluorescence-activated cell sorting (FACS) Aria was used to further separate un-stimulated naïve
T cells (CD4+CD25−) and Treg cells (CD4+CD25+). For purification of APCs, CD5 (Ly-1) MicroBeads were used to deplete CD5+ T and B cells (CAT# 130-049-301, Miltenyi Biotec).

2.3 In vitro T Cell Stimulation

All in vitro T cell stimulation was performed using anti-CD3 and irradiated APCs. Prior to co-culture, APCs were irradiated with a dose of 2500cGy using X-RAD 320 (PXi Precision X-Ray). Unless noted otherwise, purified CD4+CD25− T cells were stained with 10µM cell proliferation dye eFluor® 450 (CAT# 65-0842-90, eBioscience) in PBS for 20 min at 4°C. After three washes, 5 x 10^4 T cells were co-cultured with 2 x 10^5 irradiated APCs and 1µg/ml anti-CD3 Ab (Clone 145-2C11, CAT# 14-0031-85, eBioscience) in complete RPMI 1640 media (CAT# 11875119, Invitrogen) containing 10% FCS, 1% L-glutamine, 1% Penicillin-Streptomycin, and 57.2 mM 2-mercaptoethanol. Cells were incubated in Thermo Scientific™ Nunc™ MicroWell™ 96-well polystyrene microplates (CAT# 12-565-66, Fisher Scientific) in 5% CO₂ and 37°C incubation. Flow staining or cytokine collections were performed either on day 1 or 3 post-stimulation.

2.4 Treg Suppression Assay
Upon FACS sorting of naïve CD4+ T cells (CD4+CD25⁻) and Treg population (CD4+CD25⁺), purified naïve CD4⁺ (or CD8⁺) T cells were stained with 10 µM cell proliferation dye eFluor® 450 (CAT# 65-0842-90, eBioscience) in PBS for 20 min, followed by three washes in complete RPMI 1640 media (CAT# 11875119, Invitrogen). 5 x 10⁴ CD4⁺CD25⁻ T cells were subsequently cultured with 2 x 10⁵ irradiated APCs, anti-CD3 Ab (1µg/ml), and 5 x 10⁴ CD4⁺CD25⁺ Treg cells. For Treg suppression assay involving 1:1 to 8:1 Teff: Treg ratios, quantity of Treg cells were adjusted from 5 x 10⁴ to 6.25 x 10³ cells, respectively. For exogenous supplementation of IL-2 signaling inhibitors/agonist, recombinant IL-2 (CAT# 575404, BioLegend), recombinant IL-15 (CAT# 34-8153-82, eBioscience), anti-IL2 mAb (S4B6, CAT# 16-7020-85, eBioscience) and PC61 (anti-CD25 mAb generated by A. Elford, University Health Network) were used. Cells were incubated in Thermo Scientific™ Nunc™ MicroWell™ 96-well polystyrene microplates (CAT# 12-565-66, Fisher Scientific) in 5% CO₂ and 37°C incubation. Flow staining or cytokine collections were performed on day 1 or 3 post-stimulation unless noted otherwise.

2.5 Cytokine Analysis

Co-culture supernatants, stored in -80°C, were analyzed using IL-2 and IFN-γ ELISA kits (CAT# 88-7024-88, 88-7314-86, eBioscience). LEGENDplex™ Mouse Tₕ Cytokine Panel cytometric bead array (CAT# 740005, BioLegend) was used for T cell cytokine secretion profiling.
2.6 Surface/Intracellular Staining & Flow Cytometry

Individual cell suspensions were washed twice in FACS buffer (2% FCS and 0.05% sodium azide), followed by FcR blocking (30 min.) using anti-CD16/32 (CAT# 14-0161-85, eBioscience). For surface marker analyses, cells were subsequently stained with Abs for 30 min. on ice followed by two washes. The following antibodies were used for experiments: anti-CD25-PE (PC61), anti-CD122-PE (5H4), anti-CD4-APC (GK1.5), anti-CD8-APC/FITC (53-6.7) or anti-MHCII-APC-Cy7/FITC (M5/114.15.2), all purchased from eBioscience; and anti-MHCII-AmCyan (M5/114.15.2) was purchased from BioLegend. Anti-CD132-PE (TUGm2) was purchased from BD Biosciences. For all surface marker staining, cells were fixed using 4% PFA after washes.

