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TARGETING REGULATORY T CELL PLASTICITY WITH BCL-2 INHIBITION

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To my friends and family who have been with me through every step.

Thank you for your support.

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ABSTRACT

The specific BCL-2 small molecule inhibitor venetoclax has been shown to effectively induce apoptosis in a wide range of malignancies either alone and in combination with other drugs. Based upon these results, there has been considerable growth in its use in clinical studies used alone and in combination with chemotherapy and immune-based therapies. Lymphocytes, and T cells in particular, rely heavily on BCL-2 for survival and function. This has been determined largely by genetic deletion or overexpression of BCL-2 in murine models. However, the adaptive effects of short or long-term small molecule BCL-2 family blockade on surviving immune cell subsets and their function is not fully understood. In the current work, we aimed to better understand the effect of long-term systemic treatment with venetoclax on regulatory T (Treg) cells, which are relatively apoptotically resistant to specific BCL-2 drugging compared to other T cells. Our results indicate that long-term BCL-2 blockade by venetoclax results in Treg plasticity towards a T_H17-like functional state which aids in anti-PD-1 efficacy. Our data further highlights the importance of Treg instability on the efficacy of anti-PD-1 checkpoint blockade which will be beneficial for the clinical efficacy of immunotherapy.

CHAPTER 1

INTRODUCTION

1.1 Regulation of T cell ontogeny and homeostasis by the BCL-2 family

T cell development occurs largely in a special lymphoid organ called the thymus. Mature T cells are able to migrate from the thymus to the periphery and divide in order to maintain the T cell population outside the central lymphoid organs. Developing thymocytes pass through distinct phases in its journey to differentiate into a mature T cell, marked through changes in cell surface receptors like the CD3 complex, CD4, and CD8. T cell differentiation starts in the bone marrow with common lymphocytic progenitor (CLP) cells [1–3]. CLPs migrate from the bone marrow to the thymus via the bloodstream where they proliferate rapidly in a double negative (DN) ($CD4^-$ $CD8^-$) state. There are four stages of the DN T cell that are subgrouped by the expression of CD44 and CD25 on the cells: DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+$ $CD25^+$), DN3 ($CD44^-$ $CD25^+$), and DN4 ($CD44^-$ $CD25^-$) [1, 4]. Following the final DN stage, T cells begin to express $CD4^+$ and $CD8^+$ as they differentiate into the double positive (DP) stage. Maturation from the DP population to the mature single positive (SP) T cell populations involves positive selection of the T cell receptor alpha (TCRα) and T cell receptor beta (TCRβ) repertoire to ensure proper major histocompatibility complex (MHC) restriction and functionality, as well as negative selection to eliminate potentially autoreactive clones. During positive selection, thymocytes must be able to recognize MHC molecules to ensure that there is sufficient specificity for future recognition of antigen presenting cells (APCs) that is necessary to initiate activation signaling cascades and an immune response [1, 5]. Following positive selection, thymocytes must undergo negative selection which requires a low reactivity towards self-antigens [1, 5]. (Figure 1.1)

This differentiation process is tightly regulated by the BCL-2 family that mediate the

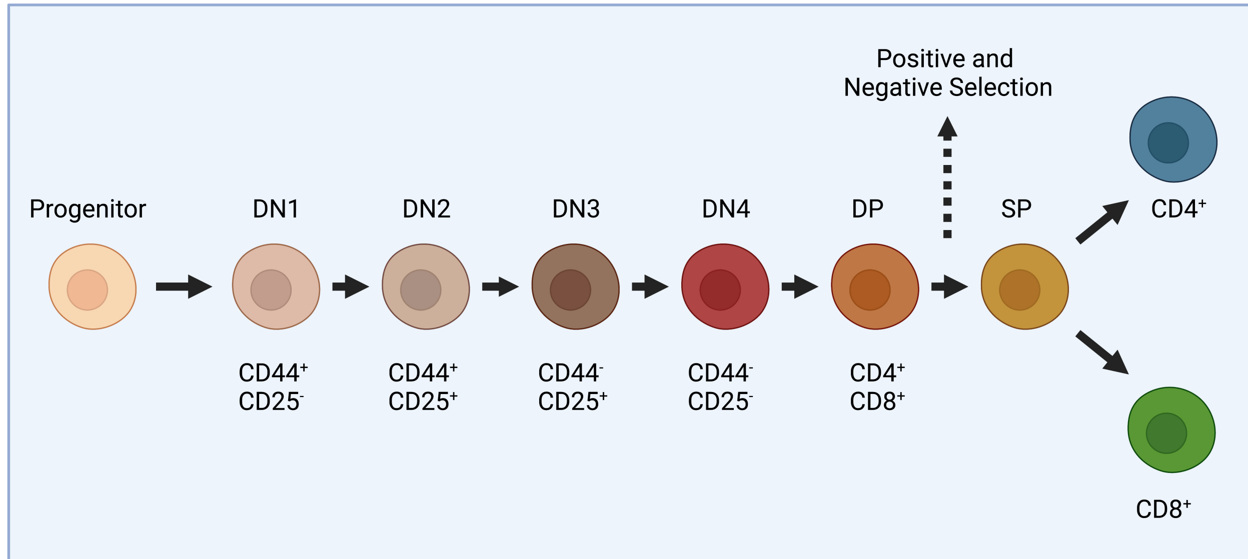

Figure 1.1: Early T cell differentiation occurs in the thymus. Common lymphocytic progenitor cells migrate from bone marrow into thymus where they proliferate through four double negative (DN) stages, cycling through differential expression of CD44 and CD25. Following the last DN stage, T cells will express both CD4 and CD8 and become double positive (DP) as they undergo a series a positive and negative selections to assess TCR affinity for MHC molecules and self antigens prior to become single positive (SP).

maturation through the DN stages, the positive and negative selections, and the differentiation into memory lymphocytes (Figure 1.2).

1.1.1 Discovery of BCL-2

BCL-2 was first discovered as part of the breakpoint region of the t(14;18) chromosomal translocation in B-cell lymphoma [6–8]. *Bcl-2* was the first gene discovered to prevent cell death and laid the groundwork for the identification of cell death mechanisms [8]. The identification of the function of BCL-2 also led to the discovery that cancer may develop by evading cell death rather than only inducing cell proliferation [8]. Since its discovery, multiple members of the same family have been identified, each falling within three main subgroups: multidomain anti-apoptotic, multidomain pro-apoptotic, or a third class defined by the presence of a single conserved death domain, BH3-region, called BH3-only proteins.

Canonically, the BCL-2 family are regulators of the intrinsic pathway of apoptosis, and it is these apoptotic signals that guide T cells through the differentiation from a common lymphoid progenitor to a mature T cell [9–11]. While the BCL-2 family consists of many proteins that each have a role in regulating T cell ontogeny, this section will focus primarily on these four players: BCL-2, MCL-1, BCL-xL, and BIM.

1.1.2 BCL-2 family roles in T cell differentiation and homeostasis

BCL-2, an anti-apoptotic protein, is shown to mediate thymic development, as *Bcl-2*$^{-/-}$ mice have smaller thymus and display immune defects due to an increase in apoptosis [11]. Further, the number of DN T cells are increased and DP T cells are reduced, which have downstream impacts on mature T cell numbers. This reveals that BCL-2 plays a direct role in the differentiation from a DN T cell to a DP T cell. Conversely, animals with BCL-2 overexpression develop autoimmunity similar to that measured in patients with systemic lupus erythematosus (SLE) [12]. Constitutive BCL-2 expression ultimately leads to increased numbers of immune cells including T cells, which also display increased longevity in culture owing to the anti-apoptotic properties of BCL-2 [12, 13]. In Tregs, *Bcl-2* was originally believed to the be the biggest contender in regulating cell survival, just as it does in conventional T cells. This was because of the dynamic expression of BCL-2 that Tregs exhibit as well as the accumulation of Treg cells in mice with forced BCL-2 expression [14]. However, it was eventually demonstrated that BCL-2 was not necessary for Treg survival.

MCL-1 also plays an important role in T cell ontogeny and homeostasis. Global MCL-1 knockout is embryonic lethal, lending to its role in hematopoietic stem cell survival [15, 16]. Animals with conditional deletion of *Mcl-1* in hematopoietic stem cells develop rapid bone marrow depletion accompanied by significantly reduced numbers of hematopoietic stem cells and other progenitor cells. T cell-specific *Mcl-1* deletion results in a significant reduction in T lymphocytes, as well as inhibition of thymocyte development at DN2/3 stage [17]. MCL-1

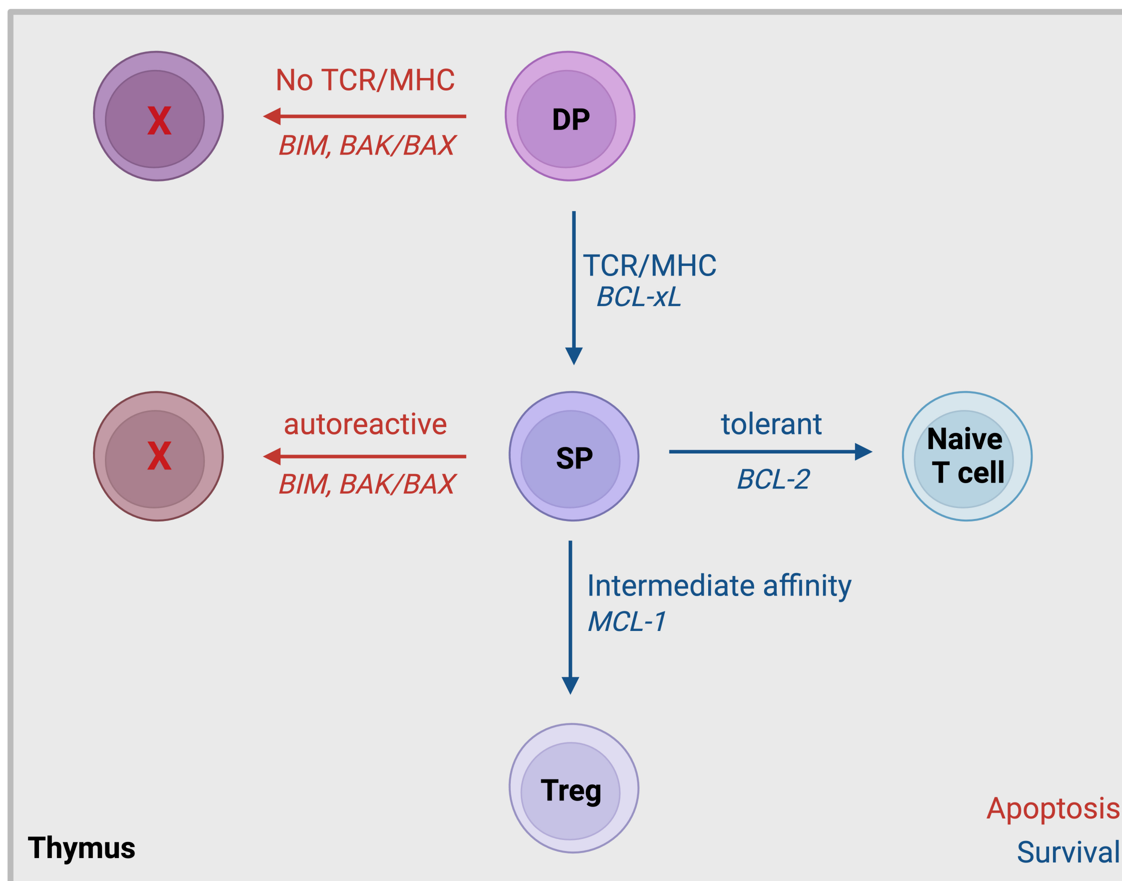

Figure 1.2: BCL-2 family regulates positive and negative selection processes during T cell differentiation. BCL-2 family regulates positive and negative selection processes during T cell differentiation. Cells that have no affinity for MHC molecules or are too autoreactive are selected against and undergo apoptosis via death signals by BIM, BAX, and BAK. Cells selected for survive through the anti-apoptotic proteins BCL-xL, BCL-2, and MCL-1. Single positive cells that are not self-reactive differentiate to become naive $CD4^+$ or $CD8^+$ T cells, while single positive cells that an an intermediate affinity to self-antigens become Tregs.

also plays a critical role in regulating Treg homeostasis, as mice with Treg-specific *Mcl-1* deletion experience global inflammation and autoimmunity [18]. Specifically, $Foxp3^{Cre}$Mcl-$1^{fl/fl}$ mice succumbed to fatal immunopathology, associated with hyper-IgE levels that were 100 times the normal concentrations, activation of $CD4^+$ and $CD8^+$ T cells and spontaneous differentiation into T_H1, T_H2 and T_H17 effector cells [18].

The role that BCL-xL plays in T cell homeostasis is not as well characterized. While loss of BCL-xL does not have a significant effect on overall T cell survival, deletion of BCL-xL in thymocytes was shown to decrease the differentiation of DN to DP T cells and ultimately reduce the number of DP thymocytes. T cells in the DP stage show sustained BCL-xL expression that is inversely correlated with BCL-2 expression, suggesting a specialized role for BCL-xL in this particular step of T cell differentiation [19]. However, these animals retain normal peripheral lymphocyte numbers, indicating that BCL-XL alone is not critical for lymphocyte homeostasis [19].

The final major component of this process is BIM, a BH3-only protein. BIM has been characterized as the most important regulator of apoptosis in T cell development, particularly in the elimination of autoreactive lymphocytes. *Bim* deficient mice display resistance to a number of apoptotic stimuli, including dexamethasone, γ-irradiation, cytokine deprivation, and ionomycin [11, 20]. Contrary to *Bcl-2* knockout, global and hematopoietic Bim deletion results in an accumulation of DP and SP T cells. This suggests that negative selection, a process that eliminates potentially autoreactive T cells, has been disrupted due to the missing apoptotic signals from BIM. Loss of BIM also leads to an accumulation of DN thymocytes that appear to have reverted back to double negative stage following both positive and negative selection [21]. The overall disruption of the selection process leads to an increased prevalence of autoimmunity. BIM has also been implicated in Treg homeostasis, particularly in the age-dependent accumulation of Tregs. Aged Tregs exhibit decreased expression of BIM compared to Treg from young mice, which causes rapid accumulation of Tregs [22].

Low expression of BIM in Tregs favors their survival and proliferation, which may be a leading cause of age-related immunosuppression.

The evidence that has been presented together show the importance of the BCL-2 family in regulating T cell development and survival. Thus far, there has been little evidence to show what role this family of proteins may be playing in regulating T cell function. However, non-canonical roles of the BCL-2 family are emerging that may suggest their importance in not only regulating cell survival, but also, the function of different T cell subsets. We will discuss these non-canonical roles in a later section.

1.2 Categorization of mature T cell subsets

Following thymic development, mature, naive T cells migrate into the periphery where they play a large role in adaptive immunity. T cells are large divided into three main subgroups: $CD4^+$ $FOXP3^+$ regulatory T cells (Tregs), $CD4^+$ $FOXP3^-$ conventional T cells (Tcons) and $CD8^+$ T cells. Tregs are differentiated in the thymus as a result of upregulation of the transcription factor FOXP3 and an intermediate affinity towards self-antigens. This mid-level affinity is greater than those of a Tcon, but low enough to escape the threshold for negative selection [23]. Treg cells have the ability to suppress effector T cells and to dampen a wide spectrum of immune responses, including those that contribute to autoimmunity. Tcons are a type of effector T cell, also known as a helper T cell. They regulate the immune response through antigen recognition and cytokine secretion to recruit other immune cells. $CD8^+$ T cells are a type of effector T cell called a cytotoxic T cell and are also able produce cytokines such as IFN-γ and TNF-α [24, 25]. However, unlike Tcons, $CD8^+$ T cells are also able to produce cytolytic molecules like granzyme B and perforins that directly induce apoptosis of its target cell [24, 26].

$CD4^+$ Tcons and $CD8^+$ T cells are categorized into naive or memory cells dependent on antigen exposure, where naive cells have not seen antigen and upregulate a collection

of extracellular markers following antigen exposure. These cell surface activation markers include CD44 and L-selectin (CD62L), CD45RA, and the chemokine receptor CCR7. In mice, CD44 and CD62L alone are able to distinguish between naive ($CD44^-$ $CD62L^+$) (T_N), central memory ($CD44^+$ $CD62L^+$) (T_{CM}), and effector memory ($CD44^+$ $CD62L^-$) (T_{EM}) subsets [27]. Cells expressing CD62L are reported to more readily proliferate in response to antigen recall [28], and readily distinguish the T_{EM} cells that present an immediate but not sustained response from the T_{CM} that sustain the immune response by homing to and proliferating in the secondary lymphoid organs to produce a supply of new effector T cells [29]. Human memory subsets are defined by additional markers with more complexity (Figure 1.3) [29–32]. Naive T cells in humans are defined as $CCR7^+$ $CD45RA^+$ [24]. These cells have multidirectional differentiation ability dependent on the microenvironmental signals and antigens present [33]. A special subset of $CCR7^+$ $CD45RA^+$ have emerged as stem cell memory T cells (T_{SCM}), defined by their expression of CD95 (Fas/APO-1). T_{SCM} cells have self-renewal properties and multipotency, representing the least differentiated memory T cell subset. These cells have been observed to have increased proliferation, survival, and anti-tumor activity compared to conventional memory T cells, and have great potential in overcoming current challenges seen in T cell based therapies [34, 35]. $CCR7^+$ $CD45RA^-$ identifies T_{CM} cells, that coexpress CD62L and are functionally distinct from the $CCR7^-$ $CD45RA^-$ T_{EM} cells [31]. Like in mice, human $CCR7^+$ $CD45RA^-$ T_{CM} cells home to secondary lymphoid organs and exhibit long lasting immune memory. T_{CM} cells have the second lowest degree of differentiation next to T_{SCM} cells, and display lower expression of effector molecules [34, 36]. Both T_{CM} cells and T_{EM} cells also express the activation marker CD45RO, which indicates that a T cell has encountered antigen. The final population of memory cells that have been characterized in humans are $CCR7^-$ $CD45RA^+$ and called terminally differentiated effector memory cells (T_{EMRA}) [30, 32, 33]. T_{EM} and T_{EMRA} cells also express a different set of chemokine receptors such as CXCR3 that allow them to migrate

into inflamed tissues and exert immediate effector function [32].

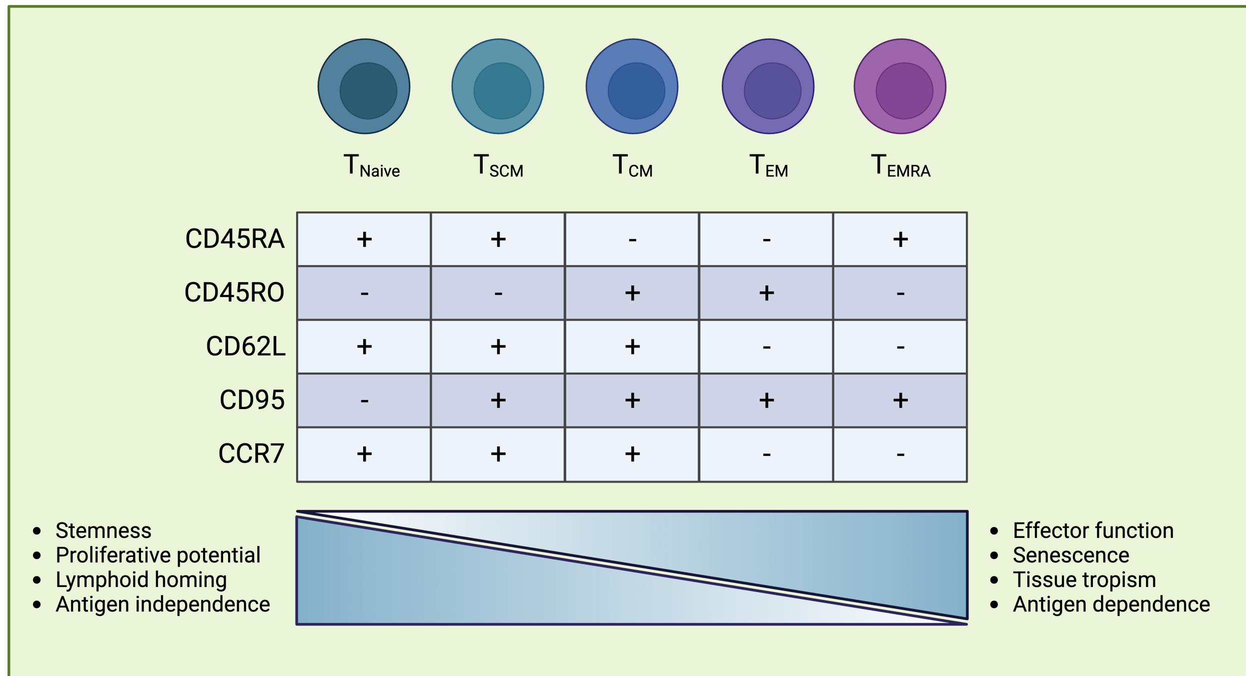

Figure 1.3: Human memory T cell compartments. While murine memory T cells can be distinguished with markers CD44 and CD62L, the distinction in humans is much more complex. At minimum, human memory T cells can be defined by expression of CD45RA, CCR7, and CD95, which can distinguish between T_N, T_{SCM}, T_{CM}, T_{EM}, and T_{EMRA} cells. Additional markers such as CD45RO and CD62L further delineate the antigen dependence, stemness, and proliferative capacities of T cells.

Another important facet of T cell differentiation that can occur concomitantly with memory status is lineage commitment [37]. Upon antigenic stimulation, $CD4^+$ $FOXP3^-$ effector T cells differentiate into more specific T_H subsets that each facilitate a different type of immune reaction [24]. Table 1.1 summarizes the main functional context, key cytokines produced and the primary transcription factor regulator of each group of these $CD4^+$ $FOXP3^-$ Tcons. In the presence of IFN-γ and IL-12, naive $CD4^+$ T cells differentiate towards a T_H1 state dependent on the the transcription factors T-bet and STAT4 [38–40]. Similarly, the presence of IL-4 in the milieu polarizes naive Tcons to a T_H2 state in conjunction with transcription factors GATA3 and STAT6 [38–40]. Cytokines secreted by T_H2 are important for the induction of humoral immune responses clearing extracellular pathogens and parasites through the

induction of immunoglobulin class switching. Finally T_H17 cells are differentiated through expression of transcription factors RORγt and STAT3 [38–40]. This particular subtype was more recently discovered and the exact role that it plays is still unclear. However, it is known that T_H17 cells can be quite pro-inflammatory through secretion of cytokines IL-17A and IL-22, which have been implicated in the pathogenicity of autoimmune diseases [38].

Table 1.1: T cell subtypes

Cell Type	**Primary Function**	**Cytokines**	**Transcription Factors**
T_H1	Cell mediated immunity (intracellular bacteria, viruses)	IFN-γ and TNF-α	T-bet, STAT4
T_H2	Humoral immunity (extracellular parasites)	IL-4, IL-5, and IL-13	GATA3, STAT6
T_H17	Cell-mediated inflammation and autoimmunity	IL-17A, IL-17F, and IL-22	RORγt, STAT3

As previously defined, Tregs also represent a population $CD4^+$ T cells whose differentiation occurs in the thymus. Unlike Tcons, Tregs are thought to be terminally differentiated and do not further divide into subsets, although there is growing evidence to suggest a degree of plasticity in Treg identity. Moreover, Tregs are not classically defined by memory status, although there is evidence to suggest that expression of activation markers and chemokine receptors are able to further stratify the functional state of Treg cells. A 2017 study reported the expression of CD45RA, CCR4, CXCR3, and CCR6 are able to distinguish four T_H-like subsets of Tregs [41]. These T_H-like Tregs still maintain FOXP3 expression, while simultaneously expressing transcription factors listed and cytokines listed in Table 1.1. The discovery that Tregs are able to become functionally similar to a T_H effector cell provided evidence towards a hypothesis that T cells have features of plasticity and has shifted the way we think about Treg function and holds significant implications in the context of human disease.

1.3 Discovery of T cell plasticity

The mature T cell lineages defined in the previous section started with the discovery of T_H1 and T_H2 subsets in the 1980s [42]. With the discovery of each new T cell subset, we gained new insight into how these cells can be characterized. These initial observations were groundbreaking as it established a paradigm that T cells further differentiate to acquire specific functions. However, this led to a firm belief that T cells were terminally differentiated. Each subset was distinct, programmed by the expression of unique transcription factors and functioned by producing specific cytokines and chemokine receptors to control different pathogens [42]. However, as technology advanced and more work was completed on these T cell subsets, it was revealed that T cells actually have the ability to alter their phenotype and repolarize towards a different lineage which fueled the hypothesis that rather than being a static population, T cells exhibit plasticity that allow them to change their fate. This has particularly been observed in T_H17 cells and Tregs, and has shifted the paradigm from "lineage stability" to "plasticity" [42].

Plasticity in T cells is defined as the ability of a single T cell to take on the phenotype and function of a different T cell subset. Reprogramming of T cells can be easily accomplished *in vitro*, seen through the polarization of $CD4^+$ $FOXP3^-$ Tcons into induced Tregs (iTregs) in specific culture conditions [43]. iTregs are also found naturally *in vivo* showing this is a physiologically relevant system. The induction of Tregs from Tcons is largely a cytokine and microenvironment-mediated phenomenon. Figure 1.4 depicts T cell plasticity under various cytokine-polarizing conditions. In studies investigating the role of FOXP3 in Treg induction, it was discovered that many hallmarks of Tregs, including expression of CD25, CD44, CTLA-4, GITR, and ICOS were all independent of FOXP3, while suppressive function and stability were FOXP3-dependent, suggesting there are elements of Treg identity that are not linked to FOXP3. This finding warrants further exploration into which facets of Treg plasticity are mediated by FOXP3, and which are not. $CD4^+$ $FOXP3^+$ Tregs have been found to

differentiate into IL-17A-producing cells when stimulated with allogeneic APCs, particularly monocytes, in the presence of IL-2 and IL-15 [44]. These Tregs will upregulate the T_H17 transcription factor RORγt and can be characterized by CCR6 expression. The effects seen in this study were inhibited by histone deacetylase (HDAC) inhibitor trichostatin A (TSA), suggesting a potential dependence on histone/protein deacetylase activity for this plasticity to occur [44].

