CD8+ T cells deficient in the c-Cbl and Cbl-b E3-ubiquitin ligases more efficiently eliminate tumor cells

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May 2023

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science in Experimental Medicine

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List of Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cells
ATP	adenosine triphosphate
BCMA	B-cell maturation antigen
CAR	chimeric antigen receptor
Cbl	Casitas B−lineage lymphoma
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CTLA4	cytotoxic T lymphocyte-associated protein 4
CRISPR	clustered regularly interspaced short palindromic repeats
CTV	CellTrace™ violet
DAG	diacylglycerol
DC	dendritic cell
DC1	conventional type 1 dendritic cell
ECM	extracellular matrix
HRP	horseradish peroxidase
IFN-γ	interferon gamma
IFNGR	interferon gamma receptor
IL	interleukin
ILC	innate lymphoid cell
IP3	inositol-1,4,5-triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
LAT	linker for activation of T cell
MAPK	mitogen-activated protein kinase
MDSC	myeloid-derived suppressor cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
NK	natural killer cell
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PI	Propidium Iodide
PI3K	phosphoinositide 3-kinase
RNP	ribonucleoprotein
SHP1	SH2 domain-containing phosphatase-1

SHP2	SH2 domain-containing phosphatase-2
SH2	Src homology region 2
SH3	Src homology region 3
TCR	T cell receptor
TGFβ	transforming growth factor beta
TIL	tumor-infiltrating lymphocyte
TME	tumor microenvironment
TNF-α	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor
WT	wildtype
ZAP70	Zeta chain of T-cell receptor-associated protein kinase 70

Abstract

CD8+ T lymphocytes are a critical component of the immune system's response against cancerous cells. CD8+ T cells can specifically recognize tumor antigens expressed on malignant cells, resulting in the delivery of cytotoxic molecules and antitumor cytokines that mediate tumor clearance. However, in clinically detectable malignancies, these tumor reactive CD8+ T cells are often rendered dysfunctional by immunosuppressive cytokines in the tumor microenvironment and suppressive cells that are recruited to tumors. Many recent advances in cancer immunotherapy have targeted these suppressive signaling pathways in T cells or improved their ability to recognize and eliminate malignant cells. c-Cbl and Cbl-b are a pair of E3-ubiquitin ligases that are expressed in T cells and function as powerful negative regulators of T cell activation. Furthermore, Cbl-b has also been identified as a key mediator of several suppressive signalling pathways in T cells. In this thesis study, we investigated the role of these Cbl proteins in the regulation of CD8+ T cell-mediated elimination of tumor cells. By knocking out both *CBL* and *CBLB* in human CD8+ T cells, we were able to improve their anti-tumor and proliferative capacity compared to wild-type and single-knockout T cells. Additionally, double-knockout CD8+ T cells demonstrated greater resistance to inhibitory signalling by TGFβ and PD-1. These results indicate that deletion of c-Cbl and Cbl-b in CD8+ T cells holds promise as a novel cancer immunotherapy strategy or could augment currently established forms of cancer immunotherapy.