Intracellular transcription factor staining was performed using Foxp3 Transcription Factor Staining Buffer Set (CAT# 00-5523-00, eBioscience) with the following antibodies: anti-CTLA-4-PE (UC10-489), Foxp3-PE-Cy7 (FKJ-16s), anti-T-bet-PE (eBio4B10) and anti-RORγt-PE (AFKJS-9), all from eBioscience; and anti-GATA-3-APC (16E10A23) was purchased from BioLegend. All data were acquired using BD FACSCanto™ II Flow Cytometer (BD Biosciences), and were analyzed on FlowJo software 7.6.1 (FlowJo LLC).
2.7 Statistical Analysis

Two-tailed paired Student’s t-test or 1-way ANOVA with Tukey’s Post-Hoc test was performed for comparisons. All data are presented as mean with standard error (n = 3) using GraphPad Prism 5 (GraphPad Software Inc.).
CHAPTER III

3 RESULTS

3.1 T cells that do not express Cbl-b are resistant to inhibition by regulatory T cells

While several studies have suggested that the absence of miR-155, p105 or Cbl-b resulted in T cells with a different sensitivity to regulation, the criteria that define Treg-resistance are not clear (Wohlfert et al. 2006, Stahl et al. 2009 & Chang et al. 2009). To further examine the importance of various molecules in Treg-resistance, in vitro Treg suppression assays were performed. MiR-155 KO, NFκB1 KO or Cbl-b KO CD4⁺CD25⁻ T cells were used to quantify their proliferative capacity as well as compare the ability of T cells from these mice to be suppressed. Consistent with Stahl et al.’s study using both murine and human CD4⁺ T cells (Stahl et al. 2009), miR-155 deficient CD4⁺ T cells appeared to be more sensitive to Treg-mediated suppression (Figure 2A). However, we also evaluated the division index (D.I.), the average number of cell division quantified in FlowJo software, and % suppression, as quantified by T cell proliferation in the presence of Tregs normalized to intrinsic proliferative capacity (% suppression = 100 – (D.I. samples/ D.I. without Tregs) x 100) (Collison & Vignali 2011 & McMurchy & Leving 2012). Our analysis showed that miR-155 KO CD4⁺ T cells have reduced
proliferative capacity by 39\% even without Tregs (Figure 2B). Taking this into account, no difference between % suppression of C57BL/6 and miR-155 KO CD4\(^+\) effector T cells was observed (Figure 2C). Therefore the absence of miR-155 alters T cell proliferation and not the ability of the T cells to be suppressed by Treg cells.

**Figure 2:** MiR-155 regulates CD4\(^+\) T cell proliferation but not Treg cell resistance. A. Proliferation of miR-155 KO CD4\(^+\) effector T cells in the presence of Treg cells, analyzed on day 3 post-stimulation. B. Quantification of miR-155 KO CD4\(^+\) T cell proliferation. Proliferation of effector T cells without Tregs was quantified based on the Division Index (D.I.) calculation by the FlowJo software. C. Measuring miR-155 KO CD4\(^+\)Foxp3 \(^-\) T cells’ cellular suppression by Tregs. Each CD4\(^+\) T cell’s intrinsic proliferation (B)
was used to normalize for the % suppression curve (% suppression = 100 – (D.I. samples/ D.I. without Tregs) x 100).

Similarly, while NFκB1 KO CD4+Foxp3− T cells appeared to be more suppressed in the Treg suppression assay (Figure 3A), the cells already had reduced proliferation when stimulated without Tregs (Figure 3B) and therefore no difference was observed between % suppression of C57BL/6 and NFκB1 KO CD4+ effector T cells (Figure 3C). Thus, miR-155 KO and NFκB1 KO T cells have a lower proliferative capacity and they are intrinsically not more sensitive to regulation.
**Figure 3:** NFκB1 regulates CD4⁺ T cell proliferation but not Treg cell resistance. **A.** Proliferation of NFκB1 KO CD4⁺ effector T cells in the presence of Treg cells, analyzed on day 3 post-stimulation. **B.** Quantification of NFκB1 KO CD4⁺ T cell proliferation. Proliferation of effector T cells without Tregs was quantified based on the Division Index (D.I.) calculation by the FlowJo software. **C.** Measuring NFκB1 KO CD4⁺Foxp3⁻ T cells’ cellular suppression by Tregs. Each CD4⁺ T cell’s intrinsic proliferation (B) was used to normalize for the % suppression curve (% suppression = 100 – (D.I. samples/ D.I. without Tregs) x 100).