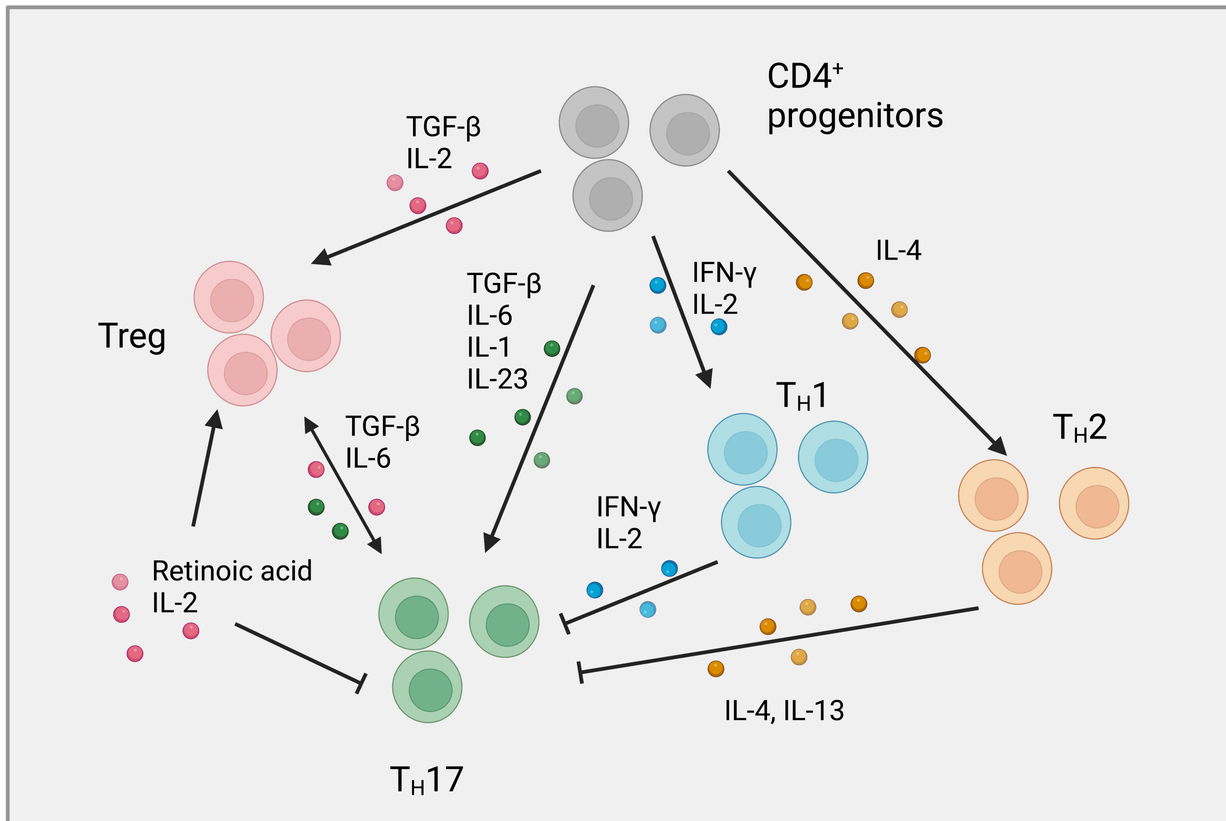

Figure 1.4: Environmental cues polarize T cells into different subsets. Under various cytokine conditions, T cells that have previously undergone differentiation and lineage commitment are able to further polarize into different T cell subsets and adopt characteristics of these subsets in a phenomenon called T cell plasticity.

The onset of plasticity is mediated by signaling at a multitude of levels outside of cytokine signaling, including cytosolic signaling, metabolism, and epigenetic/chromatin regulation (Figure 1.5). TCR ligation drives the activation of transcription factors such as activator

protein 1 (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB). Differences in TCR signaling intensity can drive $CD4^+$ into various differentiation states by tuning the receptiveness of a cell to different cytokines. PI3K signaling also plays a central role in processing extracellular cues and signal transduction into the cell. PI3K–AKT pathway is a dividing point for Tcon versus Treg differentiation, as the activation of this pathway is required for the polarization and function of Tcon lineages but is largely repressed in Tregs [42] (Figure 1.6). In Tregs, the PI3K signaling pathway is blunted by the activation of PTEN which inhibits AKT activity and rewires Treg response to IL-2 to selectively activate STAT5 which promotes FOXP3 transcription [45, 46]. PTEN deficiency has been observed to compromise Treg stability and result in their conversion to T_H1 or T_H17 cells [47, 48]. The receptor neuropilin 1 (NRP-1), which is highly expressed on Tregs, recruits PTEN to the immunological synapse and to block AKT activation and promote Treg stability. *Nrp-1* deficiency in Tregs has been shown to promote Treg instability into a T_H1-like Treg [49] (Figure 1.6). In Treg cells, expression of IFNγR has also been associated with the onset of T cell plasticity, where deletion of the receptor results in the inability of the Treg cell to update IFN-γ to result in stability of its identity [50] (Figure 1.6)

The PI3K pathway also sits closely at the center of metabolic regulation which plays a role in T cell plasticity. Following exposure to antigen, T cells shift their metabolic dependencies in order to fulfill the requirement of rapid proliferation and functional energetic needs. CD28 signaling directly controls the metabolic switch to glycolysis during conventional T cell activation through the upregulation of glucose transporter 1 (GLUT1) in a PI3K–dependent manner [51, 52]. Conversely, Tregs do not utilize glycolysis largely in part due to the inhibition of PI3K signaling, and rely heavily on fatty acid oxidation and oxidative phosphorylation [53–55]. Glutamine is also an important metabolite that shifts the balance between T_H1 and Tregs through the generation of α-ketoglutarate (α-KG), which is required for T_H1 cells but blocks Treg cell differentiation [56]. PI3K also regulates the

activity of HIF1α which induces the expression of genes required for glycolysis. HIF1α plays an important role in T_H17 cell polarization where in addition to promoting glycolysis, it also directly induces the expression of RORγt [57, 58].

The final component of T cell plasticity regulation occurs at the level of the chromatin. Histone modifications and DNA methylation can alter the accessibility of DNA to transcription factors. Chromatin reorganization creates access to key differentiation loci. Because plasticity is driven by a network of changes and not by a single protein or transcription factor, it is important to assess global alterations [59]. Key transcription factors such as STATs may help stabilize chromatin structure by recruiting chromatin remodelling enzymes and histone methyltransferases (HMTs) [60, 61]. In Tregs, DNA methylation of *Foxp3* by recruitment of TET proteins is necessary for induction and maintenance of FOXP3 expression [62]. Histone acetyltransferases (HATs) such as CREBBP and p300 are also necessary to keep the *Foxp3* locus accessible. Without these enzymes, Tregs lose FOXP3 expression and gain IL-17A [63]. Similarly, disruption of DNA methyltransferase 3a (DNMT3a) also mediates FOXP3 expression in Tregs and Tcons [64, 65].

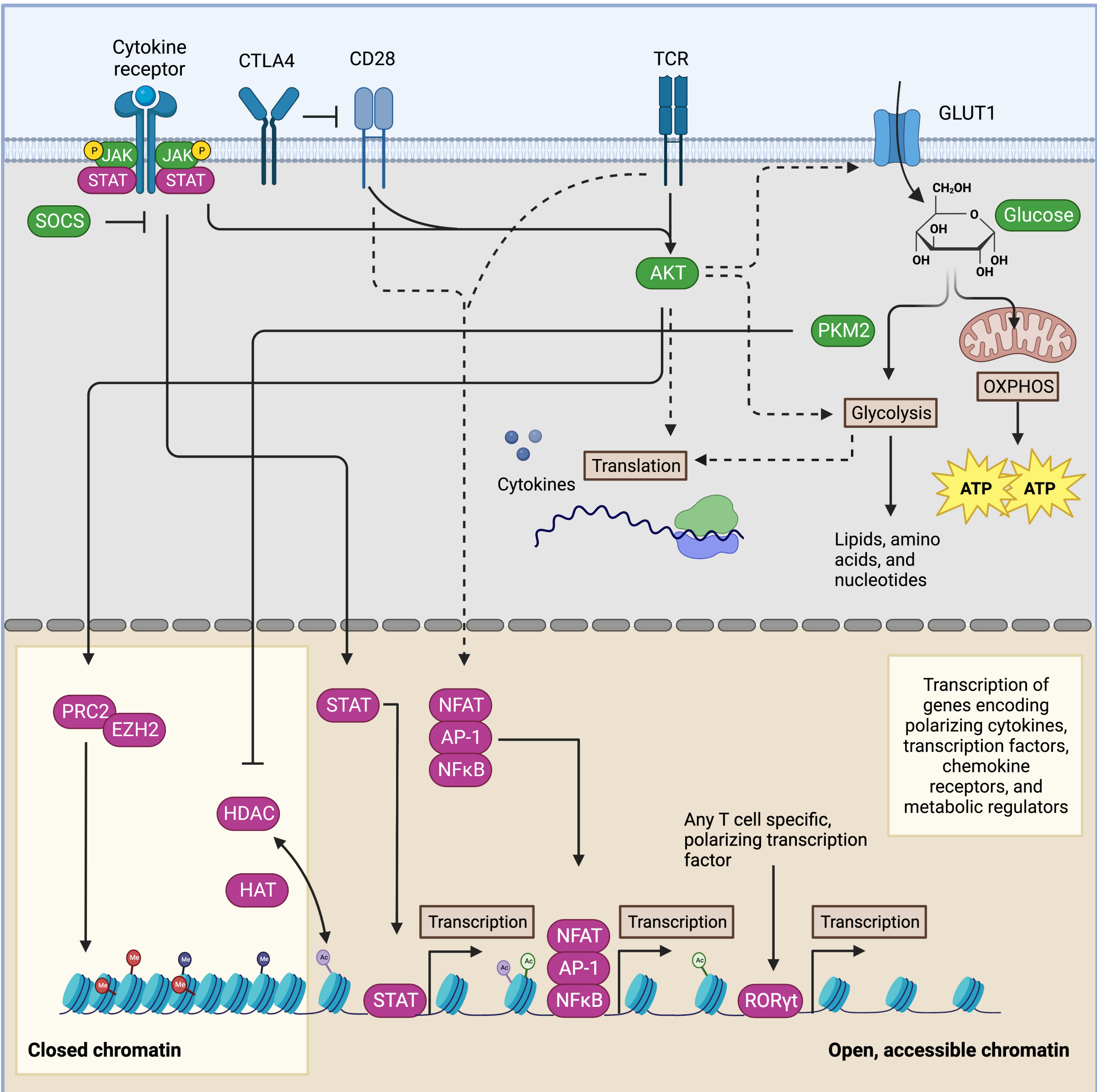

Figure 1.5: T cell plasticity is mediated by the integration of an array of signals. T cell plasticity requires a multitude of interconnected signals that work together to drive alterations. Broadly, signaling transduction initiates following extracellular receptors (blue). Protein-protein interactions are denoted with solid lines while indirect associations are indicated in dashed lines. Cytosolic proteins and molecules (green) mediate a complex network of signaling pathways. Due to the multitude of roles proteins can play in cellular processes, these pathways converge and are interconnected. Transcription factors and epigenetic regulators (purple) dictate gene transcription to mediate expression of factors necessary for T cell polarization.

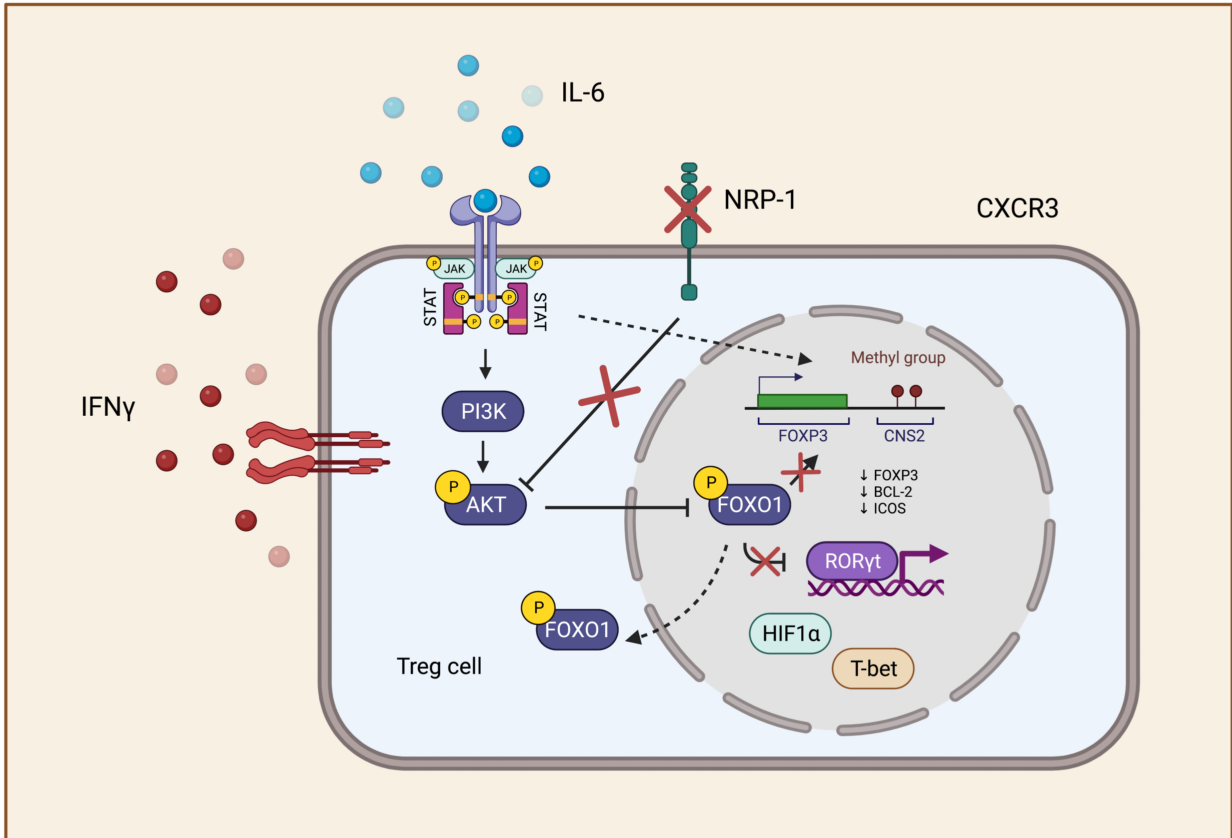

Figure 1.6: Primary mechanisms of Treg plasticity. At the level of the receptor regulation, Treg plasticity has been shown to be mediated by JAK/STAT/IL-6, NRP-1, and IFNγR signaling. In the cytoplasm, signal transduction primarily through PI3K activation has been implicated in dysregulating the balance of Tregs and T_H effector subsets through the regulation of transcription factor expression.

Currently, most of the work done regarding T cell plasticity has been conducted in $CD4^+$ T cells. However, there is evidence to suggest that $CD8^+$ T cells also have the capacity to exhibit plasticity [66]. Further, the existence of $CD8^+$ $FOXP3^+$ T cells have also been described, with opposing evidence on its specific role within different disease contexts. It is evident that there is still much we have yet to understand about T cell plasticity and their varying functions.

1.4 Tregs mediate immune tolerance through suppressive function

Canonically, Tregs are able to exert their role as mediators of peripheral tolerance through a variety of direct and indirect suppression mechanisms (Figure 1.7). Tregs can produce anti-inflammatory cytokines such as IL-10, IL-35, and TGF-β which inhibit effector cell response [67–69]. In addition, they release perforin and granzyme, which damage target cell membrane of effector T cells or antigen presenting cells leading to apoptosis [70]. Tregs are also defined by their high expression of CD25, or the IL-2 receptor, and can thereby sequester IL-2 from the microenvironment to reduce effector T cells proliferation. IL-2 starvation also prevents natural killer cells (NK cells) from proliferating and exhibiting effector functions [71, 72]. Tregs also express a variety of co-inhibitory molecules on its cell surface, such as CTLA-4, LAG-3, and PD-L1 [73]. Tregs have been shown to have a direct effect on B-cells via PD-L1/PD-1 binding and dendritic cells (DCs) via interaction through CTLA-4 and LAG-3. Specifically, CTLA-4 can suppress the co-stimulatory functions of DCs through downregulation of CD80/86 and induces upregulation of indoleamine 2,3-dioxygenase (IDO) which consequently results in inhibition of effector T cell proliferation and T cell anergy [74, 75]. Similarly, LAG-3 inhibits DC maturation through engagement of MHC II [76]. The expression of CD39 and CD73 ectoenzymes on Tregs together facilitate conversion of adenosine triphosphate (ATP) to adenosine and reduce effector proliferation through engagement with $A_{2A}R$ [77]. Tregs can also polarize monocyte populations toward M2 macrophages and prevent their differentiation in pro-inflammatory M1 macrophages [78].

However, the identification of T cell plasticity now changes the way Treg function is viewed. While a canonical Treg is able to exhibit the functions listed above, questions remain on how these functions are changed in a unstable Treg. These questions include whether or not Tregs can exist in a hybrid state of both suppression and inflammation, what the threshold of functional plasticity is, and how these plastic Treg functions under different disease conditions. Working towards finding answers to these questions will greatly improve

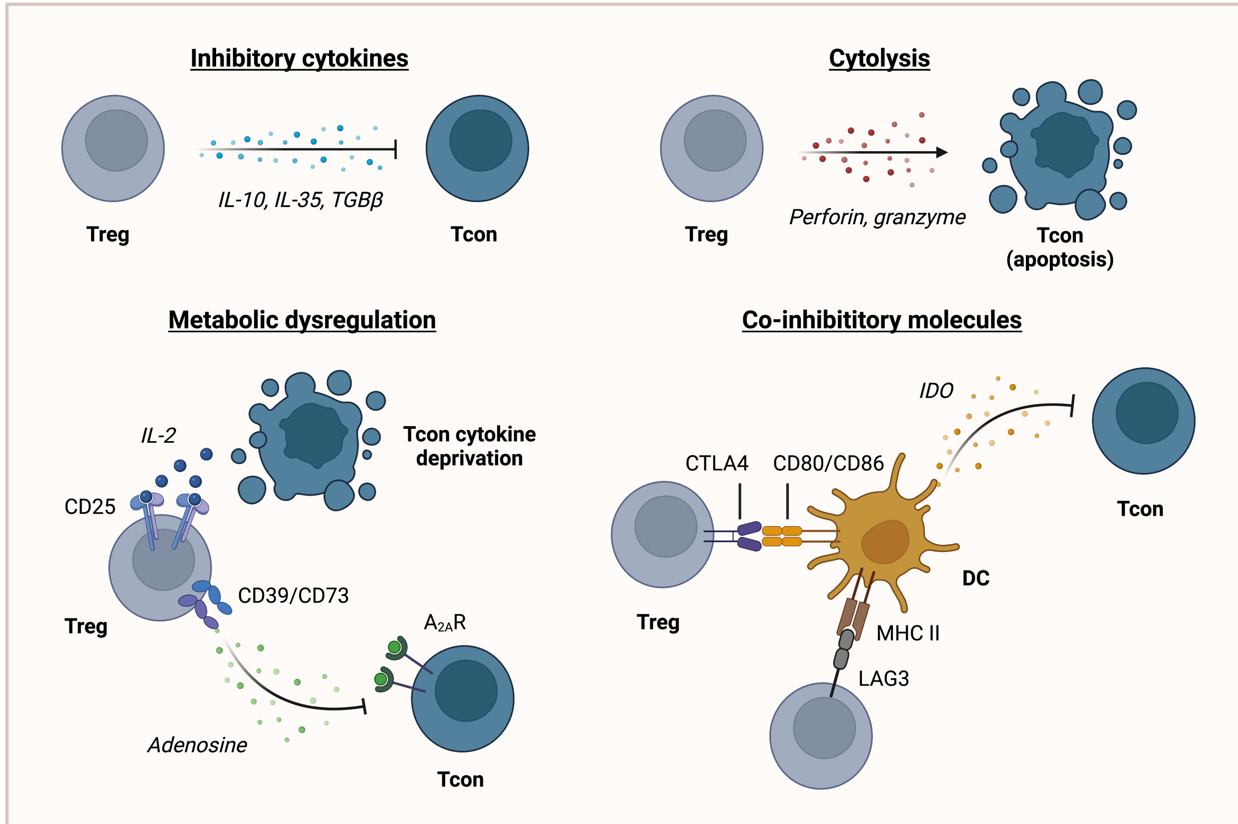

Figure 1.7: Mechanisms of Treg suppression. Tregs exert their immunosuppressive functions through a variety of mechanisms, including secretion of suppressive cytokines, cytolysis, disruption of effector cell metabolism, and expression of co-inhibitory molecules.

our understanding of the role of Tregs in various diseases such as autoimmunity and cancer, and allow us to better harness the functional power of these cells for therapeutic benefit.

1.5 Tregs in autoimmunity versus cancer

Due their ability to suppress the immune response, Tregs are essential for preventing autoimmune and chronic inflammatory diseases. These cells migrate primarily into inflammatory sites to inhibit effector lymphocyte function [79, 80]. Autoimmune disorders are caused by a lack of tolerance against self-antigens, a process mediated by Tregs. In mouse models of Treg deficiency, there is significant spontaneous autoimmune disease development, highlighting that Treg dysfunction is a common denominator. Studies in multiple autoimmune

disorders including multiple sclerosis, SLE, and inflammatory bowel disease (IBD) all reveal reduced Treg numbers coupled with impaired suppressive function [81]. Loss of FOXP3 expression has also been observed to contribute to autoimmunity and may arise as a result of the inflammatory environment that the cells are in. For example, in diabetes, a large percentage of cells displayed unstable expression of FOXP3 in inflamed tissue [82]. These cells can secrete inflammatory cytokines and induced onset of diabetes following adoptive transfer. These inflammatory cytokines may be implicated in mediating epigenetic modifications that contribute to *Foxp3* instability. In particular, TLR-2 simulation induced IL-17A production mediates histone deacetylase activity in Tregs from patients with multiple sclerosis [83]. The acquired ability for Treg cells to secrete inflammatory cytokines such as IL-17A alludes to its plasticity and fragility. The best characterized T_H-like Treg subset in the context of autoimmunity is T_H1-like Tregs. These cells upregulate transcription factor T-bet as well as chemokines CCR5 and CXCR3 and secrete IFN-γ while maintaining FOXP3 expression. This cell type has been observed in type I diabetes [84], multiple sclerosis [85], autoimmune hepatitis [86], and Sjogren syndrome [87]. Similarly, Treg cells that secrete IL-17A are known to be T_H17-like Tregs, which have been found to contribute to the pathogenesis of rheumatoid arthritis [88] and IBD [89]. There is much left to be understood about the T_H-like roles that Tregs can acquire and the implications of those functions not only in autoimmunity but also in cancer.

In addition to maintaining peripheral tolerance, Treg cells have an important role in regulating anti-tumor immunity. While the suppressive capabilities of Treg cells are indispensable for maintaining immune homeostasis, these functions can be detrimental in anti-tumor immune responses. The immune system appears to pose a barrier to tumorigenesis, made evident through the rapid formation of tumors by immunocompromised mice in comparison to immunocompromised counterparts [90, 91]. However, other studies report cancer cells are able to evade immune clearance by secreting immunosuppressive cytokines or recruiting

immunosuppressive cells such as Treg cells [92]. Tumor cells present an array of self-antigens on its cell surface, which are preferentially recognized by Treg cells, allowing Treg cells to exert their immunosuppressive functions [93–95]. Several studies correlate the infiltration of FOXP3$^+$ Treg cells into the tumor with poor disease prognosis and decreased overall survival, suggesting that the presence of Treg cells affect tumor progression [96–99]. Conversely, other studies show high Treg numbers as a favorable prognostic factor, particularly in colorectal cancer where a high density of FOXP3$^+$ Tregs in tumor tissue was associated with improved survival [100–102]. While the data on whether Tregs are a good prognostic marker in cancer is not conclusive, the literature agrees that Tregs play a large role in anti-tumor immunity. Beyond their suppression of effector T cells, Treg cells can also modulate other immunosuppressive cell populations within the TME, and depletion of Tregs may mobilize these immune subsets to promote anti-tumor immunity [103].

Due to the pivotal role that Treg cells play in immune regulation and evidence of these cells in the tumor microenvironment (TME) of many human cancers, many believe that these cells may be a major therapeutic target to induce immune-mediated tumor regression. Numerous murine studies of human cancers have shown that elimination of FOXP3$^+$ Tregs through genetic ablation or cell-depleting antibodies are sufficient to improve survival [104–106]. Although murine data suggests that targeting Tregs in cancer will be an effective treatment, there is little data to suggest this is true for human cancers, primarily due to the lack of specific Treg-targeting techniques.

1.6 Characterization of Tregs in cancer

Prior to the identification of FOXP3, Treg cells were defined by their CD4 and CD25 positivity. CD25/IL-2R is highly upregulated in regulatory T cells due to their high reliance on IL-2 for survival [107]. Early work demonstrated the presence of potential CD4$^+$ CD25$^+$ Treg cells in the cancer tissue and an increase in Treg cells in peripheral blood of cancer

patients [108–111]. However, these markers were not sufficient to delineate Tregs cells from other T cells in cancer given that activated conventional T cells also express CD25 [112, 113]. The discovery of transcription factor FOXP3 in 2003 allowed more accurate identification of Treg cells [114, 115], particularly in the context of human disease. Accordingly, researchers confirmed the presence of $CD4^{+}$ $CD25^{+}$ $FOXP3^{+}$ Treg cells in the microenvironment of various cancers [96, 97, 116, 117]. However, other markers were necessary to fully define a Treg, since conventional T cells also upregulate CD25 when activated, thus complicating the identification of Tregs. Perhaps, the inability to properly define a Treg foreshadowed much of the lack of success with early clinical trials, particularly anti-CD25 antibodies. Many of the early efforts to target Tregs as an anti-cancer therapeutic have failed due to lack of specificity for Treg cells.

Distinguishing Tregs from other circulating and tumor infiltrating lymphocytes in various cancer types has posed a great challenge, owing to the varied phenotypes of Treg cells. Treg cells are canonically characterized by an array of extracellular receptors such as CTLA-4, PD-1, GITR, ICOS, and OX40, whose expressions vary depending on cancer type [117–124]. These markers are particularly upregulated on intratumoral Treg cells but are expressed also constitutively on circulating Tregs [121, 125, 126]. Treg cells can be roughly classified into three subpopulations depending on their expression of FOXP3 and CD45RA: naive, effector, and cytokine producing [127]. Effector Tregs are believed to be a terminally differentiated, highly suppressive subset that are recruited to the tumor to inhibit immune-mediated elimination [128]. Further characterization revealed a subset of human Tregs classified as memory Tregs that phenotypically resemble conventional $CD4^{+}$ effector T cells [129]. These cells mirror expression of classical $CD4^{+}$ effector chemokine receptors CXCR3, CCR6, and CCR4. A specific subset of these memory Tregs that resemble Th-2 effector cells have been implicated in tumorigenesis of melanoma and colorectal cancer [41]. These Th-2 like memory Treg cells exhibit higher chemotaxis as well as secrete CCL17 suggesting they have enhanced

abilities to recruit other $CCR4^+$ Tregs to increase immunosuppression within the TME [41]. These observations highlight the heterogeneity of Treg cells in cancer that contribute to the difficulty of specific intratumoral Treg depletion.

FOXP3 was originally believed to be a Treg cell specific transcription factor, but further characterization revealed that human conventional T cells are able to transiently upregulate FOXP3 [127, 130], which confound the results from Treg studies. Furthermore, FOXP3 induction in non-regulatory T cells is not sufficient to confer a regulatory phenotype, suggesting that FOXP3 expression alone is not enough to define a regulatory T cell [131]. Therefore, while FOXP3 has been an indispensable marker for murine Treg research, its role in human cells has proven to be more complex, making Treg depletion in human disease more difficult [132]. Moreover, FOXP3 expression in Treg cells has been shown to be inconsistent in cancer, as Tregs lose FOXP3 expression under inflammatory conditions [65, 133, 134]. The heterogeneity of Tregs in cancer is further illustrated in colorectal cancer, where two functionally distinct populations with varying FOXP3 expression contribute to opposing prognosis predictions [97]. To further complicate matters, while expression of FOXP3 had previously been thought to be specific to the T cell lineage, a study analyzing colorectal cancer cells show that these tumors highly express FOXP3, indicating that FOXP3 expression is not restricted to Treg cells [135–138]. Stratification of patients by tumor cell expression of FOXP3 showed a correlation between FOXP3 expression in cancer cells and patient survival, however differing FOXP3 levels in Treg cells showed no significant differences in disease prognosis [138]. Because the identification of Tregs is contingent on more than just FOXP3 expression, the expression of FOXP3 by cancer cells does not pose an immediate challenge for identifying Tregs, though it emphasizes the lack of Treg specific markers that can be used for therapeutic purposes.