Résumé

Les lymphocytes T CD8+ sont essentiels dans la réponse du système immunitaire contre les cellules cancéreuses. Les cellules T CD8+ peuvent reconnaître spécifiquement les antigènes tumoraux exprimés sur les cellules malignes, entraînant la délivrance de molécules cytotoxiques et de cytokines antitumorales qui interviennent dans la l'élimination des tumeurs. Cependant, dans les tumeurs malignes cliniquement détectées, ces lymphocytes T CD8 + réactifs aux tumeurs sont souvent rendus dysfonctionnels par des cytokines immunosuppressives dans le microenvironnement tumoral et des cellules suppressives qui sont recrutées dans les tumeurs. De nombreuses avancées récentes en immunothérapie du cancer ont ciblé ces voies de signalisation suppressives dans les lymphocytes T ou amélioré leur capacité à reconnaître et éliminer les cellules malignes. c-Cbl et Cbl-b sont une paire de ligases E3-ubiquitine qui sont exprimées dans les lymphocytes T et fonctionnent comme de puissants régulateurs négatifs de l'activation des lymphocytes T. De plus, Cbl-b a également été identifié comme un médiateur clé de plusieurs voies de signalisation suppressives dans les lymphocytes T. Dans cette thèse, nous avons étudié le rôle de ces protéines Cbl dans la régulation de l'élimination des cellules tumorales par les lymphocytes T CD8+. En éliminant à la fois *CBL* et *CBLB* dans les cellules T CD8+ humaines, nous avons pu améliorer leur capacité anti-tumorale et prolifération par rapport aux cellules WT et aux cellules T avec un seul knockout. De plus, les lymphocytes T CD8+ double knockout ont démontré une plus grande résistance à la signalisation inhibitrice de TGFβ et PD-1. Ces résultats indiquent que la suppression de c-Cbl et Cbl-b dans les lymphocytes T CD8+ est prometteuse en tant que nouvelle stratégie d'immunothérapie contre le cancer ou pourrait augmenter les formes actuellement établies d'immunothérapie contre le cancer.

Acknowledgements

Firstly, I would like to thank my supervisor Dr. Hua Gu for all his knowledge, assistance, and patient guidance throughout my studies. Without his support, none of this work would have been possible. I also would like to express my appreciation for my committee members, Dr. Mathieu Ferron, Dr. André Veillette, Dr. Woong-Kyung Suh for their insights and suggestions regarding my project.

I would like to thank my colleagues: Xin Li, Weili Sun, Xiaochen Zhang, Ping Kuang, Li Zhong, and Tara Laleh for their support, training, patience, and encouragement. I am grateful for all the encouragement during hard times, their guidance, and the wonderful moments we shared throughout my time in the lab.

My heartfelt thanks also extends to all the staff of the IRCM who assisted me in completion of this project. Eric Massicotte and Julie Lord in particular were instrumental in training and facilitating my work in flow cytometry. I would also like to thank Melanie Candido for their administrative assistance in the lab.

Finally, I would like to thank my family for their constant support and unconditional love throughout all my studies. Their sacrifices have provided me with an incredible foundation to pursue my dream and goals.

Contributions of Authors

M.G. conducted the experiment and acquired all the data.

M.G. performed all the data analysis.

M.G. wrote the introduction, materials and methods, results, and discussions and future studies portions of the thesis.

Chapter 1: Introduction

1.1 Overview of Cancer Development

Cancers are a large group of diseases caused by genetic mutations in healthy cells that result in uncontrolled proliferation. As cancerous cells continue to multiply, they will invade adjacent tissues and can metastasize to other areas of the body through the circulatory or lymphatic systems, greatly increasing the severity and lethality of the disease.[1-3] Normal mammalian cells proliferate in a highly regulated manner to replace dead cells, maintain tissue integrity, and support the growth of the organism. Only a small proportion of the cells that make up the body are actively dividing, and most cells are either incapable of undergoing mitosis due to differentiating into a terminal cell fate or require external signals to re-enter the cell cycle. The most critical of these signals are known as mitogens, small proteins that are able to induce mitosis in non-proliferating cells via interaction with their cognate cell surface receptors, resulting in signal transduction through the mitogen-activated protein kinase (MAPK) pathways and the expression of key proteins such as cyclin D1 that are necessary for progression into the cell cycle.[4,5] As cells progress through the cell cycle, there are additional checkpoints that verify DNA integrity after replication and correct spindle formation prior to separation of the sister chromatids.[6,7] Detection of DNA damage will cause cell cycle arrest and the recruitment of DNA repair complexes to attempt to restore the damaged DNA. DNA damage that cannot be repaired results in prolonged cell cycle arrest and the induction of senescence or apoptosis, depending on the severity of the DNA damage.[6-8] Similarly, mitosis will be paused in healthy cells if the mitotic spindle assembly is not properly formed during the metaphase, and apoptosis will eventually be triggered if proper spindle assembly does not occur.[6,7] In order for healthy cells to

become neoplastic, they must acquire mutations that disrupt the regulation of cellular replication and cell death, allowing for the persistence of a population of cells that proliferate uncontrollably.