Consistent with previous literature, Cbl-b KO CD4⁺ effector T cells were able to rapidly proliferate in the presence of Treg cells on day 3 post-stimulation (Figure 4A) (Wohlfert et al. 2004). In contrast, the absence of Cbl-b in Treg cells did not impact the ability of Treg to suppress T cell proliferation (Figure 4A). As Cbl-b KO CD4⁺ T cells intrinsically possessed enhanced proliferative capacity, quantified by an approximate 2-fold increase in its division index (Figure 4B), % suppression calculations were performed again to exclude the confounding variable. Cbl-b KO CD4⁺ T cells demonstrated significantly lower % suppression in any given ratio of Teff to Treg cells (Figure 4C). Thus, CD4⁺Foxp3⁻ T cells from Cbl-b KO mice were resistant to the inhibitory effects of Treg, whereas CD4⁺Foxp3⁻ T cells from miR-155 KO, NFκB1 KO and p105 KO mice only displayed altered proliferation.
Figure 4: Cbl-b KO CD4⁺ Foxp3⁻ T cells display Treg cell resistance. A. Proliferation of Cbl-b KO CD4⁺ effector T cells in the presence of Treg cells, analyzed on day 3 post-stimulation. B. Cbl-b KO CD4⁺ T cell’s intrinsic proliferation quantification. Proliferations of effector T cells were quantified based on the D.I. calculation by the FlowJo software. C. Measuring Cbl-b KO CD4⁺ Foxp3⁻ T cells’ cellular suppression by Tregs. Each CD4⁺ T cell’s intrinsic proliferation (B) was used to normalize for the % suppression curve.
3.2 Cbl-b deficiency promotes Th1 phenotype

Cbl-b deficiency promotes robust pro-inflammatory response capable of generating resistance against Treg-mediated suppression. While the general pro-inflammatory phenotype was consistently demonstrated across literature, the identity of the Cbl-b CD4⁺Foxp3⁻ KO T cells remains unclear, with each study reporting different findings of helper T cell subsets (Adams et al. 2010, Oh et al. 2011 & Qiao et al. 2014). Thus, Cbl-b KO CD4⁺ T cells were stimulated without any cytokine, and T cell differentiation was monitored through transcription factor staining. Here, we report Cbl-b KO CD4⁺ T cells to be positive for T-bet (Figure 5A). Furthermore, Cbl-b KO CD4⁺ T cells secreted significantly elevated quantities of IFN-γ (demonstrating max. 18-fold increase), suggestive of a strong T₉₁ response (Figure 5B). Together with pre-existing data in the literature, we confirmed that Cbl-b deficiency promotes T₉₁ differentiation from naïve CD4⁺ T cells.
Figure 5: Cbl-b KO CD4⁺ Foxp3⁺ T cells display T₁₁ phenotype. A. Cbl-b KO CD4⁺Foxp3⁺ T cells highly express T-bet. C57BL/6 and Cbl-b deficient CD4⁺ T cells were stimulated with anti-CD3 and irradiated APCs, and intracellularly stained for transcription factors representative of each T₃ lineages 3 days post-stimulation. B. Cbl-b KO CD4⁺ T cells secrete high quantity of IFN-γ. Supernatants from Treg suppression assay in Figure 4 were collected on day 1 and 3 post-stimulation for IFN-γ ELISA (eBioscience).

3.3 Cbl-b KO CD4⁺Foxp3⁻ T cells upregulate pro-inflammatory cytokines to mediate resistance against Treg cells
Despite Cbl-b KO CD4⁺Foxp3⁻ T cells demonstrating robust resistance against Treg cells, the precise cellular mechanism is yet to be elucidated. Previous works also highlighted Cbl-b deficient T cells’ ability to secrete higher quantity of pro-inflammatory cytokines including IFN-γ and IL-2 (Adams et al. 2010). Therefore, we further characterized the potential role of these soluble factors secreted by Cbl-b KO CD4⁺ T cells in the context of Treg resistance. Stimulated in the absence of Treg cells, Cbl-b KO CD4⁺Foxp3⁻ T cells’ cytokine secretion was profiled using cytometric bead array (CBA) and was compared with the C57BL/6 control T cells. The hyper-reactive Cbl-b KO CD4⁺Foxp3⁻ T cells secreted increased level of Th1 and Th2 cytokines including IFN-γ, IL-2, IL-4, and TNF-α (Figure 6A), consistent with enhanced T-bet expression upon stimulation (Figure 5A). To further explore which cytokines were elevated in the context of Treg suppression, we performed Treg suppression assays to collect supernatant to be used for CBA. Cbl-b KO CD4⁺ effector T cells with Treg cells secreted comparable or higher level of IFN-γ, IL-2 and TNF-α in comparison to C57BL/6 CD4⁺ effector T cells stimulated without Treg cells (Figure 6B), further indicating the availability of these cytokines during Treg-mediated suppression. Lastly, to determine whether the resistance to suppression by Treg was due to the soluble factors, we performed Treg suppression assays using C57BL/6 CD4⁺CD25⁺ T cells supplemented with supernatants collected from stimulated C57BL/6 or Cbl-b KO CD4⁺ T cells. Supernatants from Cbl-b KO T cells induced T cell proliferation in the presence of Treg cells, although limited by insufficient cytokine concentration for 3 days of culture (Figure 6C). Here, we report that Cbl-b KO CD4⁺ T cells secrete a diverse repertoire of pro-inflammatory cytokines such as IL-2, IL-4 and IFN-γ, playing an important role in Treg resistance.
Figure 6: Cbl-b KO CD4\(^{+}\)Foxp3\(^{-}\) T cells display enhanced pro-inflammatory cytokine secretion. A. Cytokine secretion profile of Cbl-b KO CD4\(^{+}\)Foxp3\(^{-}\) T cells. Supernatants from stimulated C57BL/6 or Cbl-b KO CD4\(^{+}\)Foxp3\(^{-}\) T cells were collected on day 3 post-stimulation, and LEGENDplex\textsuperscript{TM} cytometric bead array (BioLegend) was performed for cytokine measurements. B. Cytokine secretion profile with or without Treg cells. LEGENDplex\textsuperscript{TM} CBA (BioLegend) was performed to monitor IFN-\(\gamma\), IL-2 and TNF-\(\alpha\) secretion by Teff in response to Treg cells. C. Supernatant-induced Treg resistance. Supernatants were collected from stimulated C57BL/6 and Cbl-b KO CD4\(^{+}\)Foxp3\(^{-}\) T cells on day 3 post-stimulation, and were added to the 1:1 mix of C57BL/6 CD4\(^{+}\)Foxp3\(^{-}\) T cells and Tregs.
3.4 IL-2 hyper-secretion by Cbl-b KO CD4\(^+\) T cells mediate enhanced CD25 expression