Furthermore, the identification of T_H-like Tregs increases the complexity of identifying the role that Tregs have in cancer. As described above, the presence of $FOXP3^+$ was

traditionally believed to be an indication of poor prognosis. However, recent studies show that the presence of T_H-like Tregs may be necessary for immunotherapy. A mouse model of Treg-specific IFNγR knockout is deficient in circulating T_H1-like Tregs and was used to assess the exact role that T_H1-like Tregs play in an MC38 murine colon carcinoma model [50]. These experiments revealed that MC38 tumors in wild type mice are fairly response to anti-PD-1 checkpoint blockade, but lose sensitivity to the immunotherapy when T_H1-like Tregs are depleted. This provides evidence to suggest that the function of Treg cells in the tumor microenvironment are not as straightforward as once believed, and gives reason to believe that although there has been an immense effort to deplete Tregs in cancer, that this may not be the best strategy.

1.7 History and challenges of clinical Treg inhibition

There has been a long history of efforts to target Tregs therapeutically. As previously described, Treg cells also constitutively express cell surface receptors such as CTLA-4, PD-1, GITR, TIGIT and OX40 [121, 125, 126]. Treg cells in the tumor microenvironment particularly upregulate immunosuppressive molecules such as CTLA-4 and PD-1, which has made these receptors particularly interesting in the context of Treg depletion (Figure 1.8). Because of the relative ease of targeting cell surface receptors, monoclonal antibodies against these molecules have been purposed to deplete Tregs from the microenvironment of the tumor. Due to the volume of antibody based approached entering the clinic, antibody therapy has yielded the highest number of FDA approved Treg targeting modalities. Nevertheless, Treg cell depletion has seen a number of adverse effects ranging from depletion of effector T cells to unintended peripheral conversion of effector T cells to Treg cells. These often lead to systemic dysregulation of immune responses, highlighting the importance of specific targeting of Treg cells in the tumor microenvironment [139].

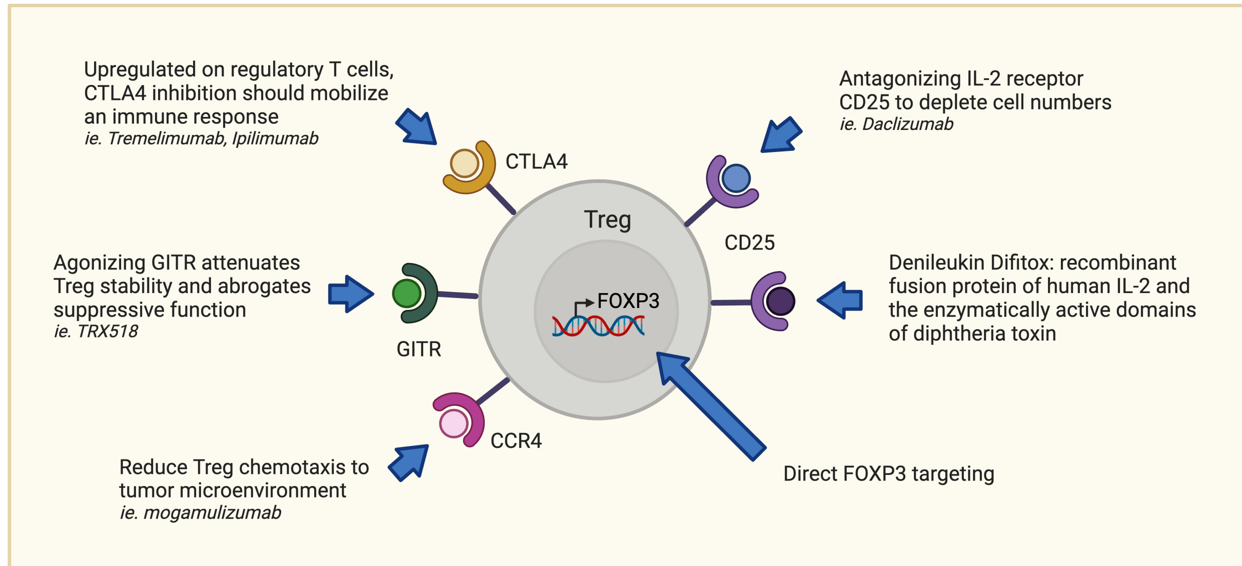

Figure 1.8: Treg targeting modalities. Historically, targeting Tregs has been limited to extracellular receptors such as CD25, CTLA-4, GITR, and CCR4. These have been met with challenges of toxicities due to the promiscuous expression of these receptors on other cell types. More recent developments have focused on targeting FOXP3 directly or modulating its expression but there is little data on the clinical efficacy of these therapeutics.

1.7.1 CD25

The first evidence of the benefit of Treg depletion to anti-tumor therapy dates back to 1999, where studies showed the elimination of CD25$^+$ cells elicits an immune response to syngeneic tumor cells in vivo [140, 141]. In 2001, reports of use of a CD25-depleting antibody in a B16-BL6 murine melanoma model showed a delay in tumor outgrowth [142]. This anti-tumor effect was further amplified through the addition of an anti-CTLA4 antibody [142]. The data suggested that targeting Treg cells using anti-CD25 antibodies can improve the efficacy of immunotherapies by enhancing the induction of effector T cells, and spurred efforts to deplete Tregs to improve anti-tumor immunity. Much of the early work in Treg depletion involved targeting CD25 [139], resulting in clinical trials for two different CD25-based Treg targeting drugs: denileukin difitox and daclizumab [143].

Denileukin difitox, which transiently depleted Treg cells, is a recombinant fusion protein of human IL-2 and the enzymatically active domains of diphtheria toxin [144]. Following up-

take via the IL-2R, the toxin is released resulting in cell death. Initial results for treatment of metastatic renal carcinoma patients suggest that denileukin difitox mediated depletion of Tregs improves vaccine-mediated T cell responses [145]. However, further clinical testing resulted in responses that were modest at best, with non-specific depletion of non-Treg lymphocytes as well [144]. The second trial was for an anti-CD25 monoclonal antibody, daclizumab, which had previously been approved for treatment of multiple sclerosis [146]. The clinical trials for daclizumab as a Treg antagonist were conducted in conjunction with cancer vaccination and resulted in stable disease in 6 out of 10 patients [142]. Daclizumab also decreased frequencies of $CD4_+$ and $CD8_+$ effector T cells, indicating the possibility of off-target effects [143]. The efficacy of daclizumab was also assessed in combination with dendritic cell vaccination for the treatment of metastatic melanoma which resulted in no significant changes in progression-free survival [147]. The limited clinical data on intratumoral FOXP3 levels following daclizumab treatment provide little evidence on whether Treg cells are truly being eliminated from the TME. Nonetheless, while clinical trials for daclizumab for Treg depletion reported no adverse events, secondary autoimmune disease as a result of daclizumab treatment led to its withdrawal in 2018 [148, 149]. Its withdrawal emphasizes the importance and difficulty of specific cell targeting, particularly in a specific region in the case of Tregs in the TME.

While unsuccessful attempts at Treg depletion via anti-CD25 have contributed to limited enthusiasm to further pursue CD25 as a target, a 2017 study described an optimized anti-CD25 antibody with enhanced Fcγ receptor binding to more specifically target Tregs in the tumor [150]. Longitudinal analysis of CD25 expression of patient samples demonstrated that its expression was restricted to $FOXP3_+$ Treg cells, despite high infiltration of $CD8_+$ cytotoxic T cells into the tumor and systemic immune modulation [150]. Treatment of tumor-bearing mice with Fc-optimized anti-CD25 did not reduce tumor burden as a monotherapy, but instead synergized with anti-PD-1 treatment. PD-1 itself has not been shown in clinic

to affect Treg cells, but is instead known to re-invigorate effector T cell proliferation and function, a likely mechanism of the synergy between these therapeutics [151–153]. While inconsistencies between murine and human data in the past indicate the need for more translatable studies to ensure efficacy in humans, the results of this study are encouraging.

1.7.2 CTLA-4

CTLA-4 became a target of high interest due to its upregulation on Tregs in the TME. A target of FOXP3, this molecule is constitutively expressed on Treg cells [99], but can also be upregulated on effector T cells following activation, portending to the possibility of non-specific immune dysregulation. CTLA-4 expression has two key roles: downregulation of effector T cells and upregulation of regulatory T cells; thus, its inhibition should mobilize an immune response, making it an attractive target for anti-tumor immunity. However preclinical data suggests CTLA-4 inhibition has a variable impact on Treg cells, with some reports of decreased cell number and function, and others of increased frequencies [154–156]. Consistent with data from anti-CD25 targeting antibodies in which non-specificity resulted in immune dysregulation, therapies targeting CTLA-4 also resulted in considerable immune-related adverse events. Clinical trials using antibodies targeting CTLA-4 such as ipilimumab or tremelimumab results in response rates of around 10% in patients with metastatic melanoma [151, 157–161]. However, immune related toxicities were also observed in nearly 30% of patients with tremelimumab [160–162] and no survival benefit was observed in Phase III trials compared to the standard of care [159].

Despite its clinical efficacy, the mechanism of ipilimumab remained poorly understood for quite some time. Beyond its binding to and subsequent occupancy of CTLA-4 receptor in order to inhibit any suppressive signals and allowing T cell activation to proceed, not much was known about how ipilimumab was potentially depleting Treg cells [163]. More recently, clinical data depict that ipilimumab treated melanoma patients have an increase

in intratumoral $CD4^+$ and $CD8^+$ T cells, but do not show any decrease in intratumoral $FOXP3^+$ Treg cells, suggesting that while ipilimumab depletes Treg cells in murine models, the same does not hold true for human cancers [164].

1.7.3 GITR

GITR is a co-stimulatory molecule that is lowly expressed by resting conventional $CD4^+$ and $CD8^+$ T cells, but constitutively by Treg cells [165–167]. Studies suggest that agonizing GITR attenuates Treg cell stability and abrogates suppressive function, making it an appealing target for anti-tumor therapy [168, 169]. In 2019, a Phase I clinical trial for a GITR agonizing antibody TRX518 was completed (Clinical Trials Identifier: NCT02628574) [170]. TRX518 reduced the number of circulating and intratumoral Treg cells but did not result in significant clinical results as a monotherapy. In *in vivo* models, the lack of response in disease progression was attributed to a highly exhaustive phenotype of effector T cells. Because GITR is a co-stimulatory molecule, effector T cell exhaustion may be due to overstimulation by the GITR agonist and might be counteracted with strategies that prevent exhaustion, such as PD-1 blockade [170]. Despite clinical data showing GITR-mediated Treg depletion, the mechanism by which GITR signaling depletes Treg cells remains poorly understood. Thus, further work is required to understand the effect of anti-GITR antibodies and GITR signaling on Treg cells.

1.7.4 CCR4

One of the mechanisms by which cancer cells evade immune destruction is through recruitment of immunosuppressive cells such as Tregs [92]. Treg cells are recruited to the TME through chemokine signaling, which led to the hypothesis that blocking chemotaxis through inhibition of chemokine receptors should attenuate Treg accumulation into the tumor microenvironment [96, 171]. Tumor cells produce the chemokine CCL22 that recognizes its

cognate receptor CCR4 on Tregs to induce chemotaxis to the TME [96, 172]. Unsurprisingly, a high proportion of Treg cells acquired from melanoma patients exhibit CCR4, providing reason to believe that CCR4 may be a good target for Treg depletion in the TME [172]. Moreover, CCR4 expression is restricted to $FOXP3^{hi}$ effector Treg cells, the highly suppressive population of Treg cells that accumulates in the tumor, and not expressed by $FOXP3^{lo}$ naive Treg cells [172]. Though targeting CCR4 appears to be an attractive strategy for targeting a subset of Tregs that are highly abundant in tumors, there is no guarantee that it will specifically deplete intratumoral Tregs given that a large proportion of Treg cells in peripheral blood are also $CCR4^{+}$ [172]. In line with preclinical data, phase I clinical trial of Treg cell depletion by the administration of anti-CCR4 monoclonal antibody mogamulizumab to patients with solid tumors resulted in increased anti-tumor immune responses in some patients, but overall did not produce a clinically significant result [173].

1.7.5 FOXP3

Currently, there are efforts to target FOXP3 directly due to its role as the master regulator of Treg identity. Ablation of FOXP3 in mice contributes to loss of Treg cell function, making it an obvious target for modulating Tregs for anti-cancer immunity. Because FOXP3 is a nuclear product, previous antibody-based targeting strategies cannot be used to eliminate FOXP3 expressing cells. The first documented attempt to target FOXP3 for anti-tumor immunity was by dendritic cell vaccination in 2007 [174]. These FOXP3 mRNA transfected dendritic cells are just as effective as anti-CD25 antibodies at depleting intratumoral Tregs and did not affect the peripheral pool of Tregs, suggesting a decreased likelihood of inducing secondary immune toxicities [174]. However, follow-up on this method of Treg targeting is lacking, perhaps owing to the limited efficacy of other dendritic cell-based vaccines in inducing tumor regression in clinical studies [175]. Since then efforts have shifted towards different FOXP3 targeting modalities.

FOXP3 peptides designed to bind to NFAT induce mild anti-tumor activity, but poor peptide stability and binding affinity have posed challenges to further developments [176]. Beyond NFAT, FOXP3 also interacts with other transcription factors important for Treg function such as RUNX1, RelA,, IRF4, STAT3, and HIF1α; however, murine models looking at genetic knockout of these factors result in varying degrees of immune dysfunction and autoimmunity [58, 177–183]. Targeting the interaction between FOXP3 and its associated transcription factors has been particularly difficult due to the large size of protein-protein interaction (PPI) interfaces and issues of drug delivery into the cell [184]. In fact, there has only been one FDA approved inhibitor of PPIs, a small molecule targeting BCL-2, highlighting the difficulty of targeting PPIs as a therapeutic [185, 186]. Nevertheless, peptides are more capable of binding to large protein surfaces with high specificity, making them a better option for targeting FOXP3 and its binding partners [187]. Previously studied peptide inhibitors of FOXP3 have been unstructured, native peptides that are easily subjected to proteolytic degradation, which would not maintain their structure long enough to enter the cell [187, 188]. The resolution to these challenges of degradation may be found in a novel class of hydrocarbon-stapled- α-helical peptides that are locked in their helical secondary structure and show increased binding affinity and resistance to degradation [187, 189].

Recently a CRISPR-screen of murine Treg cells revealed a network of nuclear factors that modulate FOXP3 expression. TCR stimulation can activate TGF-β-activated kinase 1 (TAK1)-Nemo-like kinase (NLK) signaling leading to the phosphorylation of FOXP3 and decreasing its interaction with ubiquitin ligases [190]. Knockout of NLK in murine Tregs results in reduced immunosuppressive capacity and onset of severe autoimmune encephalomyelitis [190]. Thus, while FOXP3 may appear to be an obvious target for Treg depletion, systemic targeting of FOXP3 can lead to severe autoimmune conditions.

Overall, Treg cell targeting has not seen much success. Many of the antibody-based approaches show promising results in preclinical models, but do not fare well in clinical trials.

These therapies either successfully deplete Tregs but result in severe secondary autoimmunity due to lack of specificity, or do not deplete Tregs at all in patients. It is becoming increasingly apparent that while being obvious targets, inhibiting the current markers are not sufficient methods of depleting Treg cells in human cancer. Several FDA approved anti-cancer therapeutics, particularly tyrosine kinase inhibitors and histone deacetylase inhibitors, have been evaluated for their abilities to deplete Tregs, but there have been few follow-up studies to validate these findings [191–198]. Nevertheless, there is a need to develop novel strategies to modulate Treg cells therapeutically and it may prove clinically beneficial to assess the effect of already FDA approved drugs on Tregs to minimize potential toxicities.

1.8 Potential to alter T cell function through non-canonical roles of BCL-2

Currently, there are also efforts to target T cells via targeting their metabolism. Cancer cells exhibit high metabolic activity that can induce nutrient deprivation, creating a stressful environment for infiltrating immune cells. Effector T cells, in particular, rely heavily on amino acid uptake and glycolysis for effector functions, which can be impaired due to the stressful conditions of the TME [199]. Treg cells, however, rely heavily on oxidative phosphorylation and fatty acid oxidation [53–55]. Moreover, Treg cells in the tumor microenvironment exhibit a vastly different metabolic signature than Treg cells in non-malignant tissue due to the variety of metabolic stress imposed by the TME on infiltrating immune cells [200]. Furthermore, FOXP3 reprograms Treg cell metabolism in low-glucose, high lactate conditions, promoting their survival and suppressive function in metabolically challenging environments such as the TME [201]. Solid tumors in particular demonstrate this type of metabolically stressful environment, suggesting that Foxp3 not only enables intratumoral Tregs to have a different metabolic signature from circulating Treg cells, but also provides Treg cells in the TME a metabolic advantage. Due to the metabolic reprogramming seen in T cells, targeting T cell

metabolism has become of interest in order to modulate their function for immunotherapy.

An early study investigating mechanisms of Treg suppression reports that Toll-like receptor 8 (TLR8) mediates reversal of Treg function through the MyD88-IRAK4 pathway [202]. Further studies demonstrate that TLR8 signaling can also mediate glucose metabolism of Tregs, a key process that contributes to their suppression of effector T cells [203]. Tumor infiltrating Tregs upregulate expression of key metabolic enzymes involved in both glucose and lipid metabolism compared to other tumor infiltrating lymphocytes, which allows them a survival advantage over their effector T cell counterparts and contributes to the immunosuppressive environment in the tumor [203]. These observations suggest that modulation of the TLR8 signaling pathway alters Treg metabolism to ultimately change their function and mobilize the anti-tumor immune response. Specifically, metabolic competition and cross-talk between Treg cells and effector T cells in the nutrient-deprived TME induce DNA damage through MAPK and JAK-STAT signaling that results in cellular senescence and dysfunction of effector T cells [204]. TLR8 signaling inhibits MAPK activation, thereby reversing Treg-mediated suppression by blocking induction of effector T cell senescence [205]. Correspondingly, TLR8-mediated reprogramming of Treg glucose metabolism enhanced anti-tumor immunity by preventing Treg induced effector T cell senescence [203]. TLR8 signaling further contributes to the inhibition of Treg function by downregulating key glucose transporters GLUT1 and GLUT3 to decrease glucose metabolism, as well as downregulating mTORC1-HIF1α signaling, which have significant roles in promoting Treg cell function and metabolism [57, 203, 206–208]. However, because naive Tregs can also heighten glucose consumption, further work is needed to determine whether activation of TLR8 signaling discriminately affects intratumoral Tregs or if it has a broader effect on all Tregs.

Recently, RNA-Seq analysis comparing intratumoral and circulating Treg cells from patients with breast cancer showed that Tregs in the microenvironment of breast tumors displayed heightened lipid metabolism. The data revealed that intratumoral Tregs upregulate

CD36, a glycoprotein that controls lipid uptake [209]. This upregulation confers a difference in Treg fatty acid metabolism and maintains the suppressive capacity and survival of intratumoral Tregs [209]. Interestingly, CD36 depletion does not affect the function of circulating Treg cells, suggesting that this molecule is specifically upregulated in intratumoral Treg cells. Although targeting Treg metabolism through anti-CD36 antibody monotherapy depletes Tregs in the TME, there is no significant effect on tumor regression due to effector T cell exhaustion [209]. As a result, anti-PD-1 therapy has been used in conjunction with Treg-depleting methods in preclinical studies to reinvigorate exhausted T cells [170, 209]. Co-treatment of anti-CD36 antibody with anti-PD-1 blockade significantly inhibited tumor growth and improved survival of melanoma-bearing mice. T cell exhaustion has been shown to limit the therapeutic benefit of Treg cell targeting [170], which poses an issue of how significant an impact Treg depletion itself would make in anti-tumor immunity. Nonetheless, the notion of altered Treg metabolism has become of high interest as it provides a point of distinction between intratumoral and circulating Treg cells. In fact, many metabolic studies of Treg cells in the tumor microenvironment reveal that changes in metabolic dependency provide Tregs a greater survival advantage over their effector T cell counterparts in the TME, creating a vastly immunosuppressive environment that diminishes anti-tumor immunity [53–55, 207, 210]. Intriguingly, Treg cells in the hypoxic environment of glioblastoma are able to metabolize extracellular fatty acids to support their immunosuppressive function, further supporting the idea of utilizing the differences in metabolic dependencies of Tregs in the TME to benefit tumor therapy [207].

In sum, these observations suggest that targeting Treg metabolism may be a potential answer for specifically targeting Treg cells in the tumor as opposed to systemically depleting Tregs. However, while inhibiting Treg-mediated suppression of other tumor-infiltrating lymphocytes may at least partially mobilize an anti-tumor immune response, survival capacities and functionality of effector cells within the TME is a crucial barrier. Along these lines, the

BCL-2 family also play non-apoptotic roles largely related to mitochondrial regulation and metabolic homeostasis [211], offering the potential for BCL-2 modulation to affect T cell metabolism and function (Figure 1.9). BCL-2 localizes to inner mitochondrial membrane (IMM) and has been implicated in regulation of mitochondrial respiration [212]. There is evidence to suggest that it is able to interact with multiple cytochrome c oxidase subunits. These interactions can lead to increased mitochondrial respiration and decreased cellular respiration during oxidative stress. Similarly, MCL-1 also works at the IMM and deletion of MCL-1 has been associated with disruption of the mitochondrial structure, decreases of enzymatic activities of complex I, II and III while favoring complex IV and V, as well as decreases in metabolism and respiration [211, 212]. It has been shown that mitochondrial complex III is essential for Treg-mediated immunosuppression, suggesting a close relationship between mitochondrial homeostasis and Treg function [213]. While FOXP3 expression was unaltered in complex III deficient Tregs, there is evidence to suggest that FOXP3 is closely linked to Treg metabolic regulation. In particular, FOXP3-deficiency in Tregs dysregulates mTOR signaling and leads to increases in both aerobic glycolysis and oxidative phosphorylation, the prior being more characteristic of effector T cells [206]. More recently, MCL-1 has been shown to enforce a programmatic dependency on fatty acid oxidation (FAO) in MCL-1-driven hematologic cancer cells and renders them vulnerable to FAO inhibition. Deletion of *Mcl-1* downregulates the FAO pathway, suggesting a novel role of MCL-1 in regulating cell metabolism [214].

BCL-2 family members have also been implicated in the regulation of calcium homeostasis in the endoplasmic reticulum (ER). Inositol 1,4,5-trisphosphate receptors (IP3R1–3) are ER-resident calcium channels that are essential to numerous calcium-regulated pathways (Figure 1.9). BCL-2, BCL-xL, and MCL-1 have all been shown to bind to IP3R and confers the calcium leakage caused by an allosteric shift as a result of the protein-protein interaction [211, 215, 216]. Regulation of calcium signaling has been shown to be control effector func-

tions of lymphocytes including metabolism, cytokine secretion, and cytotoxicity. Activation of T cells leads to calcium release from ER stores via IP3R channels which is then transferred to the mitochondria to enhance ATP production required for clonal expansion and secretion of cytokines [217]. Mitochondrial BCL-2, MCL-1, and BCL-xL also interact with voltage-dependent anion channels (VDACs) and differentially regulates their function at the outer mitochondrial membrane (OMM) [218]. Whether BCL-2 proteins inhibit or promote the opening of VDACs has been subject to debate [218], but the reality may be that its regulation is context dependent. Further, the calcium levels within the mitochondria can be modulated by the mitochondrial calcium uniporter (MCU) which is ultimately responsible for transporting calcium across the inner mitochondrial membrane (IMM).

The emergence of a class of small molecule inhibitors called BH3 mimetics that bind to and inhibit the function of anti-apoptotic proteins through molecular mimicry has allowed for the study of pharmacological inhibition of BCL-2 rather than genetic inhibition. These studies are more readily translatable and have greater therapeutic implications than past studies using genetic knock out, as the small molecule BCL-2 inhibitor venetoclax is FDA approved and widely used for treatment of chronic lymphocytic leukemia (CLL), small lymphocytic leukemia (SLL), and acute myeloid leukemia (AML). Venetoclax has been shown to increase generation of reactive oxygen species through inhibition of respiratory chain supercomplexes formation that ultimately led to increased effector T cell function [219]. There is also evidence to show that use of BH3 mimetics alters mitochondrial morphology which may ultimately affect mitochondrial function and cell metabolism [220]. These data together suggest that there is potential to alter T cell function via the effects that BH3 mimetics have on T cell metabolism. However, further work is required to elucidate the roles that these mimetics play in different T cell subsets.