Dysregulation of cellular replication can occur through a wide range of mechanisms. Genetic mutations can cause abnormal cells to produce mitogens that can result in autocrine signalling and proliferation.[9,10] Mutations can also increase the expression of mitogen receptors or cause mitogen receptors to signal constitutively, reducing an abnormal cell's dependence on external proliferation signals.[9,10] In many cancers, mutation or aberrant overexpression of specific oncogenic proteins such as cyclin D1, Ras, and myc also promote proliferation in the absence of mitogenic signalling by allowing mutant cells to bypass cell cycle checkpoints and eliminate the effect of negative feedback in mitogenic signalling pathways.[6,9,10] Conversely, inactivation of key tumor suppressor proteins such as p53 and Rb can also compromise cell cycle regulation in addition to many other pathways critical to maintaining proper cellular function including DNA repair mechanisms, cellular metabolism, and apoptotic signalling.[6,8-10] However, a single mutation is almost always insufficient to transform a normal cell. Many redundant signalling pathways contribute to the maintenance of cellular homeostasis and will often compensate for the effects of a single mutant gene.[9,10]

As illustrated in **Figure 1**, when a healthy cell acquires a single driver mutation that contributes to a neoplastic state, that mutant cell will generally continue to carry out its normal function while passing the mutation onto its progeny.[9-11] Even with compensatory pathways preventing

complete transformation into a malignant state, these mutant cells may still proliferate at an increased rate, uptake elevated levels of metabolites, or possess greater genomic instability.[9-11] As further proliferation occurs, one of the mutant cells may develop a second mutation that increases its capacity to survive and proliferate independently of external signalling. The offspring arising from this cell will be able to outcompete the neighbouring cells, resulting in the establishment of a clonal population of mutant cells.[9,10] Over successive generations, many additional mutations can be acquired, eventually leading to malignant cells that are able to indefinitely proliferate in an uncontrolled manner and invade both neighbouring tissues and distant regions of the body.[4,5,9,10] Throughout this process, precancerous and cancerous cells must respond to a wide range of internal and external pressures, driving the selection of the fittest mutant cells.[10]