One way that regulatory T cells have been reported to suppress effector T cells is via IL-2 consumption, leading to cytokine deprivation-mediated cell death (Pandiyan et al. 2007). We speculated that elevated secretion of IL-2 by Cbl-b KO CD4\(^+\) T cells may be responsible for sustaining T cell proliferation and that upregulation of the IL-2 receptor may also play a role in preventing Treg suppression by increasing the competitiveness to take up IL-2. Thus, we further characterized the effect of Cbl-b on IL-2 and IL-2 receptor expression, by performing IL-2 ELISA on supernatants from Treg suppression assays, and activating C57BL/6 or Cbl-b KO CD4\(^+\)CD25\(^-\) T cells without Treg cells for surface staining of CD25 on day 1 and 3 post-stimulation. In agreement with previous findings, Cbl-b KO CD4\(^+\) T cells hyper-secreted IL-2 in the presence or absence of Treg cells (Figure 6A, 7A), and displayed strikingly enhanced CD25 expression even at 1 day post-stimulation further suggesting that the T cells may have increased IL-2 uptake (Figure 7B).
**Figure 7:** Cbl-b KO CD4⁺CD25⁻ T cells upregulate CD25 in response to IL-2 during aberrant proliferation. 

A. IL-2 secretion by Cbl-b KO CD4⁺Foxp3⁻ T cells in the presence of Treg cells. Supernatants were collected from Treg suppression assay (Figure 4) on day 1 and 3 post-stimulation, and IL-2 ELISA (eBioscience) was performed. 

B. CD25 expression of stimulated C57BL/6 and Cbl-b KO CD4⁺Foxp3⁻ T cells. CD4⁺CD25⁻ T cells were stimulated for CD25 measurement on day 0, 1 and 3 post-stimulation.

A significant increase in CD25 expression (quantified by MFI) was observed by increasing TCR signaling using graded anti-CD3 concentrations or duration of stimulation, suggesting Cbl-b also regulated the threshold for IL-2Rα expression (Figure 8A, 8B).
Figure 8: Cbl-b KO CD4$^+$ T cells possess higher threshold for proliferation and CD25 expression. A. CD25 expression of C57BL/6 and Cbl-b KO CD4$^+$Foxp3$^-$ T cells in response to different level of TCR stimulation. B. Proliferation and CD25 expression kinetics. Upon CD4$^+$ T cell stimulation, proliferation and CD25 expression was measured using flow cytometry every 12 hours for 3 days.

Furthermore, when stimulated without Treg cells, Cbl-b deficiency or exogenous IL-2 supplementation specifically enhanced CD25 expression while CD122 and CD132 expression were kept at minimal expression (Figure 9A). The importance of CD25 in CD4$^+$ T cell activation was further investigated by stimulating C57BL/6 CD4$^+$ T cells with either IL-2 or IL-15. IL-15, able to interact with CD122 and CD132 but not CD25, induced low proliferation and CD25
expression (Figure 9B). Therefore, CD25 serves as the main IL-2 receptor upregulated in Cbl-b KO CD4+ T cells and together with IL-2 hyper-secretion, enhances T cell’s sensitivity to IL-2.