Given the evidence that the BCL-2 family and BH3 mimetics can modulate T cell function via metabolic alterations, it is imperative that we continue to identify other non-canonical

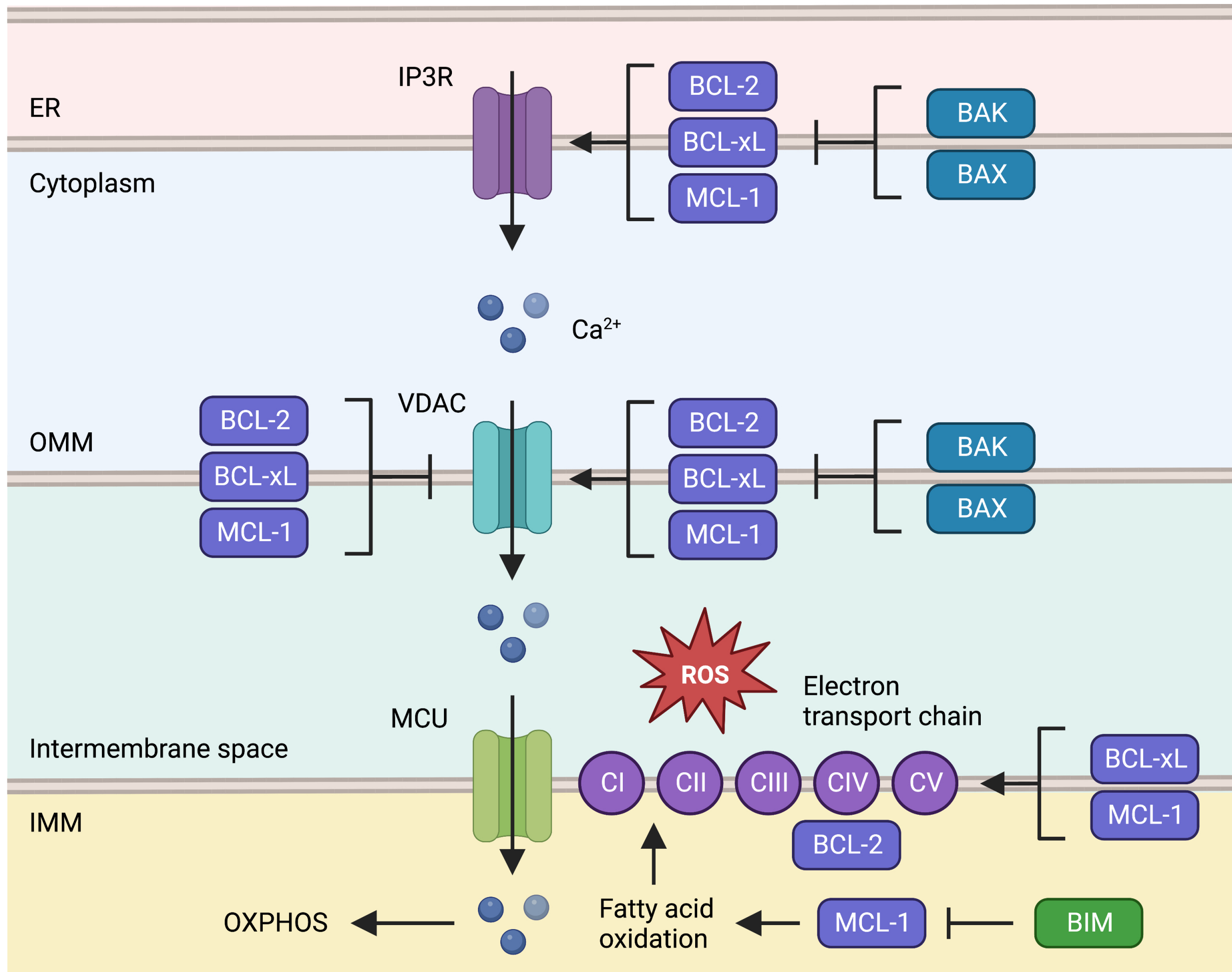

Figure 1.9: Roles of BCL-2 in cell metabolism. Anti-apoptotic proteins in the BCL-2 family favor calcium release by IP3R. The exact regulation of voltage-dependent anion channels (VDACs) by BCL-2 proteins is unclear. Other factors that regulate calcium levels in a cell includes the activity of the mitochondrial calcium uniporter (MCU) that is responsible for transportation of calcium across IMM. In the mitochondrial matrix, BCL-2 proteins also boost OXPHOS and ROS production by favoring respiratory complex IV (CIV) and complex V (CV) activity. In addition, MCL1 supports fatty acid oxidation in a BIM-dependent manner.

roles of BCL-2 and its implications on T cells. Recent evidence from our lab show that long-term BCL-2 inhibition with venetoclax in mice results in global genetic reprogramming [221]. In a congenic transplant model, mice treated with 50mg/kg of venetoclax during cell engraftment showed genetically diverse naive $CD4^+$ and $CD8^+$ T cells compared to non-treated mice through RNA-sequencing. The naive T cells in the venetoclax-treated

mice exhibit increased JAK-STAT signaling and PI3K signaling, accompanied by decreased MAPK activation [221]. The exact functional implications of these pathway alterations are yet to be delineated. This data provides preliminary evidence the BCL-2 family is able to mediate other cellular processes aside from cell metabolism that are important to T cell function. The effect that BH3 mimetics on T cells in particular are important to consider given that these drugs are used in patients as cancer therapeutics. While the main goal is to induce apoptosis of tumor cells, there may be an effect on healthy lymphocytes in patients that remains to be uncovered. Therefore, it is important that we are able to further elucidate not only the non-canonical roles of the BCL-2 family, but also the non-apoptotic functions of BH3 mimetics so that we may better understand the effects that these therapeutics have on the immune system.

1.9 Development of BH3 mimetics

As mentioned in the previous section, a class of drugs called BH3 mimetics has emerged in order to induce apoptosis through molecular mimicry. The primary motivation behind creating these therapeutics was to overcome apoptotic resistance in a variety of cancer types. These drugs are designed to mimic the BH3 domain of BH3-only protein in order to effectively bind to the anti-apoptotic proteins. This mimics the natural function of BH3-only proteins in a cell, which aid in the release of the sequestration of the pro-apoptotic proteins BAK and BAX as well as the direct or indirect activation of BH3-only proteins. These BH3 mimetics are comprised of natural products, small molecules, and peptide therapeutics that either lower the apoptotic threshold or directly initiate the intrinsic apoptotic cascade. A number of these compounds are being developed to target various anti-apoptotic proteins, with a few being in clinical trials and one with FDA approval (Table 1.2).

Early evidence that targeting BCL-2 could be efficacious in abrogating tumor growth was demonstrated using an antisense oligonucleotide targeting BCL-2 [222]. These studies dis-

covered that a *BCL-2* antisense expression plasmid ablated tumor formation by Jurkat cells, providing evidence that BCL-2 contributes to the regulation of the *in vivo* growth of these cells. This finding led to the development of antisense oligonucleotide oblimersen sodium, otherwise known as G3139 or genasense, which has shown efficacy in multiple hematological malignancies, including Non-Hodgkin's lymphoma (NHL) and imatinib-resistant BCR-ABL-positive leukemia [223, 224]. The *in vivo* efficacy resulted in a few clinical trials in NHL, CLL, and prostate cancer [225–227]. While the results of these trials varied, the overall conclusions indicate that BCL-2 could be safety inhibited in patients but only saw modest single-agent efficacy. Despite not reaching FDA approval, G3139 established proof-of-principal that *in vivo* targeting of BCL-2 holds promise providing in tumor control. There has not been more work conducted to further research on G3139 or development of any *BCL-2* oligonucletide targeting therapeutics as drug development efforts has turned to small molecule and peptide therapies that offer direct targeting modalities.

1.9.1 Small molecules

G3139 itself, being an oligonucleotide, is not considered a BH3 mimetic per say, but lessons learned from its development led to more specific targeting strategies that may increase the efficacy of BCL-2 inhibition. Initial natural or synthetic inhibitors of BCL-2, including terphenyl, purpurogallin, HA14-1, antimycin A, BH3-I, and chelerythrin, exhibited cytotoxicity or lacked sufficient pharmacological properties and binding to BCL-2 [228, 229]. The first BH3 mimetic prototypes, ABT-737 and ABT-263 (navitoclax) were developed by Abbvie and are considered to be the first true BH3 mimetics due to the lack of specificity of prior compounds. ABT-737 was discovered using a nuclear magnetic resonance (NMR)-based screening method. ABT-737 has high affinity for the BH3-binding pocket of BCL-2, BCL-xL, and BCL-w and showed *in vivo* efficacy in promoting tumor regression of solid tumors [186]. Further, ABT-737 shows no efficacy in BAK/BAX double knockout cells, indicating

the drug works through on-target binding to anti-apoptotic proteins that allows initiation of the intrinsic apoptotic pathway [230]. This is an improvement from past compounds that saw cell killing despite BAK or BAX deficiency, showing that those compounds lacked specificity. An orally bioavailable analogue of ABT-737 called navitoclax was developed and tested in clinical trials for both hematological and solid tumors [231]. However, dose escalation was significantly limited by cases of thrombocytopenia and neutropenia and halted further trials with navitoclax [232].

Navitoclax-induced thrombocytopenia was caused as a result of the BCL-xL targeting nature of the drug, as platelets have a dependency on BCL-xL for survival [233]. In order to overcome the thrombocytopenia induced by navitoclax, a new BCL-2 specific BH3 mimetic was derived from a BCL-2–navitoclax X-ray crystal structure [234]. Navitoclax was reversed engineered through the systematic removal or replacement of key binding elements to increase its specificity and affinity for BCL-2 alone [234] Characterization of this new drug, termed ABT-199 or venetoclax, revealed that it bound to BCL-2 with sub-nanomolar specificity and induced apoptosis at lower concentrations that navitoclax. The demonstrated efficacy and improved safety profile of venetoclax both in preclinical and clinical studies led to its FDA approval for the treatment of refractory 17-p-deleted CLL and AML [235, 236]. Venetoclax became the first small molecule inhibitor to disrupt protein-protein interactions and catalyzed a crusade to develop other small molecules to inhibit BCL-2 family members. To this day, venetoclax is still being tested in various combination therapies to try and improve tumor response to therapy [237, 238].

Just as navitoclax targets multiple members of the BCL-2 family, other drugs have been developed to inhibit different anti-apoptotic proteins simultaneously. One example is Obatoclax or GX15-070, which binds to all anti-apoptotic proteins (pan-BCL-2). However, the affinity towards these proteins is fairly low and it also has a mechanism of action that is non-specific to apoptosis induction, as it still kills cells deficient in BAK and BAX [228].

Obatoclax is able to overcome MCL-1-mediated apoptotic resistance [239], which may be mediated through its off-target mechanisms, including inhibition of PI3K signaling, endoplasmic reticulum stress response, and Noxa upregulation which causes dissociation of MCL-1:BAK complex [240–242]. Phase I clinical trials showed tolerability of Obatoclax in patients [243], but progression to Phase I/II did not improve patient outcomes [244, 245].

A number of small molecules targeting MCL-1 and BCL-xL are also in various stages of development. Two MCL-1 specific inhibitors have demonstrated efficacy in clinical trials: S63845 and AZD5991. S63845 is high selective for MCL-1 and leads to stabilization of MCL-1 protein *in vitro* [246]. A Phase I clinical trial was completed for S63845 in patients with AML or myelodysplastic syndrome (Clinical Trials Identifier: NCT02979366) and is currently in an ongoing Phase Ib trial for dose escalation in AML patients (Clinical Trials Identifier: NCT03672695). Similarly, AZD5991 also showed high affinity for MCL-1 and rapidly induces apoptosis in myeloma and AML [247]. This data led to the initiation of a clinical trial (Clinical Trials Identifier: NCT03218683); however this trial was recently terminated due to myocardial toxicity. Currently there are no BCL-xL specific mimetics in clinical development but the leading compound in preclinical studies is A-1331852 that potently induces apoptosis in BCL-xL dependent cancer cells [248].

1.9.2 Natural compounds

Natural products have also been explored as potential inhibitors of BCL-2. In particular, the Gossypol family is a plant-derived polyphenolic aldehyde that has been used in clinic for a long time prior to the discovery of its BH3 mimetic properties. Its negative enantiomer called AT-10 is able to bind to BCL-2, MCL-1, BCL-xL, and BCL-w with sub-micromolar affinity [228]. AT-101 demonstrated modest antitumor activity *in vitro* and *in vivo* but failed to show clinical efficacy [228, 249]. Gossypol derivatives have been created in order to reduce toxicity. Apogossypol and apogossypolone (ApoG2) lack two aldehyde groups compared to Gossypol

and are currently undergoing preclinical studies [250–252]. Studies show that BAK and BAX are required for efficacy of ApoG2, suggesting at least a partial on-target effect of the mimetic [253]. ApoG2 has been shown to trigger the mitochondrial pathway for apoptosis in primary CLL cells, suggesting its potential efficacy in CLL [253]. The benzoylsulfonide derivative of Gossypol, TW37, has seen greater preclinical efficacy [254, 255] and reached clinical trials, however no clinical data is available. Gossypol has been demonstrated to induce apoptosis in polymorphonuclear leukocytes and monocytes isolated from healthy donors, suggesting a potential for its use as an immune modulator if not an anti-cancer therapeutic [256].

1.9.3 Peptides

There is an increasing interest in the use of peptide-based BCL-2 therapeutics. Native BH3 helices are unsuitable for therapeutic use as they are quickly degraded and lack cellular penetrability [257]. Use of a chemical strategy called hydrocarbon stapling generates BH3 stapled peptides, termed "stabilized alpha-helix of BCL-2 domains" (SAHBs). SAHBs are helical, protease-resistant, and cell-permeable compared to the native peptide counterpart. A multitude of these peptides have been developed and hold the potential for specific disruption of protein-protein interactions, but none have progressed to clinical trials [258–262].

Thus far, despite the number of BH3 mimetics that have been developed, the most well characterized mechanism of action is the most obvious one: the effect of these drugs on apoptosis. The rapid and extensive development of BCL-2 family targeting therapeutics provides justification for further investigation into other effects of these drugs. Evidence of non-canonical roles of the BCL-2 family indicates that drugs that target this family of proteins may also elicit non-canonical effects as well. Therefore, it is critical that we assess these potential effects in conjunction with assessing the role of apoptotic induction in order to capture the full mechanism of action.

Table 1.2: BH3 mimetics in development

Compound	Target	Type	Stage of Development
ABT-737/ABT-263 (navitoclax)	BCL-2, BCL-xL, BCL-w	Small molecule	Phase I/II
ABT-199 (venetoclax)	BCL-2	Small molecule	FDA approved / Combinatorial clinical trials ongoing
Obatoclax	pan-BCL-2	Small molecule	Phase I/II
S63845	MCL-1	Small molecule	Phase I
AZD5991	MCL-1	Small molecule	Phase I
A-1210477	MCL-1	Small molecule	Preclinical
MIM1	MCL-1	Small molecule	Preclinical
BAM7	BAX	Small molecule	Preclinical
AT-101	pan-BCL-2	Gossypol isomer	Phase I/II
Apogossypol	pan-BCL-2	Gossypol derivative	Preclinical
ApoG2	pan-BCL-2	AT-101 derivative	Preclinical
BI-97C1 (sabutoclax)	pan-BCL-2	Apogossypol derivative	Preclinical
TW37	pan-BCL-2	Gossypol analog	Phase I/II
S1	pan-BCL-2	Small molecule	Preclinical
BIM SAHB	pan-BCL-2	Peptide	Preclinical
072RB	MCL-1, BCL-xL	Peptide	Preclinical
BIM_S2A	MCL-1	Peptide	Preclinical
MCL-1 SAHB	MCL-1	Peptide	Preclinical
NOXA BH3	MCL-1	Peptide	Preclinical
PUMA BH3	PUMA	Peptide	Preclinical

1.10 Significance: BCL-2 inhibition for immune modulation

The number of BH3 mimetics in development shows how important it is to understand how these drugs are affecting patients. As venetoclax is tested in more cancer indications for both single agent use and in combination therapies, there is an increasing need to understand

how its use not only affect malignant cells, but also healthy cells that are susceptible to BCL-2 targeting. There is a growing amount of evidence to suggest that long-term BCL-2 inhibition results in global reprogramming of T cells, which may have long-lasting effects on T cell identity and function.

The use of BH3 mimetics as immune modulators have largely focused on the compound ABT-737 in the context of autoimmunity and transplant tolerance. One of the first studies to assess the potential immuno-modulatory effects of BH3 mimetics found that ABT-737 results in lymphocyte apoptosis and ameliorates autoimmunity in several murine models [263]. ABT-737 reduces paw swelling in collagen-induced mouse models of arthritis as well as improve survival and renal function in mice with SLE [263, 264]. Moreover, in murine models of diabetes, animals treated with ABT-737 are better able to maintain long-term control of blood glucose as a result of prolonged pancreatic islet allograft survival [265]. These effects may be a result of preferentially induced apoptosis of Tcons while sparing Tregs, an effect uncovered in a murine model of graft-versus-host disease (GvHD) where ABT-737 administration results in improved survival following hematopoietic stem cell transplant [266].

Venetoclax has also been tested in patients with SLE as BCL-2 has been implicated in the disease pathogenesis. While the purpose of this clinical trial (Clinical Trials Identifier: NCT01686555) was not meant to address the efficacy of venetoclax in reducing SLE symptoms, the study ultimately showed that venetoclax depleted total lymphocytes and B cells by approximately 50% and 80%, respectively [238]. The drug was well-tolerated in women with SLE, emphasizing the potential for use of venetoclax as an immuno-modulatory agent.

Other clinical studies using venetoclax and other BH3 mimetics also suggest the potential immuno-modulatory effects in patients through lymphopenia, neutropenia, or thrombocytopenia. Patients with relapsed or refractory CLL treated with venetoclax exhibited a $>$50% reduction in their absolute lymphocyte counts (ALC). 35% of patients also reported to have

neutropenia with 6% of the patients experiencing serious infections and 2.5% developing autoimmune neutropenia [267, 268]. Administration of Navitoclax, a pan-BCL-2 inhibitor, results in significant thrombocytopenia along with neutropenia, with many patients developing infectious sequelae [268, 269]. Phase I clinical trials using G3139, a *Bcl-2* antisense oligonucleotide, in combination with doxorubicin and cyclophosphamide, found that this compound also led to global myelosuppression including neutropenia and lymphopenia [270, 271].

A large number of these studies emphasize the immune-depleting effects of venetoclax in patients through its apoptotic functions. However, there is new evidence to suggest that venetoclax may have a larger on immune cells that inducing apoptosis. A Phase III trial of venetoclax in combination with bortezomib and dexamethasone in patients with relapsed or refractory multiple myeloma (Clinical Trials Identifier: NCT02755597) showed that 4% of patients who received venetoclax developed treatment-emergent fatal infections, and 16% of patients developed pneumonia in addition to reports of neutropenia and thrombocytopenia [272]. Three treatment-related deaths were reported in the venetoclax group as a result of pneumonia or septic shock, but there was no correlation of these deaths to lympho- or neutropenia, suggesting an immuno-modulatory role of venetoclax that is distinct from its apoptotic function in immune cells. The exact role that venetoclax played in these patient outcomes are unclear. A recent preclinical study by Abbvie also described the immunomodulatory effects of venetoclax in an MC38 murine model of colon carcinoma. They showed that venetoclax treatment selectively depletes naive T cells, allowing for the enrichment of memory CD8^{+} cytotoxic T cells which synergize with anti-PD-1 immunotherapy [273]. However, the questions remains of how venetoclax may be affecting other T cells, such as Tregs, which play a large role in mediating immune responses and have been shown to be important in anti-PD-1 efficacy [50]. Together, this data further emphasizes the need to better characterize how these drugs affect the immune system outside of its apoptosis-

inducing function.

1.11 Approach: Manipulating Treg cell plasticity through BCL-2 inhibition

Here we evaluate the effects of long-term BCL-2 inhibition through venetoclax administration on Tregs. Due to the roles that Tregs play in immune regulation and the implications of non-apoptotic roles that BCL-2 may have in T cells, we asked whether or not venetoclax administration would alter Treg function in mice. Additionally, it is becoming increasingly clear that Tregs are not a homogeneous population and can be further subdivided depending on function [274, 275]. Importantly, animal models of Treg fragility show that *Bcl-2* is downregulated [49], raising an additional question of how this downregulation may be affecting the onset of Treg fragility. Given the evidence that Treg fragility is important for anti-PD-1 checkpoint blockade, it may be possible to induce this fragility with venetoclax in order to sensitize patients to immunotherapy.

In the clinic, patients are treated with venetoclax on cycles of 14, 21, or 28 days. In these studies, we look at the impact of venetoclax on the murine immune system with just 14 days of treatment. We provide proof of principle that venetoclax does, in fact, induce Treg plasticity into a T_H17-like state. These findings provide strong scientific justification for continued evaluation of the immuno-modulatory effects and how venetoclax may be used in combination with immunotherapy. Importantly, the clinical safety profile of venetoclax has been pre-established being that it is an FDA approved drug, allowing for ease of repurposing for clinical use as an immuno-modulator.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

C57BL/6 mice and Foxp3$^{\text{IRES-GFP}}$ mice were purchased from the Jackson Laboratory (C57BL/6J, C.Cg-Foxp3$^{\text{tm2Tch}}$/J). Foxp3$^{\text{GFP-Cre}}$Bcl-2$^{\text{fl/fl}}$ were generated by breeding Foxp3$^{\text{eGFP-Cre-ERT2}}$ mice with Bcl-2$^{\text{fl/fl}}$ mice. Foxp3$^{\text{eGFP-Cre-ERT2}}$ mice were gifts from Fotini Gounari, Ph.D. Bcl-2$^{\text{fl/fl}}$ mice were gifts from David Hildeman, Ph.D. Cre recombinase was activated via oral administration of 100mg/kg of tamoxifen (Sigma-Aldrich #T5648) dissolved in corn oil (Sigma-Aldrich #C8267) for five days. All animals and experiments were approved by and performed in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee of the University of Chicago.

2.2 Cell lines and culture conditions

Ex vivo T cell experiments were conducted on T cells isolated from Foxp3$^{\text{IRES-GFP}}$ mice. Spleen and lymph nodes were isolated and put into single cell suspension via physical disruption through a 40 µm filter. Cells were enriched via negative selection using CD4$^+$ T cell isolation kit following the manufacturer's protocol (Miltenyi Biotec #130-104-454). CD4$^+$ cells were stained with CD4 – APC (BD Biosciences #553051), CD25 – BV711 (BioLegend #102049) antibodies diluted 1:100 in PBS. Tregs (CD4$^+$ CD25$^+$ GFP$^+$) and Tcons (CD4$^+$ CD25$^-$ GFP$^-$) were sorted on a BD FACSAria (University of Chicago Flow Cytometry Core). On occasion, cells were stained with an additional CD44 – APC-Cy7 (BD Biosciences #560568) and CD62L – PE (BioLegend #161204) in order to select the naive T cells. Ex vivo sorted Tregs and Tcons were maintained and expanded in Advanced Dulbecco's modified eagle media (DMEM) supplemented with 10% fetal bovine serum, 100U/mL penicillin/streptomycin, 5 µg/mL Gentamicin solution, 2mM L-Glutamine, 1X Non-Essential

Amino Acids, 10mM HEPES, and 50 µM β-Mercaptoethanol. The MC38 cell line for tumor experiments was a gift from Thomas Gajewski M.D., Ph.D. and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100U/mL penicillin/ streptomycin, 2mM L-Glutamine, 1X Non-Essential Amino Acids, and 10mM HEPES.

2.3 Venetoclax

Venetoclax was reconstituted for in vivo use using 10% ethanol, 30% PEG 400, and 60% Phosal and was administered via oral gavage at a dose of 50mg/kg. Mice were treated with venetoclax for 14 days.

2.4 Anti-PD-1

Anti-PD-1 (BioXCell #BE0146) and IgG controls (BioXCell #BE0089) were diluted in PBS and administered via intraperitoneal injection every 4 days at a concentration of 10mg/kg.

2.5 Spectral flow cytometry

Cells from spleen and inguinal lymph nodes were prepared into single cell suspensions and treated with purified CD16/32 (BioLegend #101302) for 30 minutes to block non-specific FcR II/III binding. This step was done using in 20% Brilliant Stain Buffer Plus (BD Biosciences #566349) diluted in PBS. Cells were then stained for 30 minutes with extracellular antibodies at a dilution of 1:200 and Zombie Near-IR at 1:400 also in diluted Brilliant Stain Buffer Plus. Following extracellular staining, cells were washed and fixed for 1 hour at room temperature using Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (Thermo Fisher #00-5521-00). Intracellular staining was done overnight at 4°C at an antibody concentration of 1:100 diluted in 1X Permeabilization buffer (Thermo Fisher #00-8333-56). Cells were washed with PBS prior to analysis. A large panel designed for

spectral flow cytometry (Cytek Aurora) was used for the majority of flow cytometric analyses of immune populations. A full list of antibodies in this panel can be found in Table 2.1. Smaller panels were designed for more particular flow cytometric analyses, including assessments of the BCL-2 family and PI3K pathways. These antibodies are detailed in Table 2.2. Data was analyzed using FlowJo Software.

Table 2.1: Aurora immune panel

Marker	Clone	Fluorophore	Vendor
PD-1	J43	BUV395	BD Biosciences
CD86	PO3	BUV496	BD Biosciences
CD69	H1.2F3	BUV563	BD Biosciences
Ly6G	1 A8	BUV661	BD Biosciences
CD103	2E7	BV421	BioLegend
I-A/I-E	M5/114.15.2	Pacific Blue	BioLegend
CD19	6D5	BV510	BD Biosciences
CD11b	M1/70	BV570	BioLegend
CD206	C068C2	BV605	BioLegend
RORγt	Q31-378	BV650	BD Biosciences
Ki67	B56	BV711	BD Biosciences
CD4	GK1.5	Spark NIR 685	BioLegend
Ly6C	HK1.4	BV785	BioLegend
CD44	IM7	FITC	BioLegend
CD45	30-F11	Alexa Fluor 532	Thermo Fisher Invitrogen
F4/80	BM8	PerCP/Cy5.5	Thermo Fisher Invitrogen
CD8a	53-6.7	PerCP-eF710	Thermo Fisher Invitrogen
PDCA	eBio927	PE	Thermo Fisher Invitrogen
CD80	16-10A1	PE/Dazzle 594	BioLegend
CD62L	MEL-14	PE-Cy7	BioLegend
CD11c	N418	Alexa Fluor 647	BioLegend
FOXP3	FJK-16s	APC	Thermo Fisher Invitrogen
CD3	17A2	Alexa Fluor 700	BioLegend
Live/Dead	-	Zombie NIR	BioLegend
NK1.1	PK136	APC-Cy7	BioLegend

Table 2.2: Other antibodies

Marker	Clone	Fluorophore	Vendor
BCL-2	100	BV421	BioLegend
MCL-1	LVUBKM	APC	Thermo Fisher Invitrogen
BCL-xL	7B2.5	FITC	Thermo Fisher Invitrogen
BIM	C34C5	PE	Cell Signaling Technologies
Helios	22F6	PE-Cy7	BioLegend
CTLA-4	UC10-4B9	PerCP/Cy5.5	BioLegend
CD39	Duha59	Alexa Fluor 647	BioLegend
CD73	TY\11.8	APC Cy7	BioLegend
CD25	PC61	PE/Dazzle 594	BioLegend
CD127	A7R34	BV510	BioLegend
IL23R	O78-1208	BV605	BD Biosciences
pAKT (Ser473)	SNRNR	PE	Thermo Fisher Invitrogen
FOXO1	C29H4	PE	Cell Signaling Technologies
pFOXO1 (Ser256)	-	PE	Biorbyt

2.6 Intracellular cytokine staining

Cells were isolated from spleen and lymph nodes as previously described. Cells were washed divided into 96-well U bottom plate cultured with complete RPMI 1640 media, and either left unstimulated or stimulated with 50 ng/µL phorbol myristate acetate (PMA) (Millipore Sigma #P1585), 0.5 µM Ionomycin (Millipore Sigma #I9657) (PMA/Iono), and 1 µL of Golgi Stop (BD Biosciences #554724) per 1mL of media. Cells were stimulated in 37°C for 4 hours. Following stimulation, cells were washed and stained with extracellular antibodies as previously described. Cells were then fixed for 35 minutes at 4°C using Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (Thermo Fisher #00-5521-00). Following fixation, cells were washed and stained for intracellular cytokine antibodies as previously described.

Table 2.3: Cytokine antibodies

Marker	Clone	Fluorophore	Vendor
IL-17A	TC11-18H10.1	PE	BioLegend
IFN-γ	XMG1.2	BV711	BioLegend

2.7 Immunofluorescent imaging flow cytometry

Cells were isolated and stained with extracellular and intracellular antibodies as previously described. Immediately prior to flow analysis, samples were counterstained with DAPI (Miltenyi Biotec #130-111-570) according to manufacturer's protocol. Samples were analyzed on the ImageStreamX MKII via Channel 1 (Brightfield), Channel 2 (FITC), Channel 3 (PE), Channel 7 (DAPI), and Channel 11 (APC).

Table 2.4: Image stream antibodies

Marker	Clone	Fluorophore	Vendor
CD4	GK1.5	FITC	BioLegend
FOXP3	FJK-16s	APC	Thermo Fisher Invitrogen
FOXO1	C29H4	PE	Cell Signaling Technologies

2.8 RNA isolation

Cells were isolated from Foxp3$^{\text{IRES-GFP}}$ as described above and lysed with Trizol (Thermo Fisher #15596026) and total RNA was isolated from each treatment arm using the RNeasy Micro Kit (Qiagen #74004) according to manufacturer's protocol and quantified using DeNovix DS-11 Spectrophotometer. RNA was sent immediately for RNA-sequencing.