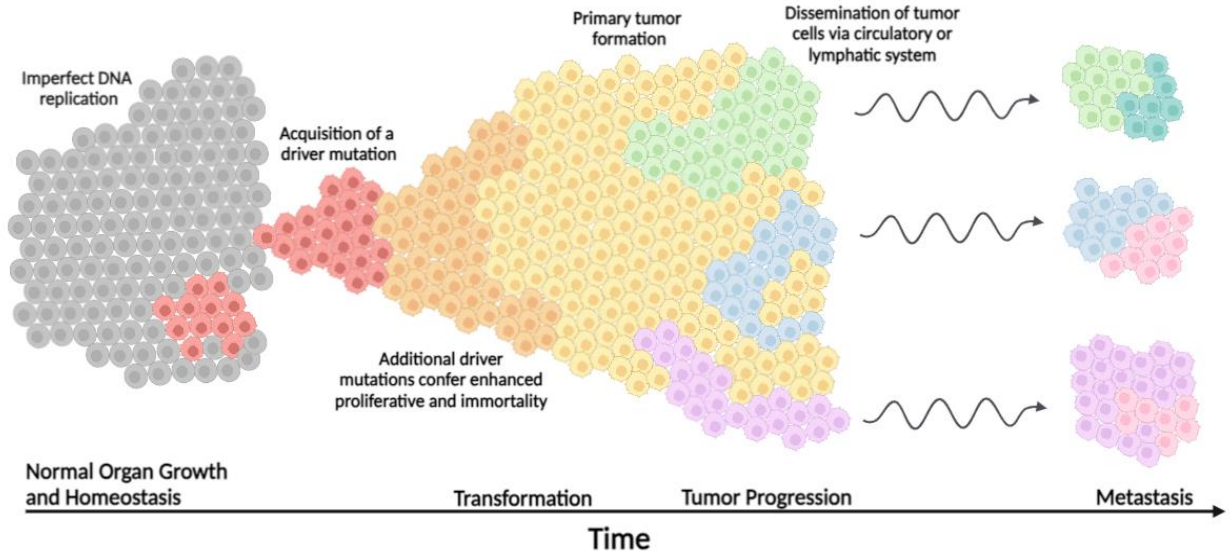


Figure 1: Clonal Expansion Model of Cancer Development

The development of cancer starts with DNA damage, resulting in the acquisition of a driver mutation that dysregulates cellular proliferation. This mutant cell continues to proliferate until an additional mutagenic event causes another driver gene mutation. Cells that have acquired this

additional driver mutations will be able to outcompete other clones, proliferating until they are the dominant clone within the neoplasm. Through subsequent cycles of mutation and clonal expansion, the cells will reach a fully transformed state and primary tumor formation occurs. At this stage, further mutations are unlikely to allow a single clone to dominate the entire tumor population. This results in competition between multiple clones within a single tumor and an increase in genetic heterogeneity.

An inherent selection pressure that all cancers must overcome is the shortening of telomeres after repeated cell divisions. Telomerase shortening will eventually trigger senescence or lead to massive genomic instability and cell death if the induction of senescence is bypassed.[9,10,12] Mutant cells that upregulate the enzyme telomerase can repair their telomeres and continue to proliferate indefinitely, while other clones that are unable to regenerate their telomeres will not progress to a fully neoplastic state.[9,10,12] In tumor-forming cancers, space and access to nutrients are two critical factors that limit the growth and proliferation of tumor cells.[9-11,13,15] Alterations to the expression of cell-cell adhesion molecules and the secretion of factors that modify the extracellular matrix (ECM) are often observed in tumor cells, enabling them to migrate locally and avoid crowding, dramatically increasing tumor growth rates.[9,13,14] Tumor cells will also frequently express angiogenic factors such as vascular endothelial growth factor (VEGF) to promote vascularization of the tumor, ensuring the delivery of additional oxygen and nutrients that are needed for tumor growth.[9,10,15] Another critical regulator of cancer growth and progression is the immune system. The relationship between cancer cells and the immune system is particularly complex in that the immune system can both promote and inhibit cancer development. Recent studies have revealed many of the mechanisms behind the seemingly contradictory role the immune system plays in cancer development.

1.2 Role of the Immune System in Cancer Development and Progression

A fundamental role of the immune system is the discrimination and elimination of non-self substances while exhibiting tolerance towards self-antigens. Cancer cells must develop mechanisms that allow them to evade immune detection and induce peripheral tolerance to survive and proliferate to a clinically detectable state.[9,16-20] In the early stages of cancer development, common mutations that lead to excessive proliferation and activation of the DNA damage response often also upregulate ligands that natural killer (NK) cells and other innate lymphoid cells (ILCs) can recognize.[16,21-23] NK cells are innate immune cells that possess the ability to rapidly respond to and kill abnormal cells through cell surface receptors such as NKG2D and DNAM-1.[16,22-24] When these receptors are bound by their corresponding ligands on a cancer cell, it causes the NK cell to become activated and form an immunological synapse.[22-24] Lysis of the cancer cell is directly mediated through the release of perforin and granzyme into the target cell.[22-24] Activated NK cells can also trigger apoptosis in cancer cells through TNF-related apoptosis-inducing ligand (TRAIL) and FasL, in addition to producing interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) that further activate cytotoxic immune cells, upregulate antigen presentation by tumor cells, and promote apoptosis and senescence in cancer cells.[16,22-24] Other subsets of innate lymphoid cells are also thought to contribute to tumor elimination at this early stage through apoptotic signalling and the production of IFN-γ and TNF-α.[24,25]