Figure 9: Cbl-b KO CD4+Foxp3 T cells selectively upregulate CD25 in response to Cbl-b deficiency or IL-2 supplementation. A. Expression of IL-2Rs upon Cbl-b deficiency or IL-2 exposure. CD25, CD122 and CD132 expression of Cbl-b KO or IL-2 supplemented CD4+Foxp3 T cells were measured on day 3 post-stimulation. B. Comparison of CD4+ T cells stimulated with or without CD25 interaction. Naïve C57BL/6 CD4+ T cells were stimulated and supplemented with IL-2 or IL-15, and proliferation as well as CD25 expression was measured 3 days post-stimulation.
3.5 IL-2 and CD25 upregulation by Cbl-b KO CD4$^+$ T cells counteract Treg suppression

To further explore the possibility that Cbl-b KO CD4$^+$ T cells overcome Treg suppression through increased IL-2 production and subsequent CD25 expression, we directly examined Treg cells and T cells during the Treg suppression assay. First, the levels of CD25 expression on CD4$^{+}$Foxp3$^+$ and CD4$^{+}$Foxp3$^-$ T cells were compared on day 0, 1 and 3 post-stimulation. Even in the presence of Treg cells, Cbl-b KO CD4$^+$ T cells expressed significantly higher level of CD25 on day 1 and 3 post-stimulation, but were still lower in comparison to the Treg cells suppressing them (Figure 10A). It was worth noting that on day 3 CD25 expression of Cbl-b KO CD4$^+$ effector T cells suppressed by Treg cells were still higher than that of C57BL/6 T cells stimulated without Tregs (Figure 10B), demonstrating Cbl-b KO CD4$^+$ T cells’ enhanced potential to capture IL-2 even in the presence of Tregs.
To confirm the direct role of IL-2 and CD25 on Treg resistant phenotype observed in Cbl-b KO CD4$^+$ T cells, a Treg suppression assay was performed with the addition of exogenous IL-2, anti-IL-2 or anti-CD25. IL-2 supplementation induced C57BL/6 CD4$^+$ T cell proliferation in the presence of Treg cell, and the addition of anti-IL2 or anti-CD25 on Cbl-b KO CD4$^+$ effector T cells reversed its hyper-proliferative state (Figure 11A). When quantified for Treg cell resistance, addition of IL-2 on C57BL/6 T cells induced approximately 5-fold decrease in % suppression, and the addition of anti-IL-2 or anti-CD25 on Cbl-b KO CD4$^+$ T cells resulted in 2-fold increase in % suppression (Figure 11B). As expected, IL-2 addition leads to increased CD25 expression on the T cells despite the presence of Tregs, whereas IL-2 or CD25 blockers showed the opposite effect (Figure 11C).
Figure 11: Enhanced IL-2 and CD25 serve as the key mediators of Treg resistance in Cbl-b KO CD4+ T cells. A & B. Treg suppression assay using exogenous IL-2 or IL-2 blockers. Treg and Teff co-cultures (at 1:1 ratio) were supplemented with IL-2 (20ng/mL), anti-IL2 (100µg/mL) and anti-CD25 (5µg/mL) on day 0 and T cell proliferation was measured on day 3 (A). D.I. calculations by the FlowJo software were subsequently used to generate % suppression measurement (B). C. CD4'Foxp3' T cells’ CD25 measurement in Treg suppression assay using exogenous IL-2 or IL-2 blockers.
Lastly, to demonstrate the importance of CD25 in Treg-resistant phenotype, Treg suppression assay was performed on C57BL/6 T cells with either exogenous IL-2 or IL-15 (lacking CD25 interaction). IL-15 did not decrease % suppression of C57BL/6 CD4<sup>+</sup>Foxp3<sup>+</sup> T cells co-cultured with Treg cells, further signifying the importance of CD25 in mediating Treg cell-resistance (Figure 12A). Accordingly, while IL-2 induces significant proliferation of effector T cells despite the presence of Treg cells, such effect was abrogated in CD25 KO CD4<sup>+</sup>Foxp3<sup>+</sup> T cells (Figure 12B). In summary, elevated secretion of IL-2 by Cbl-b KO CD4<sup>+</sup> T cells and subsequent CD25 surface-expression serve as an important cellular mechanism to counteract against IL-2 deprivation-mediated suppression by Treg cells.

**Figure 12:** Specifically IL-2Ra (CD25) plays important role in Treg resistance. A. The effect of IL-2 vs. IL-15 in Treg suppression assay. Exogenous IL-2 (20ng/mL) or IL-15 (20ng/mL) was added to the co-culture, and % suppression was quantified based on day 3 proliferation. B. The effect of IL-2Ra deficiency
in Treg resistance. Treg suppression assay was performed comparing C57BL/6 vs. CD25 KO CD4⁺Foxp3⁻
T cells. Proliferation was measured 3 days post-stimulation.