2.9 RNA sequencing and data analysis

Library preparation and RNA-sequencing were performed by the University of Chicago Genomics Facility. Samples were sequenced using Illumina NovaSeq with 100bp paired-end

reads. Alignment to murine genome (mm10) was performed using HISAT2. Counts were generated using Rsubread and data was filtered to remove samples with low counts. Differential expression analysis performed using DeSeq2. Gene set enrichment analysis (GSEA) was performed using the Broad Institute's GSEA portal.

2.10 *Ex vivo* suppression assay

Total Tregs and Tcons were isolated and sorted Foxp3$^{\text{IRES-GFP}}$ as previously described. Splenocytes were isolated from C57BL/6 mice and CD3^{+} T cell depleted using the CD3ϵ microbead kit (Miltenyi Biotec #130-094-973). CD3-depleted splenocytes were irradiated at 3000 rads. Following Treg and Tcon isolation, Tcons were labeled with CellTrace Violet (CTV) (Thermo Fisher #C34557) per manufacturer instructions. Tregs were serially diluted in a 96-well U-bottom plate with a highest cell number of 100,000 cells and co-cultured with 100,000 CTV labeled Tcons for ratios of 1:1, 1:2, 1:4, and 1:8 Tregs to Tcons. 250,000 irradiated splenocytes and soluble anti-CD3 antibody (1 µg/mL; BioXCell #BE0002) were added to stimulate the T cells. The cells were incubated for 72 hours and stained with LIVE/DEAD – Fixable Near-IR stain (Thermo Fisher #L34975) and CD4 – APC (BD Biosciences #553051). Cells were analyzed via flow cytometry and data analyzed on FlowJo software.

2.11 *Ex vivo* polarization assay

96-well U-bottom plates were pre-coated with soluble anti-CD3 antibody (1 µg/mL; BioXCell #BE0002). Total Tregs and naive T cons were isolated and sorted from Foxp3$^{\text{IRES-GFP}}$ as previously described. Splenocytes were isolated from C57BL/6 mice, T cell depleted, and irradiated as described above. Naive Tcons were labeled with CTV. 100,000 Tregs were co-cultured with 100,000 labeled Tcons in T$_{\text{H}}$17-polarizing media, which was made using

complete RPMI 1640 supplemented with the following cytokines: IL-2 (RD Systems #402-ML-100), IL-4 (RD Systems #404-ML-010), IFN-γ (RD Systems #485-MI-100), TGF-β (RD Systems #7666-MB-005), and IL-6 (RD Systems #406-ML-005). 250,000 irradiated splenocytes and soluble anti-CD28 antibody (2 µg/mL; BioXCell #BE-0015-1) were added to each well for stimulation. Cells were incubated for 96 hours at 37°C, with a media change at 72 hours. After 96 hours, cells were stimulated and stained for intracellular cytokine staining as previously described.

2.12 ATAC sequencing

Treg cells were sorted from Foxp3$^{\text{IRES-GFP}}$ as previously described. 50,000 cells were lysed with lysis buffer consisting of 10mM Tris HCl pH 7.4 (Sigma-Aldrich #T2194), 10mM NaCl (Invitrogen #AM9760G), 3mM MgCl2 (Sigma-Aldrich #M8787), 0.1% IGEPAL CA-630 (Sigma-Aldrich #I8896). Cells were pelleted immediately after lysis. A master transposition reaction mixture was made from a scaled up recipe of 25 µL 2X Tagment Buffer (Illumina #20034197), 2.5 µL Tagment DNA Enzyme (Illumina #20034197), and 22.5 µL nuclease-free H2O. Nuclei were resuspended in 50 µL of transposition reaction mix and incubated at 37°C for 30 minutes. Nuclei were purified immediately following transposition using a Qiagen MinElute Kit (Qiagen #28004). Transposed DNA was eluted in 10 µL Elution Buffer. Table 2.5 denotes the PCR reaction that was run to amplify the transposed DNA. The reaction was cycled at temperatures and times according to Table 2.6. Each reaction consisted of a unique primer pair from the Nextera Index Kit (Table 2.7).

Primer pairs were selected based off of Illumina recommendations for compatible multiplexing. Sequencing was performed by the University of Chicago Genomics Facility. Samples were sequenced using Illumina NextSeq 500 on a 75bp cassette with high complexity and 50M reads per sample. Analysis was completed in collaboration with Andrew Koh, Ph.D at the University of Chicago. Briefly, ATAC-seq reads were trimmed using SeqPurge and aligned to

Table 2.5: PCR reaction

Reagent	Volume	Vendor
Transposed DNA	10 µL	-
Nuclease Free H_2O	10 µL	-
Nextera PCR Primer 1: i7 Index	2.5 µL	Illumina #FC-131-1001
Nextera PCR Primer 2: i5 Index	2.5 µL	Illumina #FC-131-1001
NEBNext High Fidelity 2X Master Mix	25 µL	NEB #NM0541S

Table 2.6: PCR cycling conditions

Step	Temperature	Time
1	72°C	5 minutes
2	98°C	30 seconds
3	98°C	10 seconds
4	63°C	30 seconds
5	72°C	1 minute
6	-	Repeat steps 3-5, 13X
7	4°C	Infinite

Table 2.7: Nextera primer pairs

Sample	i7 Index	i7 Sequence	i5 Index	i5 Sequence
1	N701	TCGCCTTA	S503	TATCCTCT
2	N701	TCGCCTTA	S504	AGAGTAGA
3	N702	CTAGTACG	S503	TATCCTCT
4	N702	CTAGTACG	S504	AGAGTAGA
5	N703	TTCTGCCT	S503	TATCCTCT
6	N703	TTCTGCCT	S504	AGAGTAGA
7	N704	GCTCAGGA	S503	TATCCTCT
8	N704	GCTCAGGA	S504	AGAGTAGA
9	N705	AGGAGTCC	S503	TATCCTCT
10	N705	AGGAGTCC	S504	AGAGTAGA
11	N706	CATGCCTA	S503	TATCCTCT
12	N706	CATGCCTA	S504	AGAGTAGA

mm10 mouse genome assembly using Bowtie2. Reads were filtered for alignment quality > q30 and purged of duplicates using Picard. Reads mapping to the mitochondrial genome and a custom blacklist [276] were removed. Peaks were called for each sample using MACS2. For each sample, a 501bp fixed-width peak set was generated by extending the MACS2 summits +/- 250bp. Peak sets from all samples were merged, purged of overlapping peaks, and filtered with a peak score-per-million cutoff of 2 to generate an ATAC count matrix. ATAC-seq insert counts for each sample were calculated across all peaks and normalized to the number of fragments intersecting transcriptional start sites. PCA was performed on transcriptional start site-normalized data using the *MAVRIC* R package (Moskowitz and Greenleaf, 2017, manuscript submitted) for the top 35,000 peaks with highest variance. Peaks with variance significantly correlated to a given PC ($P < 0.05$) were identified using *MAVRIC*. To analyze the chromatin accessibility at known TF binding motifs in each sample, we applied ChromVAR [277] to ATAC count matrices. Enrichment of TF binding motifs was determined using HOMER with the command findMotifsGenome.pl.

2.13 Tumor model

MC38 cells were thawed and cultured as previously described. Prior to injection, MC38 cells were suspended at a concentration of 10,000,000 cells/mL of PBS. 100,000 cells were subcutaneously injected into the left flank of 6-8 week old C57BL/6 mice. Cells engrafted for 12 days when they reached tumor size of 200 cubic millimeters. Tumor volume was calculated by tumor V = (L x W x W)/2 as measured with digital calipers every other day. Mice were treated orally with venetoclax daily for 14 days.

2.14 Tumor processing and immune cell isolation

MC38 tumors were first dissected and weighed, followed by digestion for 30 minutes at 37°C in tumor digestion buffer while shaking at 200 RPM. The digestion buffer comprised of RPMI (Fisher #11875119), 2% fetal bovine serum, 200 units/mL bovine pancreas Deoxyribonuclease I (Sigma #C5138), 1 mg/mL Hyaluronidase (Sigma #H6254), and 1mg/mL Collagenase Type IV (Sigma #C5138). Digested tumors were mashed through a 70 µm cell strainer and washed twice with PBS. Following the second wash, tumor cells were resuspended in 20mL of PBS. Next, 10mL of Ficoll-Paque (Millipore Sigma #GE17-1440-02) was layered under the cell suspension to enrich for immune cells within the tumor mixture. Following a 20-minute centrifugation at 400xg, the buffy layer was taken and washed with PBS prior to antibody staining.

2.15 Statistical analysis

One-way ANOVA test with multiple comparisons was used to evaluate statistics between three or more treatment groups. Unpaired, Student's t-tests were used for statistical analyses in experiments with only two conditions. All experiments were completed with $n \geq 3$ in order to establish statistical power, with the exception of ATAC-sequencing for which $n = 2$ was used. Statistical significance was considered to be $p < 0.05$ and denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$. All statistical tests were performed using Prism 9 (GraphPad Software).

CHAPTER 3

PHARMACOLOGICAL BCL-2 INHIBITION RESULTS IN TREG PLASTICITY

3.1 Introduction

BCL-2 has been shown to have a variety of non-apoptotic roles, including regulation of metabolism, calcium signaling, mitophagy, and autophagy [211]. Most studies regarding BCL-2 have been conducted using *Bcl-2* knockdown or knockout animals which are useful for studying the functional effect of gene deletion but not translatable to patients. The FDA approval of the BCL-2 inhibitor venetoclax allows us to more effectively assess how pharmacological, rather than genetic, inhibition of BCL-2 affects cellular processes. Among its functions, BCL-2 also plays a large role in T cell differentiation, regulating the positive and negative selection processes that occur. However, not much is known about the roles that BCL-2 plays in mature T cells beyond maintaining survival via inhibition of apoptosis. It has been shown that BCL-2 is downregulated in mouse models of Treg plasticity [49], raising the question of how this downregulation may be contributing to cell plasticity. We hypothesize that inhibiting BCL-2 with venetoclax will destabilize Treg identify and shift the cell towards a T_H-like phenotype.

3.2 Results

3.2.1 Homeostatic BCL-2 blockade results in the upregulation of a T_H17 like gene signature in Tregs but not Tcons

To assess the potential effects of venetoclax on Treg stability, we administered 50mg/kg of venetoclax daily via oral gavage for 14 days to Foxp3$^{IRES\text{-}GFP}$ mice. We then sorted out CD4$^+$ CD25$^+$ GFP$^+$ Tregs and CD4$^+$ CD25$^-$ GFP$^-$ Tcons (Figure 3.1A) and cells were

sent for RNA-sequencing (n = 4). We first looked at key transcription factors that regulate T_H lineage commitment, such as *Rorc* (RORγt), *Tbx21* (T-bet), and *Gata3* as well as genes that have been show to mediate Treg stability, such as *Tcf3* and *Tcf7l1* which encode for the E-proteins (E2A) (Figure 3.1B). We observed that the *Tcf3*, *Tcf7l1*, and *Tcf7l2* are downregulated following venetoclax treatment. Tregs lacking E2A have been shown to further differentiate into effector-like cells accompanied by upregulation of IRF4, ICOS, CD103, KLRG-1, and RORγt [278]. Interestingly, the gene encoding for RORγt is also upregulated following venetoclax. RORγt is typically expressed in T_H17 cells in conjunction with STAT3. This increased expression of *Rorc* suggests that venetoclax-treated Tregs are becoming T_H17-like, which are defined by their co-expression of transcription factors FOXP3 and RORγt. Further assessment of other genes that are typically upregulated in T_H17 cells, including *Il23r*, *Batf*, *Irf4*, and *Stat3* also show higher expression in Tregs following venetoclax treatment (Figure 3.1C). Together, these changes suggest that Tregs are adopting a T_H17 gene signature following venetoclax.

We validated changes in T_H17 gene signatures through FACS analysis of intracellular transcription factor RORγt within the FOXP3$^+$ Treg cell population (n = 6). Indeed, BCL-2 blockade increases both the proportion and number of FOXP3$^+$ RORγt$^+$ Treg cells in the spleen and lymph nodes (Figure 3.1D-F). The changes in pro-inflammatory cytokine expression further validate the suggestion from the RNA-seq analysis that Venetoclax induces Treg to T_H17 cell plasticity. To assess whether these changes observed were specific to Tregs, we analyzed expression of *Stat3*, *Rorc*, and *Il23r* in Tcons via RNA-sequencing following treatment as depicted in Figure 3.1A. Further analysis of these genes in Tcons reveals that these genes that were upregulated in Tregs remain unchanged in Tcons, suggesting that the upregulation of the T_H17 signature by venetoclax is specific to Tregs.

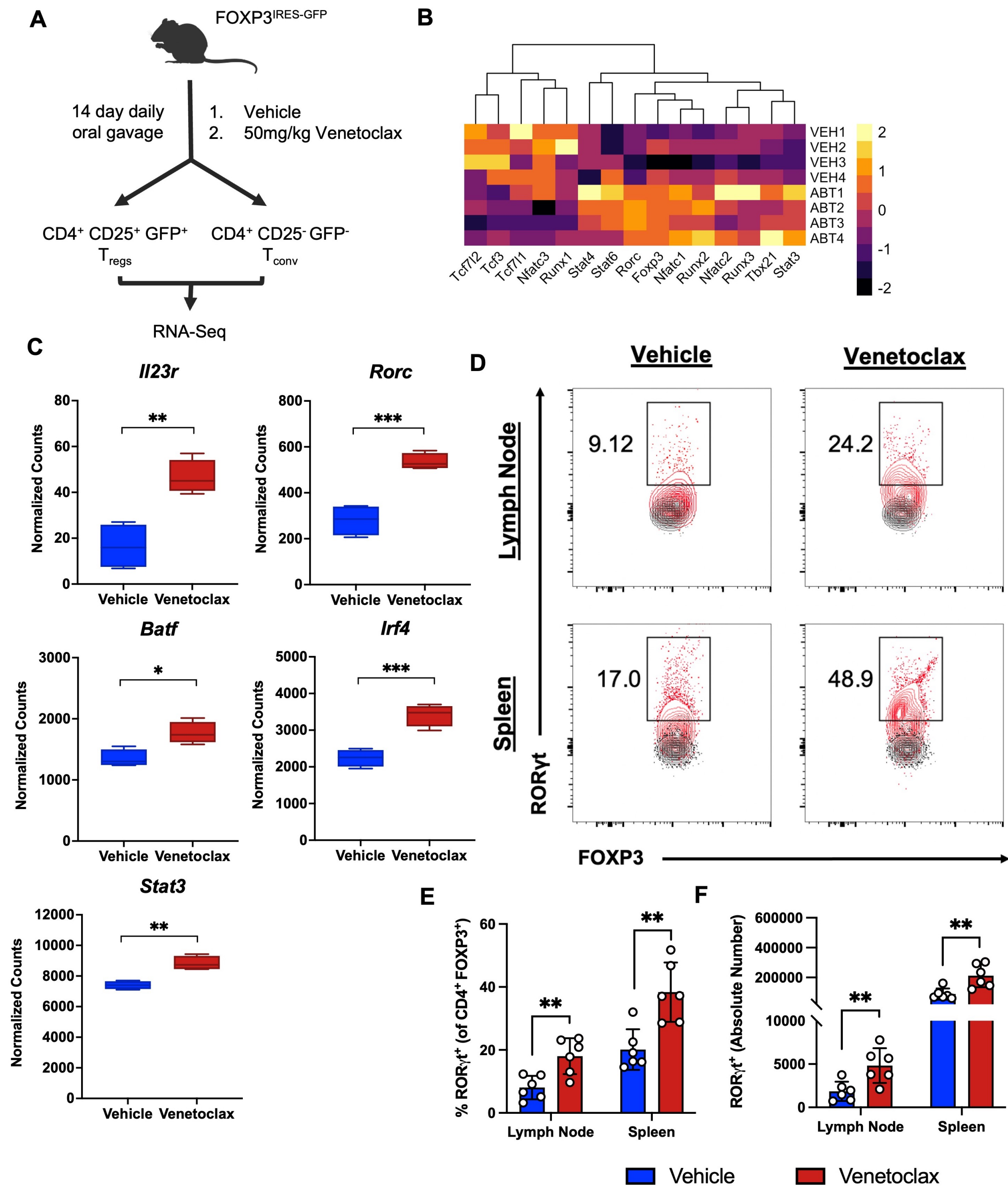

Figure 3.1: BCL-2 inhibition drives increase in T_H17-like Treg cells (A) Treatment schematic for RNA-sequencing. (B) Analysis of normalized gene counts for transcription factors important in T cell lineage commitment. (C) Normalized gene counts for genes important in T_H17 differentiation and stability. (D) Representative flow plots for intracellular RORγt staining. (E) Proportion and (F) absolute numbers for of RORγt$^+$ Tregs. Data represented as means $\pm$ SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

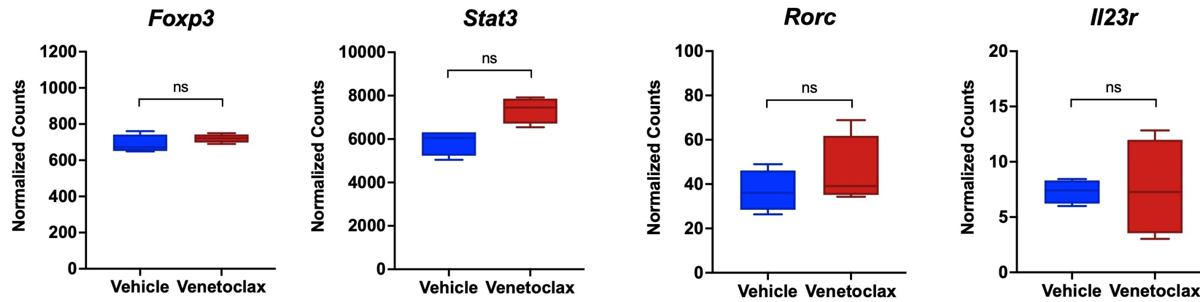

Figure 3.2: Upregulation of T_H17-related genes is specific to Tregs. Analysis of Foxp3, Stat3, Rorc, and Il23r in RNAseq data of Tcons following same venetoclax treatment reveals no change in expression of these genes. Data represented as means ±SD. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.2.2 Changes in RORγt expression of Treg cells are not a result of venetoclax-mediated selection for memory cells

Venetoclax has lympho-depleting effects on the immune system (Figure 3.3), particularly in the depletion of naive T cells and concomitant enrichment of memory cells [221, 273]. It has been previously observed that the memory compartment of Treg cells in humans largely comprises of Th-like cells that phenotypically and functionally mimic conventional $CD4^+$ T cells [41]. Due to the selection of memory cells following venetoclax treatment, we questioned whether the increases in RORγt^+ T_H17-like Treg cells are due to the increase in memory cell population. We first wanted to confirm that venetoclax was also enriching the memory cell population of Tregs. Because Tregs are not canonically characterized by memory status, we assessed a variety of activation markers, including CD44, CD62L, and CD69. Among these, CD44 most accurately separates murine memory cell populations from naive populations and also displayed the best separation between "naive" ($CD44^{lo}$) and "memory" ($CD44^{hi}$) Treg cells. Analysis of CD44 with and without venetoclax reveals that there is also an enrichment of $CD44^{hi}$ cells in both the lymph nodes and spleen following venetoclax exposure ($n = 6$) (Figure 3.4A,B). We next asked whether or not these changes in memory proportion can

explain the changes seen in RORγt expression in Tregs. We observe that RORγt$^+$ Tregs reside in both the CD44^{hi}and CD44^{lo}subsets, as well as an increasing trend of RORγt$^+$ Treg cells in both naive and memory populations as well (Figure 3.4C). This suggests that while the memory enrichment may contribute to the increase in RORγt$^+$ Tregs, it does not entirely account for the total increase as the RORγt$^+$ Tregs are not exclusively CD44^{hi} cells and also reside in the CD44^{lo} compartment which are actively being depleted by venetoclax.

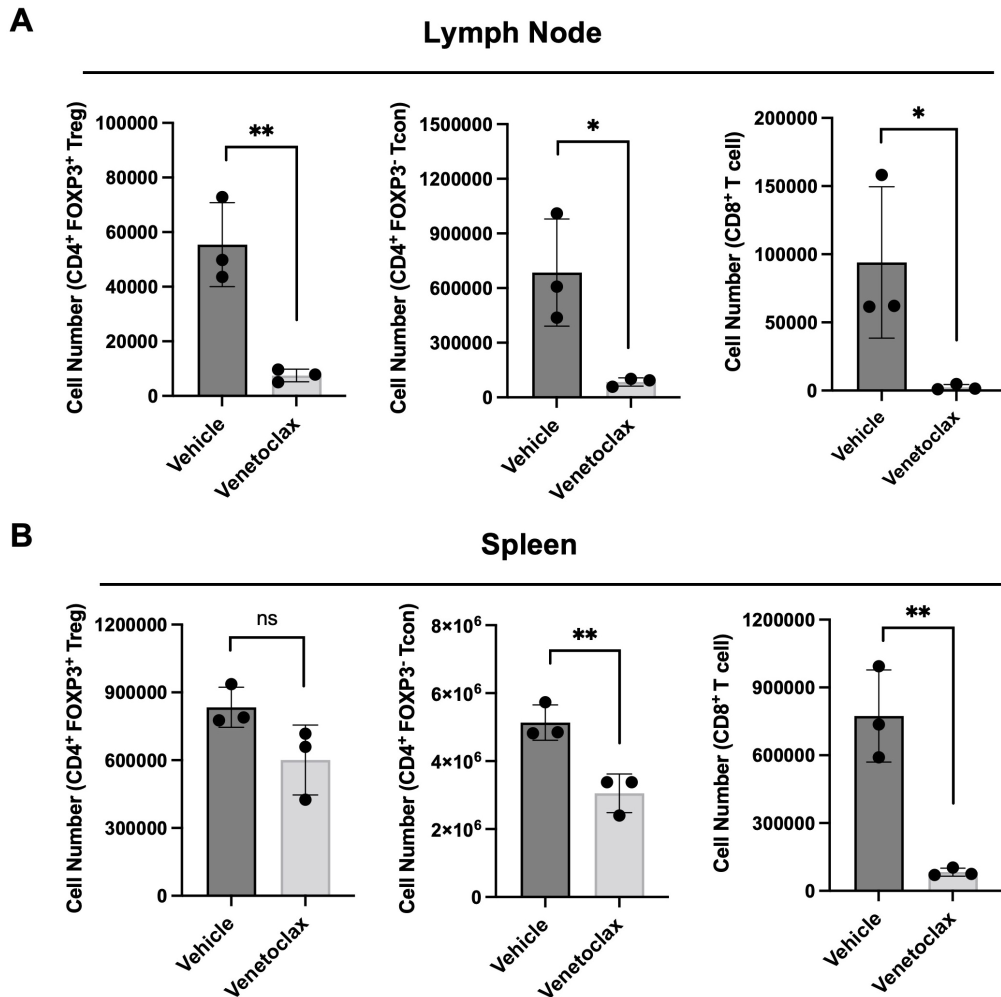

Figure 3.3: Venetoclax treatment results in lymphodepletion Numbers of in CD4$^+$ Treg cells, CD4$^+$ Tcon cells, and CD8$^+$ T cells (A) lymph nodes and (B) spleen. Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

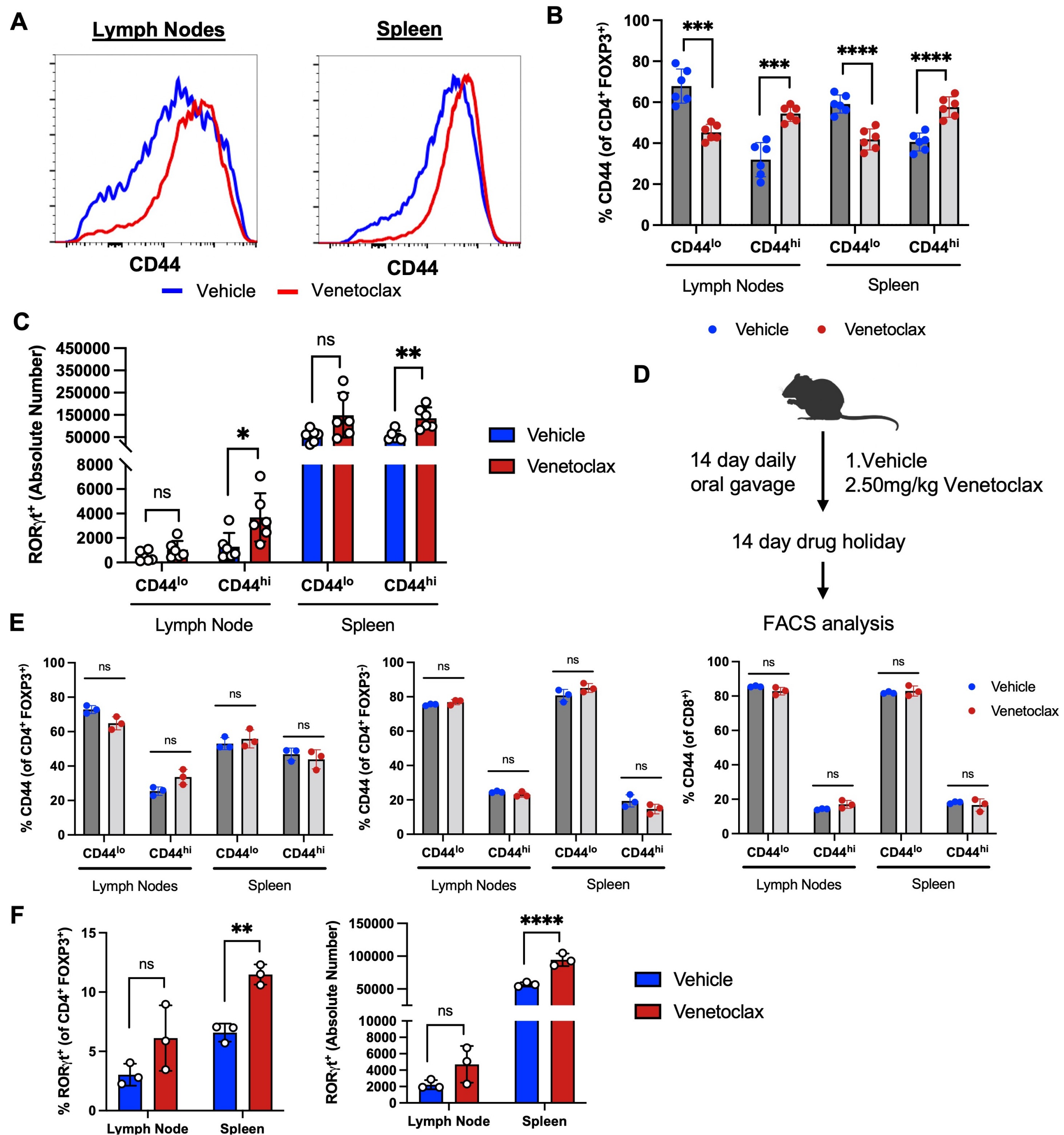

Figure 3.4: Increase in RORγt Tregs is not due to memory cell selection. (A) Representative histograms of CD44 expression of Tregs in LN and spleen with and without venetoclax. (B) Quantification of changes in proportions of CD44^{lo} and CD44^{hi} cells. (C). Absolute numbers of RORγt^{+} Tregs in CD44^{lo} and CD44^{hi} populations. (D) Schematic of treatment to assess phenotype persistence (E) Normalization of CD44 proportions in CD4^{+} FOXP3^{+} Tregs (left), CD4^{+} FOXP3^{-} Tcons (middle), and CD8^{+} T cells (right). (F) Persistence of RORγt^{+} increase in LN and spleen following removal of venetoclax. Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

We next assessed whether removal of the drug could result in normalization of naive cells and reversal of the RORγt$^+$ phenotype. To this end, we treated C57Bl/6 mice at the same dosing schedule, but followed treatment with a 14 day drug holiday (n = 3) (Figure 3.4D). The terminal-phase elimination half-life of venetoclax was previously found to be between 14.1 and 18.2 hours [279], therefore by 14 days of drug removal, there should be very little to no trace of venetoclax remaining in the body. At completion, we analyzed the FOXP3$^+$ Treg cells for expression of FOXP3 and RORγt and memory status. While the observed lymphodepletion following 14 day treatment as well as changes in naive versus memory populations were reverted to homeostatic levels in CD4$^+$ Tregs, CD4$^+$ Tcons, and CD8$^+$ T cells (Figure 3.4E), changes in RORγt$^+$ expression did not revert back in the spleen (Figure 3.4F). Due to the half-life of venetoclax, it is very unlikely that by 14 days, there will be functional amounts of drug left to sustain these effects. This provides further support that the Treg cell plasticity observed is independent of Venetoclax-induced selection of memory cells.