Myeloid-derived innate immune cells such as dendritic cells (DCs) and neutrophils are recruited to tumors through chemokines secreted by innate lymphoid cells and tissue-resident immune cells to aid in tumor clearance through phagocytosis of dying tumor cells.[16,25,26,27] Recruited

macrophages can further contribute to tumor killing after activation by IFN-γ to induce cytotoxicity in tumor cells through the production of nitric oxide and reactive oxygen species.[25,26] However, the most critical role these cells play in the destruction of tumors is the processing and presentation of tumor antigens to T cells to facilitate an adaptive immune response. It is thought that cancer cells are initially highly immunogenic due to the expression of mutated proteins, aberrant expression of proteins not normally expressed in somatic cells, or even the expression of viral oncogenic proteins incorporated into the genome.[16,18,20,21] Antigens derived from these proteins can be acquired by professional antigen presenting cells (APCs) and trafficked to secondary lymphoid organs to prime the adaptive immune response.[3,16-18,21,25] In mice, a particular subset of DCs known as conventional type 1 DCs (cDC1s) play a key role in the trafficking of tumor antigens and subsequent activation of naive CD8+ and CD4+ T cells.[17,25,28,29]

Naïve T cells whose T cell receptors (TCRs) specifically bind the tumor antigens presented by cDC1s on major histocompatibility complex (MHC) molecules receive the necessary signals for T cell activation.[17,30,31] The TCR itself lacks intracellular signalling domains and is associated with the CD3 receptor which possesses several intracellular signalling domains known as immunoreceptor tyrosine-based activation motifs (ITAMs).[30-32] When the TCR engages an MHC molecule bearing its cognate antigen, the CD8 or CD4 co-receptor molecule will also bind the TCR-MHC complex, activating lymphocyte-specific protein tyrosine kinase (Lck) through phosphorylation and recruiting it to the TCR.[30,31,33] Lck then phosphorylates the ITAMs on the signalling domains of CD3, recruiting the Zeta chain of T-cell receptor-associated protein kinase

70 (ZAP70).[34] ZAP70 phosphorylates various adaptor proteins that result in the activation of PLCγ1 and production of the intermediate signalling molecules inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG).[30,35,36] These molecules will initiate further downstream signalling pathways, resulting in nuclear translocation of the transcription factors NFκB, NFAT, and AP-1 in addition to cytoskeletal reorganization.[30,31,37] In parallel with TCR signal transduction, costimulatory signalling also occurs through the binding of CD28 by CD80 and CD86 expressed on cDC1 cells.[17,28-30] Once engaged by its ligand, the intracellular region of CD28 becomes phosphorylated, allowing for the binding of the p85 subunit of phosphoinositide 3-kinase (PI3K).[30,31,37] The p85 subunit then recruits the catalytic p110 subunit of PI3K, leading to the generation of phosphoinositide 3-kinase (PI3K) and recruitment of AKT, Vav, and PKCθ.[30,31,38] Activation of these enzymes leads to further downstream signalling that promotes the activity of the key transcription factors seen in TCR signalling: NFκB, NFAT, and AP-1.[30,31,38] The combination of both signals results in complete T cell activation of both CD4+ and CD8+ T cells. cDC1 cells also express other costimulatory ligands and secrete cytokines such as IL-12 and type 1 interferons that aid in the differentiation of activated CD8+ T cells into tumor antigen-specific cytotoxic T cells.[28] Activated CD4+ T cells provide additional help to both cDC1s and activated CD8+ T cells through the expression of CD40L and secretion of IL-2 and IL-21 respectively to ensure a robust anti-tumor response.[28]