3.6 Cbl-b KO CD4⁺Foxp3⁻ T cells mediate Treg resistance
despite Treg expansion

While the overproduction of IL-2 and increased CD25 expression by Cbl-b KO CD4⁺ T cells contributed to the Treg resistant phenotype, the overall effect on Treg cells has yet to be explored. As such, a Treg suppression assay was performed using either C57BL/6 or Cbl-b KO CD4⁺CD25⁻ T cells. By gating on CD4⁺ population in the experimental set-up, we found that a higher proportion of CD25⁺Foxp3⁺ population was presented on day 1 post-stimulation and only a 2-fold decrease in % Foxp3⁺ T cells were observed with Cbl-b KO CD4⁺ T cells on day 3 post-stimulation (Figure 13A). Consistent with previous data, despite the inability for C57BL/6 Tregs to proliferate in vitro, Cbl-b KO CD4⁺ T cells were sufficient in partially driving Treg expansion in the Treg suppression assay (Figure 13B).
Figure 13: IL-2 secretion by Cbl-b KO CD4<sup>+</sup>Foxp3<sup>+</sup> T cells positively contribute to Treg expansion. A. Quantification of Treg and Teff population in Treg suppression assay. The effect of Cbl-b KO CD4<sup>+</sup> effector T cells on the proportion of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells were analyzed after gating on the CD4<sup>+</sup> population. B. Proliferation of Treg vs. Teff cells in 1 to 1 mix. Treg cells were also stained with proliferation dye to compare its responsiveness to C57BL/6 or Cbl-b KO effector T cells.

On day 3 post-stimulation, Cbl-b KO CD4<sup>+</sup> T cells increased the level of CD25, Foxp3 and CTLA-4 on Treg cells, which have previously been correlated with enhanced Treg functions (Figure 14A-C).
Figure 14: The effect of Cbl-b KO CD4+ T cells on functional markers of Treg cells. CD25 (A) and Foxp3 (B) expression of Tregs were quantified on day 0, 1 and 3 post-stimulation. Tregs’ CTLA-4 staining and measurements were performed 3 days post-stimulation (C).

Finally, we performed Treg suppression assay with either IL-2 or IL-2 blockers (anti-IL-2 or anti-CD25) and monitored Treg phenotype. IL-2 addition also increased CD25 expression on Foxp3+ T cells, and the use of IL-2 blockers decreased CD25 expression on Tregs (Figure 15). Overall, while IL-2 secreted by Cbl-b KO CD4+ T cells also expanded Treg cells, such expansion was still ineffective in restricting the effector T cells.

Figure 15: Exogenous IL-2 supplementation and Treg resistance also enhance Treg phenotype.

Treg suppression assay was performed using exogenous IL-2 (20ng/mL), anti-IL2 (100µg/mL) and anti-CD25 (25µg/mL), and CD4+Foxp3+ T cells’ CD25 expression were quantified on day 3 post-stimulation.
3.7 IL-2 hyper-secretion as the CD4\(^+\) T cell specific mechanism

While Cbl-b deficiency has been previously studied in the context of T cell activation and initiating a robust pro-inflammatory response, the distinct roles comparing Cbl-b deficient CD4\(^+\) or CD8\(^+\) T cells is under-appreciated. In our study, Cbl-b KO CD4\(^+\)Foxp3\(^-\) T cells overproduced IL-2 which subsequently played an important role in counteracting Treg suppression. To determine whether such a phenotype is also consistent in Cbl-b KO CD8\(^+\) T cells, we stimulated C57BL/6 or Cbl-b KO CD4\(^+\) and CD8\(^+\) T cells to directly compare their IL-2 and CD25 expression. Despite stimulation without Treg cells, Cbl-b KO CD8\(^+\) T cells expressed lower IL-2 and CD25 expression on day 3 post-stimulation, in comparison to C57BL/6 CD4\(^+\) T cells (Figure 16A, 16B). Therefore, we suggest our proposed mechanism to be specific to Cbl-b KO CD4\(^+\) T cells in isolated conditions.

**Figure 16:** Absence of Cbl-b leads to increased expression of IL-2 and CD25 predominantly in CD4\(^+\) T cells. A. IL-2 secretion by Cbl-b KO CD4\(^+\) vs. CD8\(^+\) T cells. Supernatants were collected 1 and 3 days post-stimulation (using anti-CD3 and irradiated APCs) for IL-2 and analyzed by ELISA (eBioscience). B. CD25 expression on C57BL/6 vs. Cbl-b KO T cells, day 3 post-stimulation.
CHAPTER IV

4 DISCUSSION

4.1 Exploring the role of Cbl-b deficiency and IL-2 in Treg resistance

T cell regulation plays an important role in various immunopathologies as well as the development of treatment strategies, thus multiple studies were directed at understanding resistance to Treg-mediated immunosuppression. Up to date, resistance to regulation by Treg cells has previously been observed in the context of intracellular immunoregulatory molecules (such as miR-155 and Cbl-b) and cytokine signaling pathways, but the underlying mechanisms behind Treg resistance have not been fully established. In this study focused on CD4+ T cells, we first explored the role of miR-155, NFkB1 and Cbl-b in determining T cells’ ability to escape suppression by regulatory T cells. Throughout the literature, Treg resistance was defined in various ways (in the form of proliferation measured by CFSE stain or ³H-thymidine incorporation) along with each using different quantification methods. In our study, miR-155 and NFkB1 deletion altered T cell proliferation but they had no influence over the suppression by Treg cells. We found that only Cbl-b played a role in Treg cell resistance. Mechanistically, we report that Cbl-b KO CD4+ T cells hyper-secrete IL-2 and the subsequent upregulation of CD25
increases T cell exposure and sensitivity to IL-2, counteracting IL-2 deprivation-mediated suppression by regulatory T cells (Figure 17A, 17B).