3.2.3 Conditional Bcl-2 knockdown in Tregs confers Treg instability

Due to previously observed BCL-2 downregulation in unstable Treg cells [49], we asked whether BCL-2 has a direct role in mediating Treg plasticity. We observed in our flow analysis of BCL-2 that there exists a bimodal distribution of BCL-2 expression in Treg cells that has not been previously described. Previously, a similar bimodal distribution has been characterized about BIM expression where BIM levels correlated to Treg aging and persistence [22]. There exists a higher proportion of BCL-2^{hi} cells compared to BCL-2^{lo} (Figure 3.5A). The population of BCL-2^{hi} Tregs significantly decreases in LN following venetoclax treatment (Figure 3.5A). Further, analysis of RORγt$^+$ in BCL-2^{hi} and BCL-2^{lo} Treg cells reveals that BCL-2^{hi} Tregs in the LN have significantly lower baseline levels of RORγt$^+$ cells (Figure 3.5B), suggesting a potential role of BCL-2 in maintaining Treg

lineage stability. It is unclear how venetoclax is driving this change in BCL-2^{lo} versus BCL-2^{hi} proportions in lymph nodes, specifically if it's an apoptosis-mediated shift. It is possible that the relative depletion of BCL-2^{hi} cells, which has proportionately lower levels of RORγt^{+} cells, is enriching for the BCL-2^{lo} population with more RORγt^{+}. However, due to the increase in the number of RORγt^{+} cells overall, it is unlikely that this is the result of an apoptotic selection.

To address this question, we generated Foxp3$^{\text{GFP-Cre}}$Bcl-$2^{\text{fl/fl}}$ where conditional *Bcl-2* knockout occurs in Tregs following tamoxifen administration (n = 4). 60% *Bcl-2* knockdown is observed in Tregs but not Tcons or CD8^{+} cells of tamoxifen-treated mice (Figure 3.5C). Tamoxifen-induced BCL-2 knockdown in Tregs was sufficient to increase the proportion of RORγt^{+} Tregs, further validating the role that BCL-2 plays in maintaining Treg stability (Figure 3.5D). However, the percentage of BCL-2^{lo} cells following venetoclax treatment does not nearly recapitulate the knockdown seen with the Foxp3$^{\text{GFP-Cre}}$Bcl-$2^{\text{fl/fl}}$ mice, despite changes in RORγt^{+} proportions being equal or higher than those seen in the conditional knockout mice. This suggests that while high BCL-2 expression aids in Treg stability, it certainly is not the only contributing factor to Treg to T_H17 plasticity.

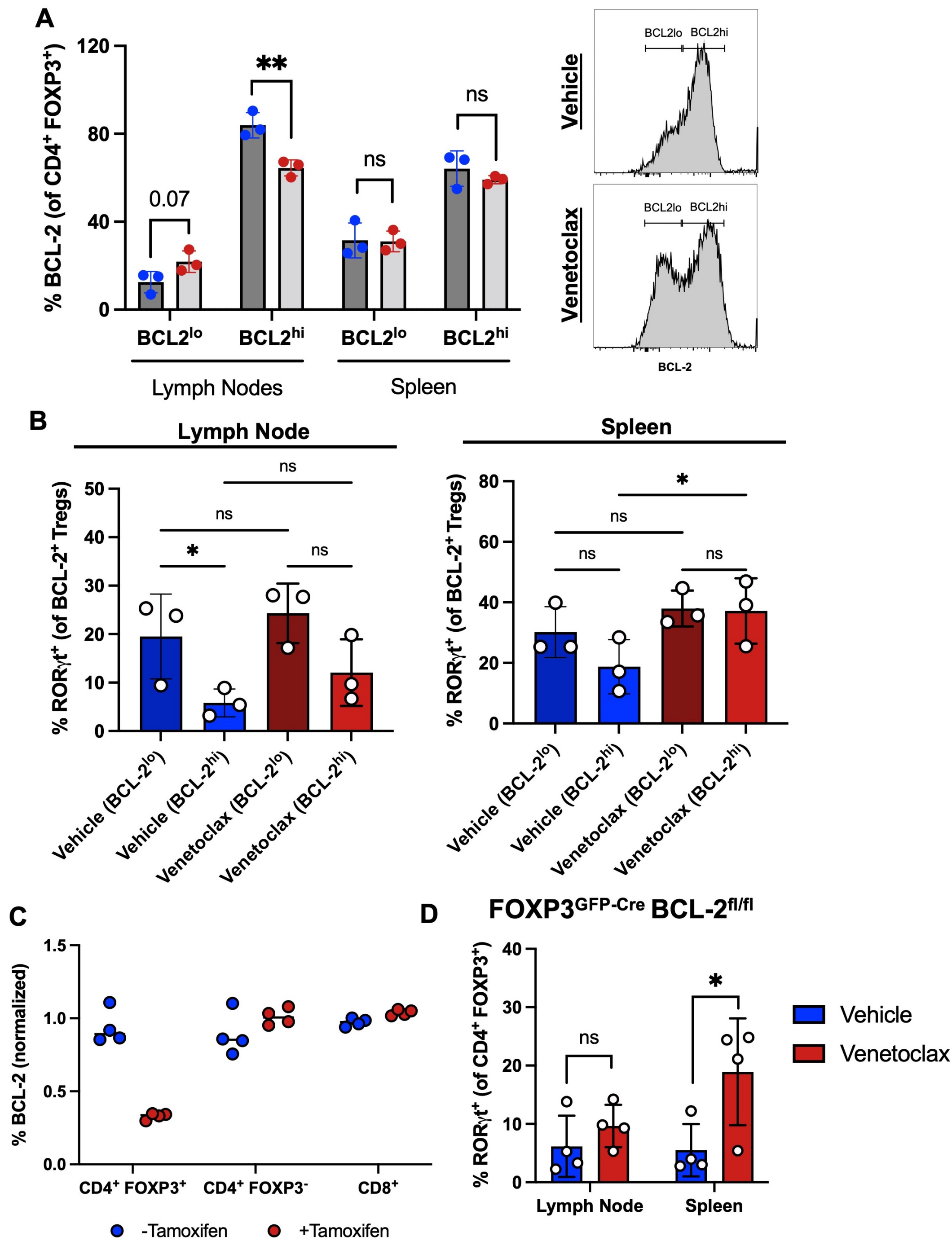

Figure 3.5: High BCL-2 expression stabilizes canonical Treg phenotype. (A) Proportions of BCL-2^{lo} and BCL-2^{hi} Treg cells in LN and spleen. (B) Proportion of RORγt^{+} Tregs in BCL-2^{lo} and BCL-2^{hi} populations. (C) *Bcl-2* knockdown in CD4^{+} FOXP3^{+} Tregs, CD4^{+} FOXP3^{-} Tcons, and CD8^{+} T cells following tamoxifen administration in Foxp3$^{\text{GFP-Cre}}$BCL-2$^{fl/fl}$ mice. (D) Proportion of RORγt^{+} Tregs in LN and spleen in *Bcl-2* knockdown cells. Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

3.2.4 T_H17-like Treg cells express increased IL-17A cytokine

Next we inquired whether Venetoclax induced T_H17 functional profiles in the RORγt$^+$ Treg cells. Previous studies show that while T_H17-like Treg cells still maintain their suppressive function, they have been shown to consistently produce IL-17A as well as IFN-γ in various cancer types to promote a pro-inflammatory environment [89, 280]. To assess Treg suppressive function, we conducted an *in vitro* suppression assay where CTV-labelled Tcons were co-cultured with varying concentrations of treated and untreated Tregs. This assay allows for the quantification of the Treg's suppressive effects on Tcon proliferation. Consistent with previous findings [280], venetoclax-induced T_H17-like Tregs showed no difference in their capacity to suppress Tcon proliferation compared to vehicle treated Tregs (Figure 3.6A).

However, despite being functionally suppressive *in vitro*, it is the ability for T_H-like Tregs to secrete pro-inflammatory cytokines that marks its role in autoimmune pathogenesis [84, 85, 88]. Therefore, we assessed the expression of IL-17A and IFN-γ in Tregs following venetoclax treatment (n = 4). Importantly, we found that FOXP3$^+$ Treg cells produce pro-inflammatory cytokine IL-17A following venetoclax exposure upon stimulation with PMA and ionomycin (Figure 3.6B). These minimal but significant changes in IL-17A expression have been shown to contribute to the pathogenesis of autoimmune disease and IBD [88, 89]. This data suggests that venetoclax-treated Tregs are not only phenotypically similar to a T_H17 cell, but functionally similar as well.

We also assessed the ability for the RORγt$^+$ IL-17$^+$ Tregs to polarize naive Tcons as a proxy for the potential effects on the microenvironment as much of the functional effect that Tregs have lies in their ability to interact with other cells. Thus, we sorted and co-cultured CD4$^+$ CD25$^+$ GFP$^+$ Tregs and CD4$^+$ CD25$^-$ GFP$^-$ CD44$^-$ CD62L$^+$ naive Tcons in polarizing conditions to measure the expression of intracellular cytokines in the naive Tcons (n = 7). Following 96 hours of co-culture, naive Tcons co-cultured with venetoclax treated Tregs expressed significantly higher levels of IL-17A compared to Tcons cultured with

vehicle Tregs (Figure 3.6C). Together, these data show that venetoclax not only induces IL-17A expression in Tregs, but these effects on Tregs can result in expression of IL-17A in other T cells. Because IL-17A is a pro-inflammatory cytokine, it will be important to assess the effects of venetoclax-induced Treg plasticity in a disease model.

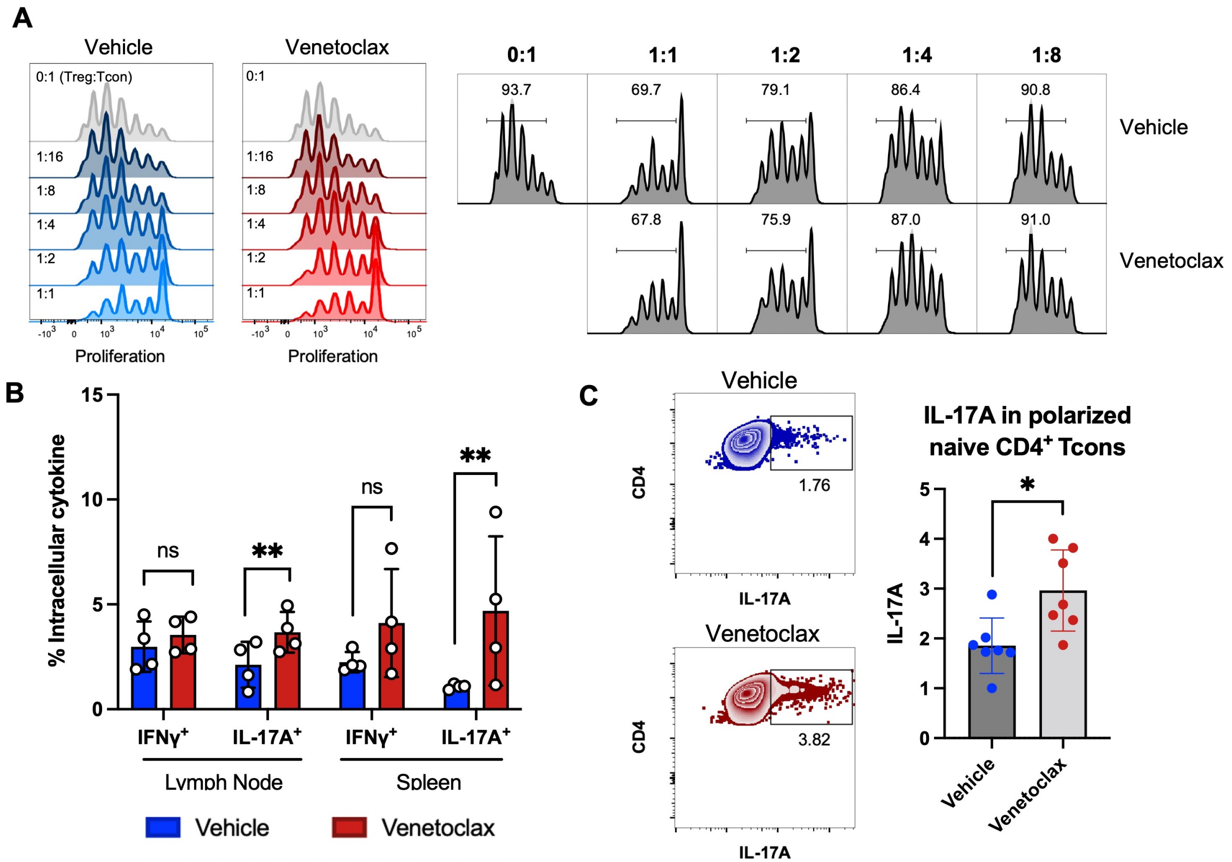

Figure 3.6: Venetoclax induced T_H17-like Tregs express IL-17A and have pro-inflammatory effects on naive T cells. (A) *In vitro* suppression assay. Briefly, CTV-labeled Tcons were co-cultured with treated Tregs at varying ratios. Tcon proliferation was assessed using CTV dilution. (B) Intracellular cytokine staining for IFN-γ and IL-17A. (C) *Ex vivo* polarization assay. Intracellular cytokine expression of IL-17A in naive Tcons following 96 hour co-culture with treated Tregs. Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

3.2.5 Venetoclax treatment results in increased T_H17-like Treg cells in the TME of MC38-bearing mice

Next, we asked how venetoclax treatment affects Tregs in the tumor-infiltrating lymphocyte population (TILs). Previously, Venetoclax was shown to synergize with anti-PD-1 therapy to control tumor progression in a MC38 colon adenocarcinoma model, attributed to an increase in effector CD8$^+$ T cells [273]. However, the potential role of Treg cells in anti-tumor immunity was not elucidated in this model, given that there is evidence to show the importance of Treg plasticity in anti-PD-1 efficacy [50, 275]. To determine whether venetoclax increases the population of RORγt$^+$ Treg cells in this tumor, we performed flow cytometry analysis on tumors excised 26 days following tumor inoculation (14 days after initial Venetoclax dosing) (n = 5) (Figure 3.7A). Of note, colorectal cancers are characterized by high infiltration of RORγt$^+$ Treg at baseline, which has been associated with significant inflammation and tumor progression [89, 281]. Following treatment, tumors excised from mice that received venetoclax weighed significantly less those that received vehicle (Figure 3.7B). Consistent with previous findings, mice that received venetoclax displayed slower tumor growth that their vehicle counterparts (Figure 3.7C). Notably, we observed a significant increase in the percentage and number of RORγt$^+$ Treg cells in the tumor (Figure 3.7D).

CD8$^+$ T cell infiltration has been shown to support the recruitment of FOXP3$^+$ Treg cells into the tumor in various cancer types, and a lower ratio of CD8$^+$ T cells to Treg cells has been associated with poor clinical outcomes due to suppression of an immune response by the recruited Treg cells [117]. Thus, we calculated the CD8:Treg ratio in the TILs and revealed that Venetoclax treatment modestly decreased the ratio of CD8$^+$ T cells to Treg cells, and even further significantly decreased when comparing CD8$^+$ T cells to RORγt$^+$ Treg cells (Figure 3.7E). Decreased CD8:Treg ratios suggest either decreased CD8$^+$ T cells or increased Treg presence in the tumor, both of which are typically associated with immune inhibition and decreased anti-tumor immunity. Increased tumor control in our model despite

lower CD8:Treg ratios suggest an aberrant interaction between $CD8^+$ T cells and Treg cells.

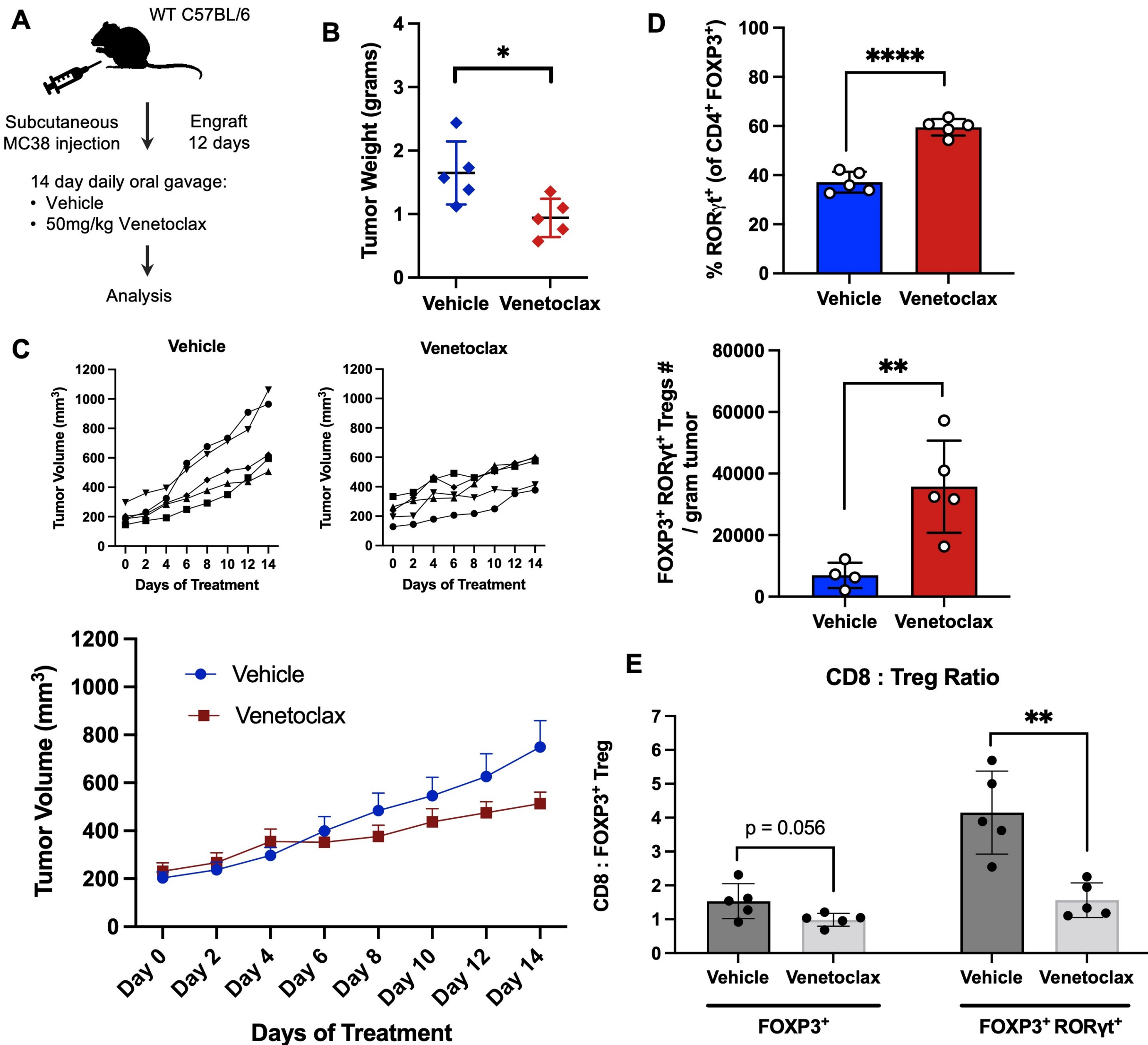

Figure 3.7: BCL-2 inhibition leads to increase in T_H17-like Tregs in MC38 tumor model. (A) Experimental set up. (B) Tumor weights at endpoint. (C) Tumor growth curves during venetoclax treatment. (D) Proportion (left) and numbers per gram of tumor (right) of $ROR\gamma t^+$ Tregs. (E) CD8:Treg ratios. Tumor data represented as means ± SEM. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. All other data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

3.2.6 Treg-specific Bcl-2 knockdown synergizes with anti-PD-1

Next, we wanted to assess the role of Tregs itself in this MC38 model. To do this, we conditionally knocked down *Bcl-2* in Tregs specifically as we previously showed that *Bcl-2* knockdown in the spleen was sufficient to confer the induction of T_H17-like Tregs. The hypothesis here was that Treg-specific BCl-2 knockdown would induce T_H17-like Tregs in the tumor microenvironment, and that this would synergize with anti-PD-1 without venetoclax treatment. We inoculated $Foxp3^{GFP\text{-}Cre}Bcl\text{-}2^{fl/fl}$ with MC38 cells subcutaneously (Figure 3.8A). In addition to a single agent venetoclax, we also included single agent anti-PD-1, combination of venetoclax and anti-PD-1, or single agent anti-PD-1 with tamoxifen administration to induce Treg specific *Bcl-2* knockdown. Single agent anti-PD-1 was sufficient to delay tumor progression (Figure 3.8B), but did not result in any complete responses (Figure 3.8C). The combination of venetoclax and anti-PD-1 together resulted in 2 out of 5 complete responses, although average tumor sizes were similar to those of anti-PD-1 alone (Figure 3.8C,D). The greatest tumor control was seen in single agent anti-PD-1 with conditional *Bcl-2* knockdown, resulting in 4 out of 5 complete responses (Figure 3.8C,D). The data here shows that Tregs themselves play a large role in the response to anti-PD-1.

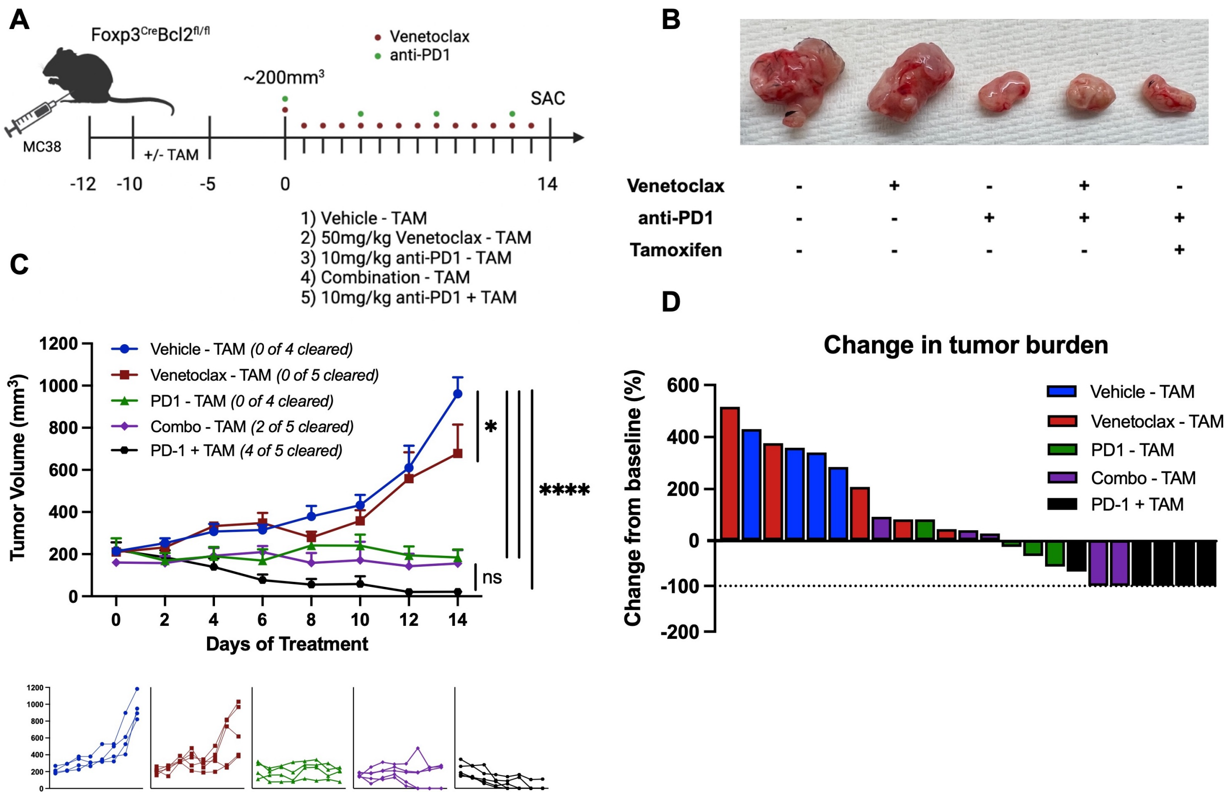

Figure 3.8: Treg-specific *Bcl-2* knockdown synergizes with anti-PD-1 in MC38 tumor model. (A) Experimental set up. (B) Representative tumor images from each condition. (C) Tumor growth curves during treatment with single agent venetoclax, single agent anti-PD-1, combination of venetoclax and anti-PD-1, or single agent anti-PD-1 with Treg specific *Bcl-2* knockdown. Combined growth curves (top) and individual growth curves (bottom). (D) Waterfall plot of change in tumor burden from baseline depicting complete responses in 4 out of 5 mice who received anti-PD-1 in conjunction with Treg specific *Bcl-2* knockdown. Tumor data represented as means ± SEM. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. All other data represented as means ± SD. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3.3 Conclusions

In this chapter, we discussed the effects of long-term BCL-2 inhibition via venetoclax treatment on the homeostasis on Treg cells. RNA-sequencing first revealed the upregulation of a T_H17 gene signature in Tregs following 14 day venetoclax treatment. This gene signature was accompanied by the downregulation of E-protein genes, which have been shown to play

a role in Treg stability. This was further validated via intracellular flow for RORγt, the key transcription required for T_H17 differentiation. We also confirmed that venetoclax treated Tregs express IL-17A, a key finding to validate the functional effect that venetoclax has on Tregs. The functional alterations were further assessed through a polarization assay to quantify the potential effects the T_H17-like Tregs may have on the immune microenvironment. Finally, we assessed whether these changes observed in normal mice were recapitulated in tumor-bearing mice. To this end, we used an MC38 tumor model of murine colon carcinoma and treated the mice with the same dose of venetoclax. We found that venetoclax also increases RORγt$^+$ Tregs in the tumor microenvironment, with altered CD8:Treg ratios suggesting a potential role of T_H17-like Tregs in the anti-tumor immune response. Further, we observed that conditional *Bcl-2* knockdown in Tregs cells is sufficient to confer synergy with anti-PD-1 checkpoint blockade in the MC38 model, providing further evidence that BCL-2 modulation affects the roles that Tregs play in the efficacy of cancer immunotherapy.