After activation and differentiation, tumor antigen-specific T cells will traffic to the tumor and eliminate cancer cells in an antigen-dependent manner.[16-18,21] In the initial stages of the adaptive immune response, cytotoxic CD8+ T cells are thought to play the most prominent role in the

elimination of tumor cells through both direct cell-to-cell killing and secretion of cytotoxic cytokines.[16,19,39-42] Tumor cells are targeted by cytotoxic CD8+ T cells through the expression of MHC class I molecules, which present fragments of endogenous proteins. Tumor antigens presented by MHC molecules can be recognized by the TCRs of cytotoxic CD8+ T cells, initiating the formation of an immunological synapse.[39,41,43-45] TCR exhibit remarkably high sensitivities for their cognate antigen-MHC molecules, allowing cytotoxic CD8+ T cells to efficiently target tumor cells for destruction.[46] Immunological synapse formation triggers the exocytosis of perforin to disrupt the membrane of the tumor cell and granzymes, which enter into the target cell through the broken membrane and trigger apoptosis through caspase cleavage.[39,43-45] Cytotoxic CD8+ T cells also express FasL, that can bind Fas receptors on tumor cells to trigger apoptotic cell death.[39,44] CD8+ tumor-infiltrating lymphocytes (TILs) also secrete large amounts of IFN-γ, IL-12, and TNF-α that upregulate MHC class 1 expression, trigger cell death, and promote the survival and tumor killing activity of other immune cells.[40,47] Th1 polarized CD4+ T cells can also secrete many of these cytokines and produce high levels of IL-2 that promotes the survival of TILs. These interactions between tumor cells and both innate and adaptive immune cells are summarized in **Figure 2A**.

Despite the high efficacy of the immune response in eliminating abnormal cells, some tumor cells may possess mutations that allow them to survive the initial immune response. The mechanisms by which tumor cells prevent destruction by the immune system are varied, but many limit the effectiveness of CD8+ TILs.[16,21] TCR-antigen-MHC interactions are a critical component of T cell activity, and the affinity of the TCR for a presented antigen has been shown

to impact the quality of T cell activation and effector function.[30,45,48,49] Therefore, tumor cells that undergo mutations that result in the expression of less immunogenic antigens may not be completely eliminated by CD8+ TILs.[16,49] Tumor cells may also downregulate the expression of MHC class I molecules, transporter proteins associated with antigen presentation, or non-essential mutant proteins to limit the presentation of tumor antigens, thereby mitigating recognition by cytotoxic CD8+ T cell.[39,45] Downregulation of MHC class I molecules does render tumor cells more vulnerable to the cytotoxic activity of NK cells; however, tumor cells can also acquire additional mutations that result in the expression of ligands for immune checkpoint receptors such as programmed death-ligand 1 (PD-L1), CD155, and CD112.[16,39,41,50] These ligands can bind to immune checkpoint molecules such as programmed cell death protein 1 (PD-1) and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains that are expressed on NK cells and activated T cells, abrogating the cytotoxic activity of these cells.[16,39,45,47,50]