**Figure 17:** Proposed mechanism: Cbl-b KO CD4+ T cells promote Treg resistance through enhanced IL-2 production. A. Graphical illustration of wild type Treg and wild type Teff in a suppression assay compared with B. Cbl-b KO T effector cells co-cultured with wild-type Treg cells.

In conjunction with hyper-secretion of IL-2 by Cbl-b KO CD4+ T cells to reverse IL-2 deprivation-mediated suppression by Tregs, Cbl-b and IL-2 signaling pathways’ direct role in limiting inhibitory signals have been implicated in numerous studies, particularly in the context of restricting the TGF-β signaling pathway. Cbl-b directly ubiquitinates SMAD7, a negative regulator of the TGF-β signaling pathway, and therefore lack of Cbl-b results in robust pro-inflammatory T cell response even in the presence of TGF-β. Accordingly, SMAD7 has also been implicated in generating CD4+ T cells that are resistant to Treg-mediated suppression.
In response to Cbl-b deficiency, elevated IL-2 signaling further promotes PI3K/Akt, STAT1, STAT3 and STAT5 signaling pathways, which not only enhances T cell activation but may restrict inhibitory signals. For instance, SMAD7 can also be upregulated in response to JAK1/STAT1 signaling pathway (Nagarajan et al. 1999), and STAT3 was recently shown to interact with SMAD3 to restrict TGF-β signaling pathways (Wang et al. 2015). Thus, while prevention of IL-2 deprivation by Treg cells serves as an important mechanism in mediating Treg resistance, it may work in concert with previously described mechanisms, highlighting the multi-functional roles of Cbl-b and IL-2 and generating resistance to inhibitory signals.

Beyond the role of Cbl-b KO CD4+ T cells’ ability to induce IL-2-mediated Treg resistance, other pro-inflammatory cytokines also may play an important role. As demonstrated in Figure 6A, Cbl-b deficiency promotes elevated secretion of immunomodulatory cytokines including IFN-γ, IL-4, TNF-α and IL-17A. With multiple studies suggesting the role of diverse pro-inflammatory cytokines in inducing resistance to Treg cell-mediated suppression, other cytokines secreted by Cbl-b KO CD4+ T cells should not be neglected. With many cytokines sharing overlapping JAK/STAT and PI3K pathways, pro-inflammatory cytokines secreted by Cbl-b KO CD4+ T cells may amplify the proliferative response and T cell activation. Nonetheless, the use of anti-IL2 or anti-CD25 neutralizing antibody was sufficient in significantly reducing Cbl-b KO CD4+ T cells’ proliferative capacity (Figure 11A). Thus, within the diverse pro-inflammatory cytokines secreted, we report IL-2 to be one of the most potent players in inducing Treg cell resistance.
While Cbl-b deficiency mediates robust pro-inflammatory response and resistance to Treg-mediated suppression, Treg activities were also enhanced (Figures 13-15). Our observation that Treg also expanded under conditions where T cells developed resistance to Tregs was novel but perhaps not unexpected. The role of IL-2 in Treg expansion has been reported in numerous studies, where systemic injection of IL-2 also increases Treg activities (Lemoine et al. 2009 & Beyer et al. 2012). IL-2R signaling is crucial for STAT5-driven Treg cell function (Chinen et al. 2016), and because Cbl-b KO CD4+ T cells confer resistance through overproduction of IL-2, Treg cell expansion is indeed inevitable. Consistent with our model, when EL4 exogenous tumor cells were transplanted into the flanks of C57BL/6 or Cbl-b KO mice, Cbl-b deficient mice displayed higher tumor-infiltrating Tregs despite the increased anti-tumor immune response and overall survival (Chiang et al. 2007). With any form of pro-inflammatory T cell response, Treg enhancement is often inevitable. Thus, while enhanced quality and quantity of Treg cells are worth noting, the overall immunological phenotype is dependent on the balance between inhibitory and activating mechanisms, not strictly based on the number of cells.