In order to fully understand how venetoclax is inducing Treg plasticity, we used Foxp3$^{\text{GFP-Cre}}$Bcl-2$^{\text{fl/fl}}$ mice to assess the role of BCL-2 in Tregs. Conditional knockdown of *Bcl-2* in Tregs was sufficient to induce upregulation of RORγt, suggesting a direct role of BCL-2 in maintaining Treg stability. However, the upregulation of RORγt in *BCL-2* knockout mice was not as potent as seen in that of venetoclax treated mice, suggesting additional mechanisms of venetoclax-induced plasticity apart from BCL-2 inhibition that have yet to be explored. In the next chapter, we will discuss potential mechanisms as to how Treg plasticity may be occurring following venetoclax treatment.

CHAPTER 4

POTENTIAL MECHANISMS OF BCL-2 MEDIATED TREG PLASTICITY

4.1 Introduction

Originally, $CD4^+$ T cells were believed to be terminally differentiated once they have undergone lineage commitment, whether that commitment is a Treg or a T_H helper subset [282]. However, it has become increasingly clear that this characterization is insufficient to describe the complexity of $CD4^+$ T cell differentiation, particularly in humans, and T cell subsets are not all terminally differentiated cells, but rather exhibit characteristics of plasticity. In the previous section, we described the phenomenon of Treg plasticity towards a T_H17 cell as a result of BCL-2 inhibition by venetoclax. These cells are characterized by an increase in RORγt and IL-17A expression, as well as their ability to polarize naive T cons. Here, we will discuss the potential mechanisms of Treg plasticity in our system, and begin to deduce how this plasticity may be occurring.

Past studies done on mechanisms of Treg plasticity include upregulation of epigenetic regulation of FOXP3, PI3K-AKT-FOXO1 activation, and IL-6 signaling. Due to the complexity of cell plasticity, there exists the possibility that these previously described mechanisms have some degree of interplay. For example, IL-6 has been shown to induce Treg destabilization by aiding in the methylation of the *Foxp3* locus in a *Dnmt3a*-dependent manner [65]. Therefore, it is important to assess a variety of potential pathways and the connections between these pathways in order to fully delineate how venetoclax-mediated Treg plasticity is occurring. We use ATAC-sequencing as a preliminary screen to check for potential epigenetic alterations, assess for intracellular protein expression of PI3K signaling, as well as look for changes in IL-6 production. It has been shown that monocytes are a large contributor to the presence of circulating IL-6. Further, we previously discussed that differential expression

of BCL-2^{lo} versus BCL-2^{hi} mediates Treg stability, where higher proportions of BCL-2^{hi} Tregs are more stable, and BCL-2^{lo} have higher percentages of RORγt$^+$ Treg cells. Thus, it will also be important to elucidate this mechanism and how this may affect other pathways involved in Treg stability and plasticity.

4.2 Results

4.2.1 ATAC-sequencing reveals global changes in chromatin accessibility and increased accessibility of RORγt binding motifs

In order to assess the potential epigenetic mechanism of venetoclax-mediated Treg to T_H17 plasticity, we conducted ATAC-sequencing to look at changes in chromatin accessibility (n = 2). Analysis of accessibility allows us to better delineate at what level at which these changes in Tregs may be occurring. Interestingly, analysis of accessibility showed that there are global changes in state of open and closed chromatin in Tregs following venetoclax treatment (Figure 4.1A), suggesting that there are larger epigenetic alterations that may be affect Tregs outside of just plasticity. Deviation scores were calculated using chromVAR which first computes a "raw accessibility deviation" that represents the difference between total number of fragments mapping to peaks containing the motif and the total expected number of fragments based on the average of all cells [277]. This is then used to compute a z-score that is corrected for bias that indicates the gain of loss of accessibility of a specific motif relative to the average. Deviation scores for binding motifs of key transcriptions factors involved in Treg plasticity, including *Batf*, *Irf4*, and *Stat4* show changes in accessibility between vehicle and venetoclax treated samples (Figure 4.1B). Specific analysis of RORγt and FOXP3 show that accessibility of RORγt motifs are amongst the most significantly increased following venetoclax treatment (Figure 4.1C,D) and accessibility of FOXP3 motifs are decreased (Figure 4.1D). Further, the transcription factor with the greatest decrease in accessibility of its binding motifs is

Foxo1, alluding to changes in PI3K-AKT-FOXO1 signaling (Figure 4.1C,D). Comparison of motif enrichment in venetoclax-treated Tregs versus untreated Tregs compared to *in vitro* induced $\mathrm{T_H}$17 cells versus Tregs show a correlation between enrichment of binding motifs of transcription factors important for $\mathrm{T_H}$17 cell differentiation (Figure 4.1E).

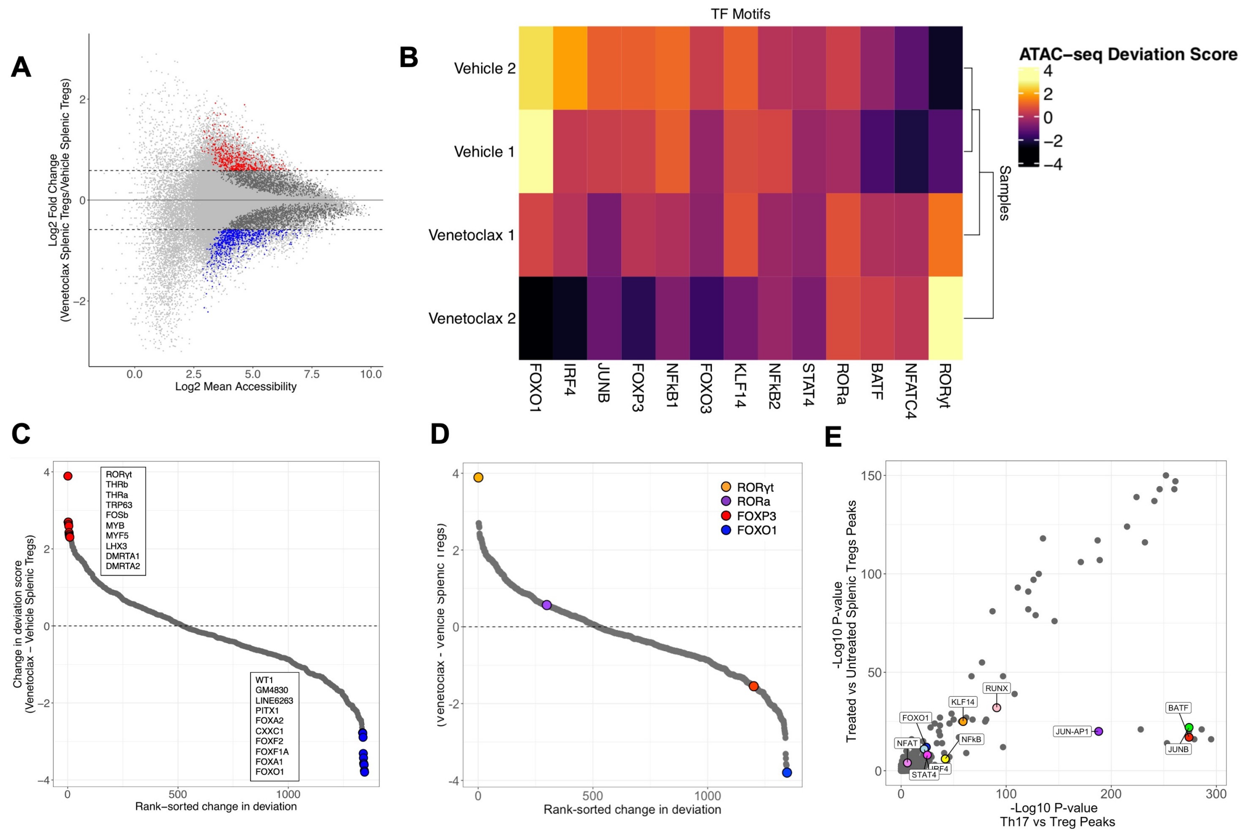

Figure 4.1: Changes in chromatin accessibility of venetoclax- treated Tregs reflect $\mathrm{T_H}$17 signature. (A) Global accessibility changes in splenic Tregs. Light grey points (77,715) represent all peaks in peak set. Darker gray points (3,826) are peaks that correlate with principal component 1 when plotting treated versus untreated samples which were used for further analysis. Red points (486) are peaks that have a fold change of 1.5 or greater and blue points (667) have a fold change of 0.66 or less. (B) Binding motifs of $\mathrm{T_H}$17-related transcription factors show changes in accessibility between vehicle and venetoclax Tregs (C) Top 10 open and closed genes in venetoclax-treated Tregs compared to vehicle. (D) Accessibility of binding motifs for *Rorc*, *Rora*, *Foxp3*, and *Foxo1*. (E) Correlation between enrichment of binding motifs of transcription factors important for $\mathrm{T_H}$17 cell differentiation in venetoclax treated versus vehicle Tregs and bona fide $\mathrm{T_H}$17 cells versus induced Tregs.

4.2.2 BCL-2 inhibition in T cells activates PI3K signaling

FOXO1 has been implicated in regulation of Treg development and function. Specifically, FOXO1 is able to stabilize FOXP3 transcription and facilitate the nuclear export of other transcriptional modulators such as STAT3 [283]. Further, FOXO1 binds to the DNA binding domain of RORγt and inhibits RORγt-mediated transcription of T_H17 related genes [284]. Previously, we saw activation of downregulation of FOXO1 signaling in naive T cells following congenic transplant [221]. We first confirmed this finding via intracellular flow staining of three nodes along the PI3K pathway: phospho-AKT serine 473 (pAKT) to assess direct PI3K activation, total FOXO1 and phospho-FOXO1 (pFOXO1) (n = 5). PI3K inhibits FOXO1 via pAKT-mediated phosphorylation at serine 256 after which FOXO1 is excluded from the nucleus. Venetoclax treatment resulted in increased pAKT expression, indicating that PI3K was indeed active in both LN and spleen of $CD4^+$ $FOXP3^-$ Tcons (Figure 4.2A) and $CD8^+$ T cells (Figure 4.2B). Interestingly, we also saw an increase in total FOXO1 levels (Figure 4.2). However, this was accompanied by an increase in pFOXO1, suggesting that FOXO1 is being inhibited by pAKT but may be aggregating in the cytoplasm prior to being degraded.

We next focused in on Tregs to assess PI3K activation in Tregs. We observed a significant decreased in accessibility of FOXO1 binding motifs in our ATAC-sequencing data (Figure 4.1C,D), suggesting a change in FOXO1 signaling in Tregs. To confirm the PI3K-activating effects of venetoclax in Tregs, we first looked at *Pik3ca*, *Akt1*, and *Foxo1* gene counts from the RNA-seq (Figure 3.1A) and found increases in RNA expression in all three genes upon BCL-2 inhibition (Figure 4.3A). Similar to other T cells, pAKT, FOXO1, and pFOXO1 are all increased in Tregs as well following venetoclax treatment (n = 5) (Figure 4.3B), confirming that PI3K activation is a T cell lineage-agnostic consequence of BCL-2 inhibition, contrary to the upregulation of T_H17-related genes. Due to the increases seen in total FOXO1 levels, which is indicative of FOXO1 aggregation, we conducted ImageStream analysis to assess where FOXO1 was localizing. Five mice were used in each treatment group but data was

extracted from multiple cells at the single-cell level within each sample with a total of 142 and 188 data points for vehicle and venetoclax treatments in the lymph node, respectively 114 and 81 data points for vehicle and venetoclax treatments in the spleen. Co-staining of FOXO1 and DAPI showed increased FOXO1 in the cytoplasm of Tregs in both lymph nodes and spleen (Figure 4.3C,D).

Finally, to further understand how BCL-2 inhibition affects Treg cells and the role of PI3K upregulation and subsequent FOXO1 inhibition, we re-analyzed our RNA-sequencing data on Foxp3$^{\text{IRES-GFP}}$ mice treated daily with Venetoclax for 14 days ($n = 4$) and looked specifically at *Foxo1*-regulated genes [285]. Analysis of *Foxo1*-regulated genes reveals dysregulation of FOXO1 signaling in the Venetoclax treated Treg cells (Figure 4.3E). Genes that exhibited lower expression in vehicle Tregs, such as *Il7r*, *Igf1r*, *Socs3*, *Cd83*, and *Jak2* are upregulated with BCL-2 inhibition (Figure 4.3E). Conversely, genes that had higher expression in vehicle-treated Tregs, including *Klf2*, *Bach2*, *Ccr7*, and *Pdk1*, are downregulated (Figure 4.4E).

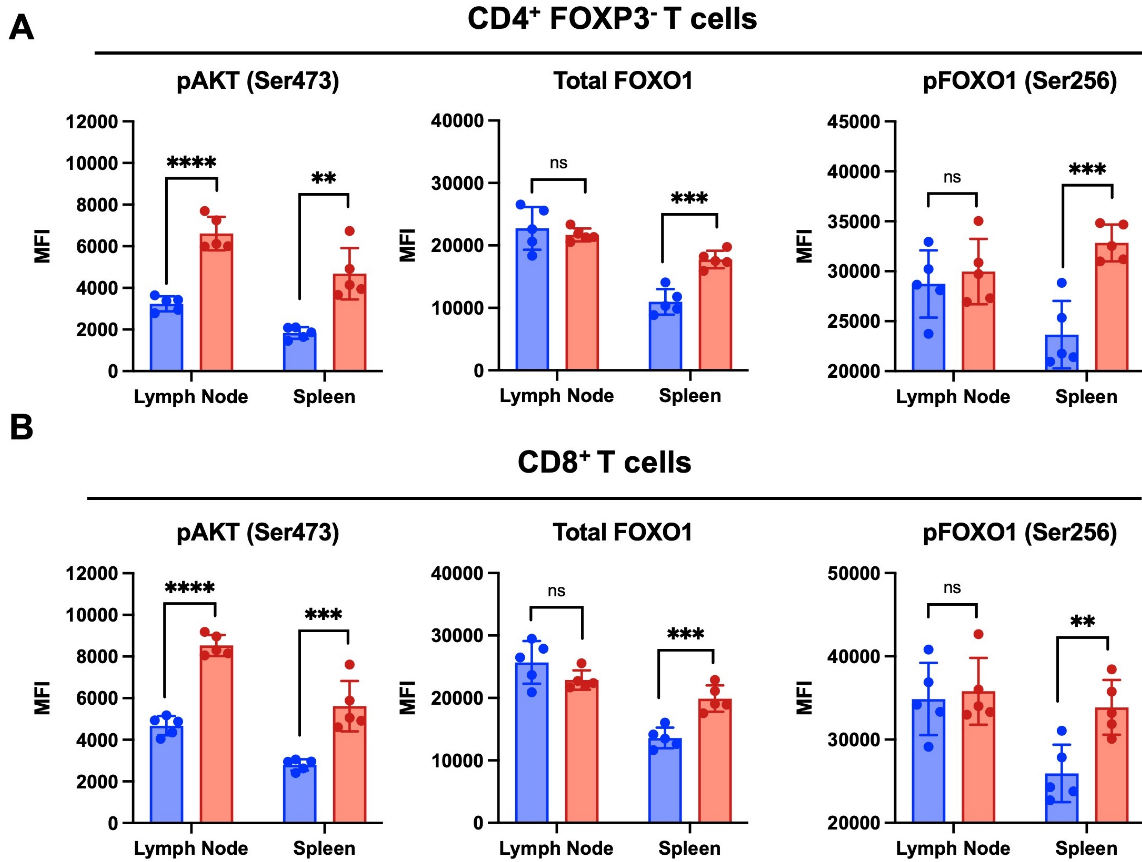

Figure 4.2: Activation of PI3K pathway in CD4$^+$ FOXP3- Tcons and CD8$^+$ T cells following venetoclax treatment. Phospho-AKT (Ser473) (left), total FOXO1 (middle), and phospho-FOXO1 (Ser256) (right) expression in (A) CD4$^+$ FOXP3- Tcons and (B) CD8$^+$ T cells. Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Figure 4.3: Activation of PI3K pathway in Tregs following venetoclax treatment. (A) Gene expression of *Pik3ca*, *Akt1*, and *Foxo1* (B) Phospho-AKT (Ser473) (left), total FOXO1 (middle), and phospho-FOXO1 (Ser256) (right) expression in Tregs. (C) FOXO1 localization in cytoplasm. (D) Representative images from ImageStream analysis of FOXO1 aggregation in spleen. (E) Heat map of FOXO1-regulated genes shows disruption of FOXO1 signaling. Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4.2.3 Increased numbers of monocytes following venetoclax

Because venetoclax is a systemic drug and there is so much cross-talk between different immune populations, it is imperative to assess its effect on other cells in the immune system. In order to assess how venetoclax may be affecting the immune system as a whole, we conducted a comprehensive immune panel to assess potential changes in immune cell populations as indicated in Table 2.1.

Analysis of immune populations in the spleen (n = 3) show that numbers of $CD11b^{+}$ $Ly6C^{+}$ $F480^{-}$ $MHCII^{lo}$ monocytes is significant increased following venetoclax treatment (Figure 4.4A). We also observed a significant decrease in natural killer (NK) cell populations (Figure 4.4C), but no significant changes in macrophages, dendritic cells (DCs), neutrophils or B cells (Figure 4B, D-F). Upon this observation, we also analyzed the monocyte population in mice treated with venetoclax followed by a drug holiday (n = 3), and found that percentage and numbers of monocytes remain significantly increased despite removal of drug (Figure 4.4F). As previously discussed, the terminal half-life of venetoclax is around 18 hours, and therefore it is very unlikely that there will be enough drug remaining in the body to sustain these effects. Finally, we also see an increase in the number of monocytes per gram of tumor in the MC38 tumor model (Figure 4.4H). Together, this data suggests that venetoclax is causing a substantial increase exclusively in the number of monocytes.

There have not been many studies completed on the effect of BCL-2 inhibition on monocytes in particular, however anecdotal data from patients show that CLL patients treated with a combination of venetoclax and ibrutinib display an increase in HLA-DR on monocytes compared to patients who received ibrutinib alone [286]. There may be a deletional effect of venetoclax on the monocyte population via apoptosis that may account for the increases observed, but this does not explain the increases in absolute number that we observe in our data. While a small sample size, this data together with our observations suggest there may be a functional effect of venetoclax on the monocyte population. Monocytes are a

key producer of IL-6, a cytokine that has previously been implicated in modulating FOXP3 expression by regulating methylation at the CNS2 region [65]. Future studies will require the assessment of IL-6 produced by monocytes following venetoclax administration, and how this change might affect Tregs and other cells in the immune microenvironment.

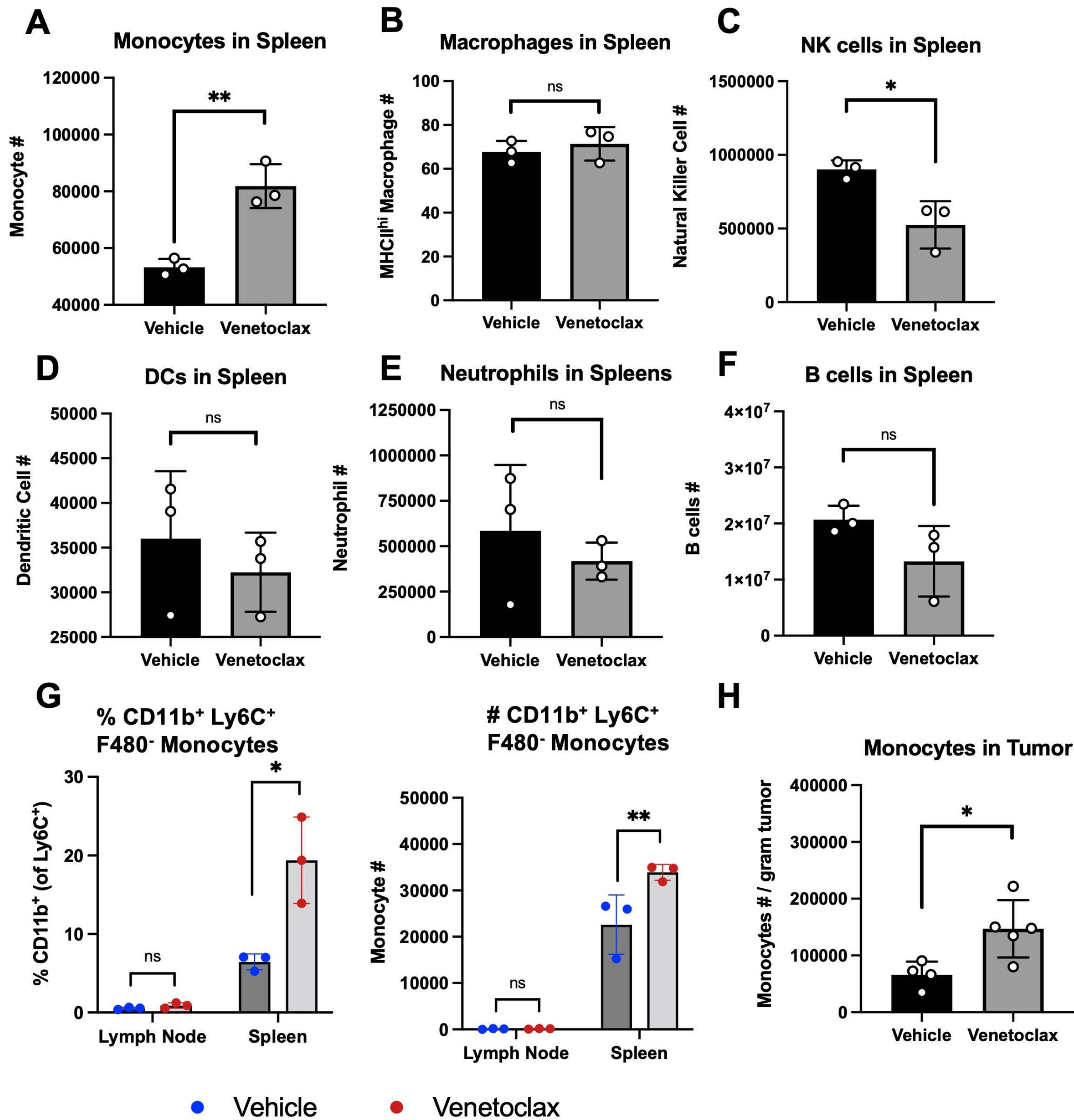

Figure 4.4: Changes in non-T cell immune populations. Numbers of (A) monocytes, (B) macrophages, (C) natural killer cells, (D) dendritic cells, (E) neutrophils, and (F) B cells in the spleen following venetoclax treatment. (G) Percentage (left) and absolute number (right) of monocytes following drug holiday. (H) Number of monocytes in MC38 tumor. Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4.3 Conclusions

The mechanism of Treg plasticity is quite complex. Many different mechanisms have been previously described to induce Treg effector differentiation, including methylation of FOXP3, PI3K activation, and IL-6 signaling. Of these, PI3K activation has been the most well studied, although methylation of FOXP3 have been shown to occur in Treg plasticity in patients. We discussed three potential mechanisms that may play a role in venetoclax-mediated Treg plasticity (Figure 4.5). First, ATAC-sequencing revealed that there are global accessibility changes following venetoclax treatment accompanied by increased accessibility of RORγt and decreased accessibility of FOXP3 binding motifs, suggesting a potential epigenetic mechanism at play. Further, FOXO1 binding motifs are observed to have the most significantly decreased accessibility, suggesting an interplay between epigenetics and the second mechanism explored: PI3K activation. Analysis of PI3K activation in T cells overall shows that PI3K is indeed active, with a subsequent inhibition of FOXO1 through phosphorylation at serine256. Finally, we see a significant increase in the number of monocytes present both in normal mice as well as in the tumor microenvironment of MC38-bearing mice. Monocytes are known to secrete IL-6, which has previously been found to contribute to FOXP3 methylation that can confer Treg instability.

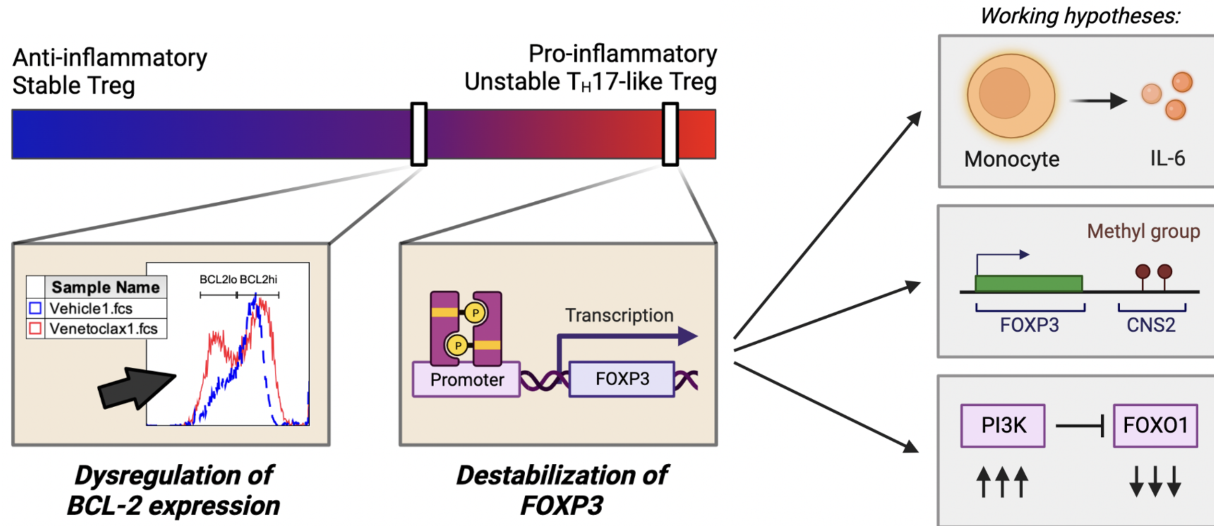

Figure 4.5: Graphical abstract for non-canonical role of venetoclax on Tregs. The first data chapter discusses the phenotypic and functional effects of systemic venetoclax administration on Tregs. We see an increase in RORγt$^+$ Treg cells that also express IL-17A and are able to polarize naive CD4$^+$ Tcons to also express IL-17A, providing evidence that these cells are unstable and adopting a $\mathrm{T_H}$17 phenotype. We attribute this instability partly to the dysregulation of BCL-2 protein expression. However, it does appear that venetoclax exerts an exert that is not specific to BCL-2 inhibition Tregs that allows further plasticity to occur. We hypothesize this may be a result of destabilization of FOXP3, caused by IL-6 signaling, FOXP3 methylation, or PI3K activation.

It is possible that all three changes contributes to the onset of Treg plasticity following venetoclax treatment, considering that inhibition of FOXO1 signaling may have an epigenetic component, and that IL-6 can contribute to DNA methylation. IL-6 result in PI3K activation, furthering supporting the hypothesis that all three of these changes observed may be converging to induce T cell plasticity. It has been established that many of these processes are integrated due varying roles that a singular pathway can play within a cell. Figure 4.6 delineates our hypothesis for how the changes discussed might be interconnected. Due to the complexity of T cell plasticity, further studies will be required to fully establish the exact connection between these three observations and their role in mediating the upregulation of $\mathrm{T_H}$17-related genes.

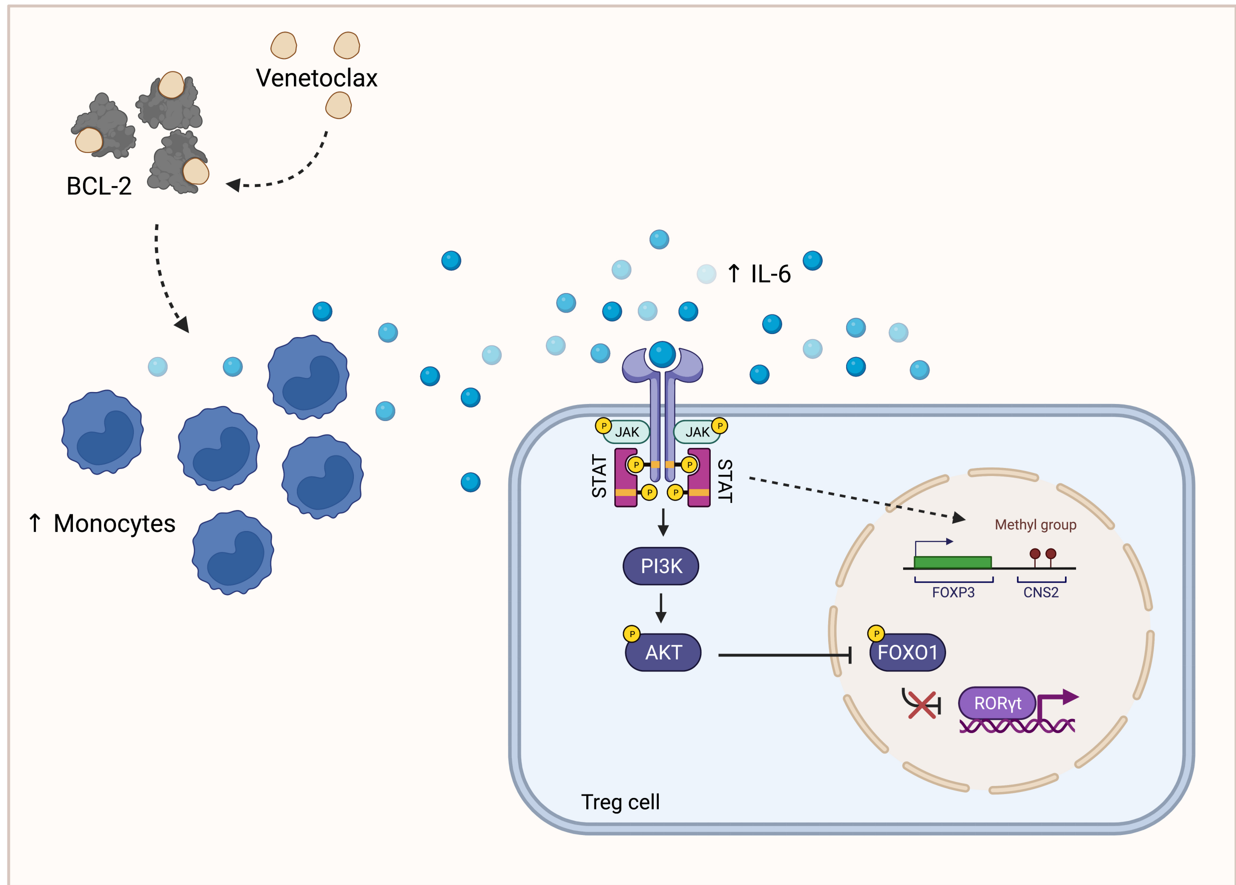

Figure 4.6: Multiple hypothesis integration. Our data provides evidence that venetoclax induces Treg cell plasticity by upregulation of RORγt. Despite the use of a BCL-2 specific inhibitor, we observe several non-BCL-2 factors that may contribute to this plasticity. These factors may all converge upon IL-6 mediated signaling. First, we observe the increase in CD11b^{+} Ly6C^{+} F480^{-} monocytes in the microenvironment. The second observation is the upregulation of PI3K signaling and subsequent inhibition of FOXO1. Finally, we see global changes in chromatin accessibility as well as the specific decrease in accessibility of FOXP3 binding motifs. Further investigation reveals that IL-6 signaling is a common denominator in all three changes observed. Monocytes are a primary secretor of IL-6 in the periphery. IL-6 signaling activates PI3K in T cells and also has been shown to contribute to *Foxp3* methylation. Future studies will investigate further into the validity of these hypotheses.

CHAPTER 5

DISCUSSION

5.1 Conclusions

The presence of Tregs has been associated with a worse prognosis in many cancers; thus identifying novel targeting techniques may prove clinically beneficial. Despite efforts to target Tregs therapeutically to improve anti-tumor immunity, major challenges have hindered clinical efficacy. These barriers have primarily involved the lack of specific, non-redundant targeting modalities on Tregs and the adverse side effects that result from systemic Treg depletion. The general role that Tregs play as mediators of peripheral tolerance lends to the belief that systemic inhibition of Tregs even with a specific target will lead to onset of autoimmunity. Tregs have historically been considered terminally differentiated, characterized by their high expression of cell surface markers CD4 and CD25 and transcription factor FOXP3. However, more recent studies indicate that Tregs can further differentiate into effector T_H subsets, in a phenomenon called Treg fragility or Treg plasticity, in which Tregs with unstable FOXP3 express are able to upregulate effector T cell transcription factors such as T-bet, GATA3, and RORγt. These T_H-like Tregs have been implicated in the pathogenesis of a multitude of autoimmune diseases, which are defined by an overactive immune system. The activation of the immune system is attractive component for cancer immunotherapy and therefore further studies on how T_H-like Tregs develop may allow us to induce this phenotype in cancer to create a pro-inflammatory, immune-activated microenvironment suitable for immunotherapy.

5.2 Future directions

5.2.1 FOXP3 methylation following venetoclax treatment

Epigenetics are critical to the stability and maintenance of Treg identity. It has been observed that complete demethylation of the Treg cell-specific demethylated region (TSDR) is required for optimal expression of FOXP3 [287]. Methylation of *Foxp3* has been observed in T_H1-like Tregs in the microenvironment of patients with multiple sclerosis and type I diabetes [84, 85]. This pathway is dependent on the transcription factor BLIMP-1, which prevents IL-6-mediated activation of DNMT3a which methylates the CNS2 regions of FOXP3 to downregulate its expression [65] (Figure 5.1A). Intriguingly, our RNA-seq analysis shows that the expression of the gene encoding BLIMP-1, *Prdm1*, is significantly increased (Figure 5.1B), suggesting a potential mechanism to keep the system in homeostasis following venetoclax administration. We also observe an insignificant increase in *Dnmt3a* RNA expression which may suggest that the system is being pulled towards a pro-methylation state, but is being kept at homeostasis by the increased expression of BLIMP-1.

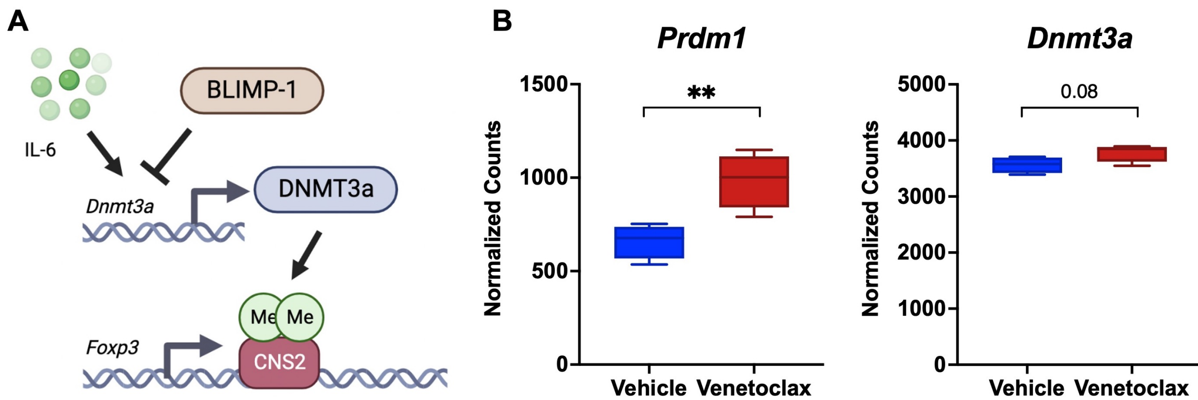

Figure 5.1: IL-6 and BLIMP-1 mediate FOXP3 methylation through regulation of DNMT3a (A) Schematic of IL-6- and BLIMP-1-mediated regulation of FOXP3 methylation through DNMT3a. (B) Normalized gene counts of *Prdm1* which encodes for BLIMP-1 (left) and *Dnmt3a* (right). Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

To confirm the role of epigenetics in our system, we intend to conduct assays to first assess for methylation of FOXP3. This can be done using a methyl-sensitive PCR or a chromatin immunoprecipitation (ChIP) assay. Another approach would be to conduct ChIP-sequencing, which would provide information on genome-wide profiles of transcription factors, histone modifications, DNA methylation, and nucleosome positioning.

5.2.2 Exploiting upregulated pathways as targets to further drive Treg instability

Another remaining question is whether or not we can fully shift a Treg cell to a T_H17 cell therapeutically. Due to the nature of T cell plasticity, there are measures we can take to further direct the Treg towards a T_H17 phenotype. The balance between Tregs and T_H17 cells teeters on a fulcrum regulated by different pathways on each side. For example, while we observe the upregulation of transcription factor RORγt in Tregs following venetoclax, the majority of Treg cells maintain their low expression of RORγt. Further, our studies only evaluate the FOXP3-expressing Tregs cells, however, it would be interesting to evaluate if there are cells that have fully lost FOXP3 expression that we do not capture in our data. Preliminary evaluation of the RORγt expression in $CD4^+$ $FOXP3^-$ Tcon cells reveal that there are no changes in RORγt expression in non-FOXP3 cells, suggesting that there may not be cells that gain RORγt and lose FOXP3 following venetoclax. Genetic studies suggest this is possible, but the question is how we can do this therapeutically.

The upregulation of BLIMP-1 suggests that there is a "push and pull" mechanism to Treg plasticity. BLIMP-1 has previously been observed to be a regulator of Treg identity by preventing methylation of FOXP3 [65]. Our hypothesis is that both sides of the spectrum are playing tug-of-war, where effects of the drug are driving Tregs towards the T_H17-like state, while upregulation of BLIMP-1 may be pulling the Treg back towards a canonical Treg identity. Exploration of the hypothesis that BLIMP-1 inhibition in combination with

venetoclax would further drive Treg plasticity would be compelling. BLIMP-1 inhibition would theoretically remove the tether that holds Treg identity in place and allow for full release into a T_H17 cell state. BLIMP-1 is just one example of how Tregs may be maintaining this balance between identity and plasticity, and therefore further work will be needed to identify other regulators that can be exploited to alter Treg identity.

5.2.3 Elucidate the mechanism of BCL-2 mediated PI3K regulation in Tregs

Earlier we described the activation of PI3K in all T cells following treatment with venetoclax. We originally believed this to be a potential survival mechanism in response to apoptotic signals via venetoclax, however, it appears this activation may be more complex. In preliminary studies in Foxp3$^{\text{GFP-Cre}}$Bcl-2$^{\text{fl/fl}}$ mice, PI3K activation increases without tamoxifen treatment where BCL-2 is intact in all T cells. This is consistent with the phenotype observed in wild-type mice. However, upon tamoxifen induction where *Bcl-2* is knocked down in Tregs only, all T cells lose PI3K activation despite BCL-2 still being expressed in $CD4^+$ $FOXP3^-$ Tcons and $CD8^+$ T cells (Figure 5.2). The maintained expression of BCL-2 in $CD4^+$ $FOXP3^-$ Tcons and $CD8^+$ T cells indicates that venetoclax retains its target in these cell types, and therefore is able to exert its apoptotic affects. If our hypothesis regarding the activation of PI3K being a survival mechanism, we would have expected to PI3K activation in Tcons despite tamoxifen treatment. This suggests that activation of PI3K is not a general T cell survival mechanism in response to venetoclax but rather a Treg-mediated response. Therefore, it will be important to confirm this observation as well as further elucidate why this activation of PI3K in all T cells is dependent on BCL-2 expression specifically in Tregs.

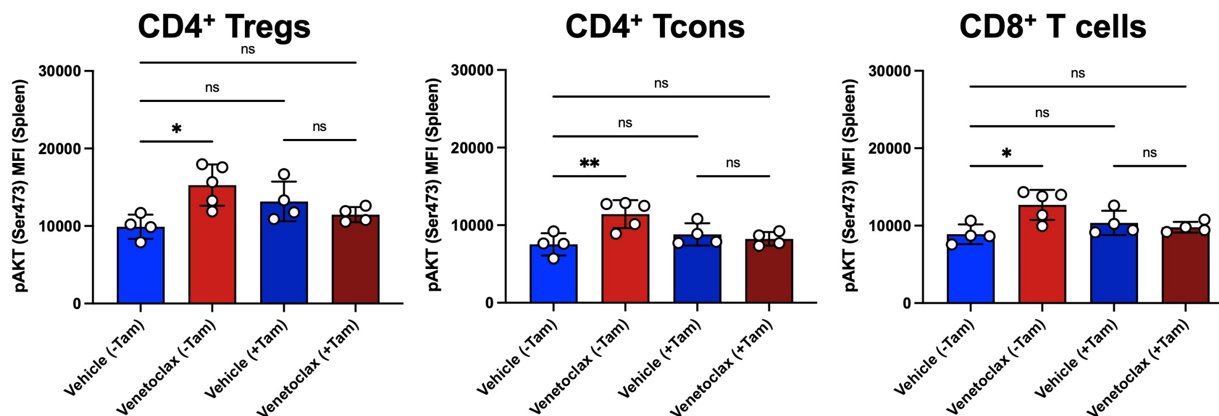

Figure 5.2: PI3K activation T cells in Foxp3$^{\text{GFP-Cre}}$Bcl-2$^{\text{fl/fl}}$ animals. PI3K activation represented by activation of pAKT (Ser473) in CD4$^+$ FOXP3$^+$ Tregs (left), CD4$^+$ FOXP3$^-$ Tcons (middle), and CD8$^+$ T cells (right). Data represented as means ± SD. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Moreover, it is also interesting that knockdown of BCL-2 itself in Tregs does not confer PI3K activation, but the effect is specific to the effect of venetoclax in Tregs as venetoclax administration in Tregs without *Bcl-2* does not activate PI3K. This raises the question of how venetoclax is causing PI3K activation in a BCL-2 dependent manner that is not recapitulated with genetic knockdown. In *Bcl-2* genetic knockout, the BCL-2 protein will no longer be present, but with venetoclax treatment, BCL-2 protein is intact but is dissociated from BH3-only proteins. This suggests that the dissociation of BCL-2 from BH3-only proteins in Tregs but not other T cells is perhaps inducing a non-canonical function of this protein. Thus, it is important to consider investigating how venetoclax may be driving other non-canonical functions of BCL-2 in T cells. We will expand on this concept in another future directions.

5.2.4 Effects of venetoclax-induced PI3K activation on T cells

Due to its role as a master regulator of many cellular processes, PI3K has many downstream signaling pathways outside of regulating FOXO1 activity. Thus, PI3K activation following venetoclax treatment has many implications beyond the potential role it plays in regulat-

ing T cell plasticity [288]. PI3K is a critical regulator of cell metabolism, particularly in upregulating the glucose receptor GLUT1 following T cell activation [51, 289]. mTOR is also a major signaling pathway downstream of PI3K that regulates anabolic metabolic reprogramming in T cells. mTOR complex 1 (mTORC1) is needed for cell cycle entry and also regulates metabolic changes that occur upon T cell activation. Raptor is an essential component of mTORC1 that plays a critical role in regulating *de novo* lipid synthesis and oxidative phosphorylation. Deficiency of *Raptor* in Tregs leads to impaired lipid biosynthesis and mitochondrial respiration [206]. Rictor, an essential component of mTORC complex 2 (mTORC2), has also been implicated in regulating Treg function as *Rictor* knockout is able to rescue *Foxp3* deficiency in Tregs [290].

It would be worthwhile to evaluate the effects of venetoclax on T cell metabolism, and how PI3K activation following venetoclax differentially affects metabolism of $CD4^+$ $FOXP3^+$ Tregs, $CD4^+$ $FOXP3^-$ Tcons, and $CD8^+$ T cells, as these three cell types, despite all be T cells, rely on different metabolic pathways for survival and function. Tregs are generally more reliant on oxidative phosphorylation, while Tcons are more dependent on glycolysis, both of which can be regulated by PI3K signaling. However, we expect that the exact metabolic dependencies of these cells are much more nuanced and continent on the particular subset of T cells they fall into. Given that the BCL-2 family also have non-canonical roles in regulating cell metabolism, it is very possible that there is interplay between these two pathways on regulation of T cell metabolism. These effects have the potential to have a profound impact on the field of cancer immunology, as T cell function is incredibly dependent on their metabolism.

5.2.5 Differential effects on lymphoid tissues

Our data show that effects of venetoclax have more potent effects on Treg plasticity in the spleen than the lymph node. This suggest that 1) there is something biologically different

about Tregs in the spleen compared to lymph nodes that confers differential responses to venetoclax, or 2) there is a tertiary factor in the microenvironment of the spleen that further drives Treg plasticity. Due to the increase in monocytes seen in the spleen following venetoclax, it is possible that this change is responsible for the higher degree of plasticity in splenic Tregs. It has been observed that activated $CD14^+$ monocytes promote T_H17 responses [291]. Therefore, it will be important to elucidate how effects of venetoclax on the microenvironment affect Treg plasticity.

However, intrinsic differences in Tregs of different lymphoid organs have previously been described and therefore could also be a contributing factor. Compartmentalization of Tregs have found to determine suppressive function as well as expression of certain chemokine receptors such as CCR4, CCR7, and CXCR4 [292, 293]. To explore the possibility that biological differences between Tregs in different tissues respond differently to venetoclax treatment, we will perform single-cell RNA-sequencing on venetoclax-treated Tregs isolated from various lymphoid tissues in order to parse out potential differences between these cells.

5.2.6 Comparison of genetic Bcl-2 downregulation versus functional BCL-2 inhibition

Our data comparing the effects of venetoclax to the effects of BCL-2 genetic knockdown seem to suggest that there is some degree of overlap between genetic versus functional BCL-2 downregulation. However, it is important to consider that while these may result in similar outcomes in the context of Treg plasticity, our results do not capture the whole story. As we alluded to in an earlier future directions, the reality is that pharmacological inhibition of BCL-2 using venetoclax likely results in free floating BCL-2 and BIM proteins that have been dissociated from one another in the cell through the competitive binding of venetoclax. Questions remain regarding how these dissociated proteins are behaving in the cell, and whether they are able to exert other functional effects or if they get degraded. Analysis

of protein levels of BCL-2 and BIM following venetoclax treatment seem to suggest that there is some degree is degradation due to decreased protein expression. However, both BIM and BCL-2 exhibit bimodal distributions of protein expression that would suggest even following venetoclax treatment and protein degradation, there remains a population of high expressing cells. It would be interesting to further elucidate how these high-expressing cells are behaving and if they have any non-apoptotic effects in the cell.

5.2.7 Translation to other cancer models

Additional studies in other tumor models would provide further justification for using venetoclax as an immune modulator by mediating Treg plasticity. The MC38 model is characterized by a high baseline level of T_H17-like Tregs, and thus it would be interesting to see whether venetoclax would induce similar changes in other models that do not already have T_H17-like Tregs. Our work in normal, non-tumor-bearing mice would suggest that these changes are non-discriminative towards the tumor type.

Moreover, it would also be of interest to assess how the onset of Treg to T_H17 would affect "cold" versus "hot" tumors and how this may play a role in the efficacy of immunotherapy in various cancer indications. Most cancers express antigens that are able to be recognized by APCs, however, certain tumors lack the proper microenvironmental signals to attract immune infiltration [294]. Cancers that have high T cell infiltration are classified as "hot" tumors, and cancers with little to no T cell infiltration are "cold" tumors. Cold tumors pose a large barrier to immunotherapy, which has largely been dependent on the presence of $CD8^+$ T cells for efficacy. At a foundational level, cold tumors, despite being antigenic, can occur as a result of a few conditions: 1) deficit of APCs in the TME, 2) absence of T cell activation, 3) impaired trafficking or 4) lack of infiltration into the tumor [295]. The induction of Treg instability in the tumor microenvironment may be a possible resolution to some of these challenges, as they would create a more pro-inflammatory environment that

may confer trafficking of T cells to the TME. In particular, T_H17 cells stimulate CXCL9 and CXCL10 to recruit other T cells to the microenvironment [296] (Figure 5.3). The secretion of IL-17A by T_H17 cells as well as the increase in monocytes could also contribute add to the inflammation and recruitment of T cells to the TME (Figure 5.3). This would be contingent on there being at least some T cells in the microenvironment, or the ability of the Tregs themselves to be able to traffic to the TME following induction of plasticity. It may be promising to assess the ability of venetoclax treatment to turn a cold tumor into a hot tumor and if this can be used in combination with immunotherapy that would otherwise not work in the cold tumor.

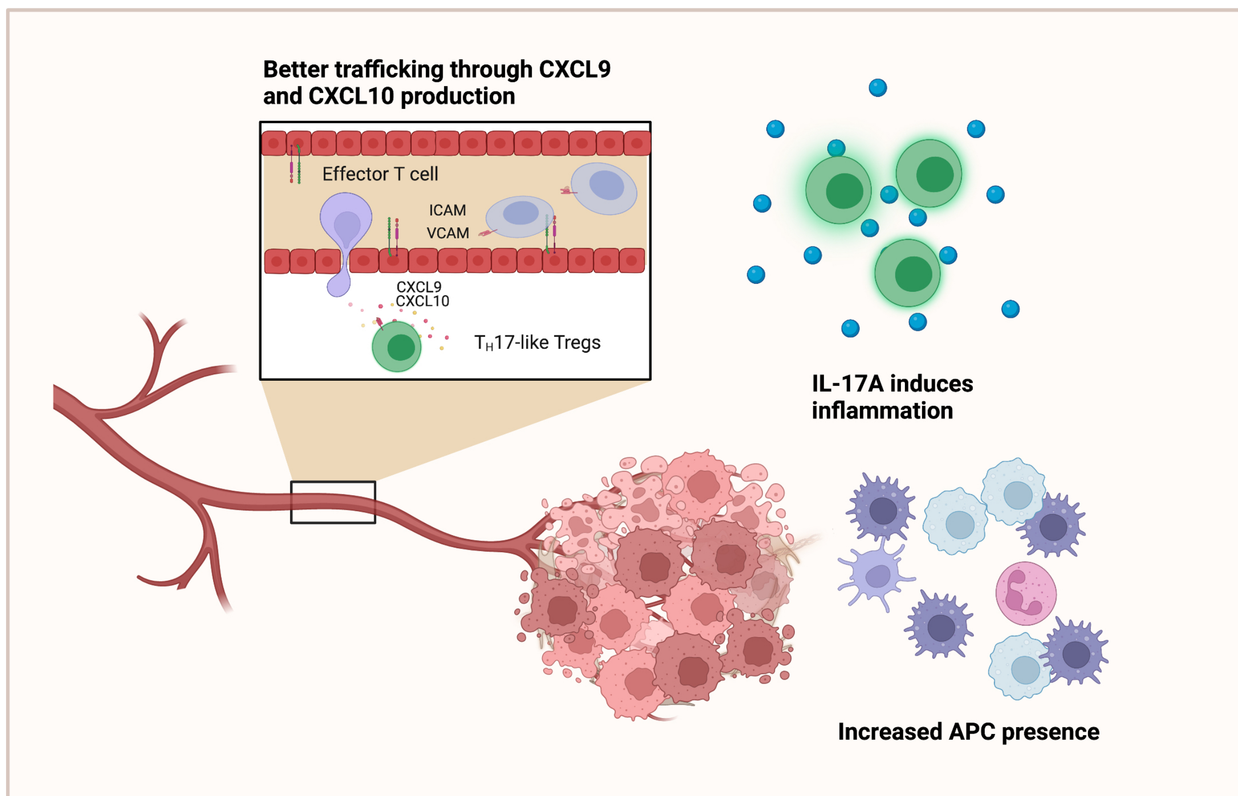

Figure 5.3: Turning a cold tumor into a hot tumor. Potential contributions of venetoclax treatment to the T cell infiltration into tumor microenvironment. Increases in T_H17-like Tregs may produce a more pro-inflammatory environment. T_H17 cells also stimulate CXCL9 and CXCL10 production which aid in the recruitment and trafficking of other immune cells. Venetoclax-mediated increases of monocyte populations may also contribute to increased T cell activation within the TME.

5.3 Considerations

Since its discovery, T_H17 cells have been characterized as a main contributor to autoimmunity. Its discovery also led to the characterization of these cells types in various cancer types and often associated with a worsened prognosis due to its pro-inflammatory properties. On the contrary, Tregs have been viewed as the opposite to T_H17, possessing anti-inflammatory suppressive capacities and being associated with the alleviation of autoimmunity. Despite the evidence suggesting a pro-tumorigenic role of T_H17 cells, recent advances in the cancer immunotherapy suggest that T_H17 cells may play a significant role in anti-tumor immunity.

There is anecdotal evidence supporting the beneficial role of IL-17A producing cells to immunotherapy response in patients [297]. Specifically, a male patient with a history of mild psoriasis and Crohn's disease was receiving pembrolizumab (anti-PD-1 checkpoint blockade) for metastatic colon cancer. The first two rounds of pembrolizumab resulted in 50% reduction in carcinoembryonic antigen (CEA). However, upon the third round, the patient displayed a severe psoriatic rash as well as an exacerbation of Crohn's symptoms [298]. He was given secukinumab, an FDA-approved IL-17A blockade for treatment of psoriasis, to alleviate the psoriatic rashes, which resolved the adverse effects, but profoundly reduced the anti-tumor efficacy of pembrolizumab. This suggests that IL-17A producing cells are critical for the therapeutic benefit of anti-PD-1 therapy. This data supports the preclinical data seen where the presence of T_H1-like Tregs were necessary for efficacy of anti-PD-1 therapy in MC38 [50]. In melanoma patients, presence of IL-17A producing cells is an indicator of anti-PD-1 response [299]. This same analysis showed that monocytes were the biggest predictor of efficacy to pembrolizumab, another cell type that increases upon venetoclax treatment. Both pieces of evidence suggest that while T_H17 cells have been thought to worsen cancer prognosis at baseline, their presence may play an important role in the efficacy of cancer immunotherapy.

While it is difficult to pinpoint the exact role that Tregs are playing in the tumor models studied, the synthesis of the data we gathered combined with previous studies on Treg instability as well as the evidence from patient data together suggest that Tregs are playing a role that is much more complex than originally believed. We surmise that shifting the balance of Tregs and T_H17 cells in certain human cancers will have positive outcomes for use with immunotherapy. One potential method of inducing Treg instability is through venetoclax administration, which dysregulates BCL-2 expression and exerts modulatory effects on the immune system that create an environment suitable for Treg plasticity. Safety and toxicity of venetoclax have already been established in multiple clinical trials, making it a relatively easy therapeutic to repurpose for immuno-modulation.

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