4.2 Cbl-b and IL-2 mediated Treg resistance in disease settings

The role of Cbl-b in pathological conditions is poorly established. In humans, while defects in cbl-b was correlated with susceptibility to various autoimmune diseases such as type I diabetes and multiple sclerosis (Bergholdt et al. 2005 & Sanna et al. 2010), specific cellular and immunological mechanisms driving the pathological condition are yet to be elucidated. Evidence suggests hyperactive CD4+ T cells and IL-2 may play an important role in the pathogenesis of autoimmune conditions such as systemic lupus erythematosus (Gómez-Martín et al. 2013 &
Banica et al. 2014). In mouse, with recent papers outlining the role of Treg cells in inducing IL-2 deprivation-mediated T cell suppression \textit{in vivo} (Chinen et al. 2016), we hypothesize activation of Cbl-b deficient CD4$^+$ T cells to be sufficient in counteracting Treg cell-mediated suppression \textit{in vivo}. With most literature focused on exploring the role of Cbl-b in CD8$^+$ T cells or NK cells, further work is required to understand the CD4$^+$ T cells and their roles in mediating immune response in physiological conditions.

4.3 Translational potential of targeting Cbl-b in T cells for cancer immunotherapy

The Treg cell resistant mechanism employed by Cbl-b KO CD4$^+$ T cells in our study offers insights into the potential clinical applications for many immunological diseases. For instance, resistance to immunosuppression gained attention in the field of cancer immunotherapy, to generate robust T cell response that is more durable in the immunosuppressive tumor microenvironment. CD4$^+$ effector T cell activity and sufficient IL-2 exposure often serve as a pre-requisite for a robust anti-tumor CD8$^+$ T cell response, and prevention of T cell suppression that is established in the tumor microenvironment (Antony et al. 2005). As such, the ability of Cbl-b deficient CD4$^+$Foxp3$^-$ T cells to secrete massive quantity of pro-inflammatory cytokines (IL-2, IFN-$\gamma$, and TNF-$\alpha$), and its ability to overcome CTLA-4 and TGF-$\beta$ signaling supports the \textit{in vivo} therapeutic potential of Cbl-b KO CD4$^+$ T cells (Li et al. 2004 & Wohlfert et al. 2006). Thus, directly targeting Cbl-b in CD4$^+$ T cells may offer solution to generate a robust anti-tumor response and Treg cell-resistance.
Alternatively, the addition of high dose IL-2 may enhance existing adoptive T cell therapy by providing a protective mechanism against Treg function, while supporting T cell survival. ACT using tumor infiltrating lymphocytes, pioneered by the Rosenberg group, has demonstrated promising clinical outcomes in multiple malignancies including metastatic melanoma (Rosenberg et al. 2011). To promote TIL survival and function, multiple studies used the high dose IL-2 treatment regimen, resulting in tumor regression of 50% or more (Dudley et al. 2008, Besser et al. 2010, Radvanyi et al. 2012 & Pilon-Thomas et al. 2015). Furthermore, in the clinical investigation by Rosenberg et al., majority of the patients showing complete tumor regression showed ongoing complete regressions beyond 3 years (Rosenberg et al. 2012), further highlighting the clinical significance of the treatment strategy. The addition of high dose IL-2 treatment regimen after TIL therapy may render transferred TILs resistant to Treg suppression, while IL-2 treatment alone may be sufficient to avoid inhibitory effects of Treg cells. Such claim requires further validation, but demonstrates the significance of IL-2 in generating anti-tumor T cell response in the presence of Treg cells. Despite the superior tumor regression achieved through the high does IL-2 treatment regimen, it is also accompanied by toxicities including anemia and thrombocytopenia (Pilon-Thomas et al. 2015). Thus, the future treatment strategies should address the systemic side-effects often observed in high-dose IL-2 usage.

Lastly, despite the limited number of studies investigating Cbl-b in the context of cancer immunotherapy, Cbl-b may play a crucial role in currently existing immunotherapies. Our group has previously demonstrated the role of IL-7 in boosting immunotherapy through modulation of key immunoregulatory molecules including Cbl-b and subsequently rendering Treg cells
ineffective (Pellegrini et al. 2009). Alternatively, IL-2, also currently used in clinical trials in conjunction with adoptive T cell therapy, involves propagation of multiple T cell signalling pathways, one of which includes enhanced PLC-γ1, PKCθ, and PI3K-Akt activities. Interestingly, PKCθ plays an integral role in negative regulation of Cbl-b through poly-ubiquitination and subsequent degradation upon CD28 co-stimulation (Gruber et al. 2009). Thus, molecules known to modulate PKCθ activity may potentially down-regulate Cbl-b as part of their mechanism. While there exists preliminary evidence suggesting the indirect regulation of Cbl-b in several different immunotherapy strategies, more work is required to precisely validate the importance of Cbl-b in currently used immunotherapy. In summary, our studies have defined a mechanism to render T cells refractory to the inhibitory effects of Tregs while highlighting the potential to promote the immune response by IL-2 overproduction through targeting Cbl-b or other related signaling pathways within CD4⁺ T cells. Such a strategy could potentially be incorporated in many treatment modalities including the Chimeric Antigen Receptor (CAR) -T cell therapy or TIL therapy to generate robust anti-tumor immunity.
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