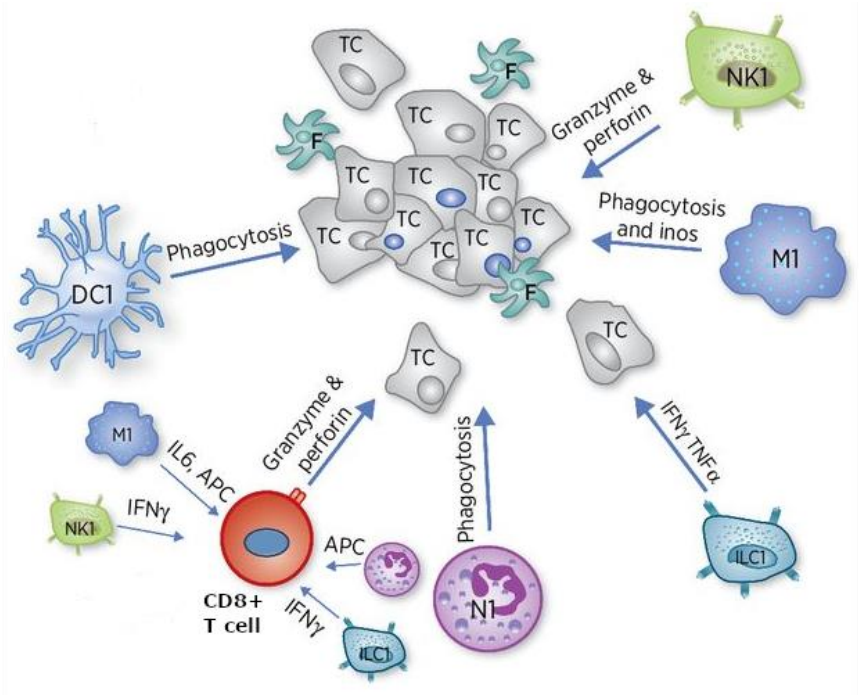
Tumor cells can also mediate immunosuppression of cytotoxic cells through the expression or induction of inhibitory and Th2 promoting cytokines such as TGFβ, IL-10, IL-4, and IL-13, in addition to the maintenance of a hypoxic and low pH environment and the secretion of various growth factors in the tumor microenvironment (TME).[25,47,51] Tumor-associated macrophages are polarized by these signals to a tumorigenic state where they express high levels of growth factors, IL-10, and promote angiogenesis through the secretion of VEGF.[16,25,26,51] Other innate myeloid cells such as neutrophils and myeloid-derived suppressor cells (MDSCs) also provide pro-tumorigenic signals in the form of IL-4, IL-6, G-CSF, TGFβ and VEGF, further inhibiting the activity of CD8+ TILs.[3,25,27,51] MDSCs also release reactive oxygen species and nitric oxides,

which serve to further negatively regulate the cytotoxic activity of NK cells and CD8+ T cells in the TME.[51] Although the effect of different ILC subsets within established tumors is still relatively poorly understood, immunosuppressive cytokines are thought to limit IFN-γ production by type 1 ILCs and the secretion of IL-4 and IL-13 by type 2 ILCs is likely to contribute to a Th2-biased immune response in the TME.[24,25]

The TME can also suppress the activity of cDC1 cells through the presence of VEGF, IL10, and TGFβ, inhibiting their maturation into effective antigen-presenting cells, limiting expression of costimulatory molecules, and upregulating the expression of immunosuppressive molecules such as PD-L1, TGFβ and IL-10.[17,25,51] This in turn limits the quality and magnitude of the cytotoxic CD8+ T cell response against tumor cells and can cause CD8+ T cells to become dysfunctional.[17,47] Furthermore, DCs that do acquire antigen in the TME often promote a regulatory or Th2 response when priming naïve CD4+ T cells, resulting in the expansion of regulatory T (Treg) cells and Th2-biased T cells.[17,25,51] These T cell subsets can be recruited to the TME by chemokines produced by tumor-associated macrophages and other tumor-associated immune cells, where their survival and proliferation are promoted by the presence of TGFβ and IL-4.[17,25] Th2 CD4+ T cells contribute to the maintenance of inflammation through IL-6 production and inhibition of cytotoxic activity through the secretion of IL-10.[25,42,52] Treg cells inhibit the activity of CD8+ TILs through a wide range of mechanisms, including conversion of extracellular adenosine triphosphate (ATP) into immunosuppressive adenosine, triggering dysfunction in CD8+ TILs by sequestering IL-2 through expression of high levels of membrane-bound and soluble IL-2 receptor (IL-2R), downregulating costimulatory molecules on APCs to

limit activation and differentiation of CD8+ T cells, and secretion of high levels of inhibitory cytokines such as IL-10 and TGFβ.[44,47,51] The immunosuppressive interactions of the TME are summarized in **Figure 2B**. The heavily immunosuppressive environment of the TME, in concert with the frequent expression of inhibitory immune checkpoint ligands and chronic exposure to high levels of tumor antigens, often render CD8+ TILs exhausted and unable to proliferate or carry out effector functions.[47,53] Exhausted CD8+ T cells also upregulate the expression of multiple immune checkpoint molecules such as PD-1, TIM-3, TIGIT, and LAG-3 that are stimulated by tumor cells and recruited immune cells in the TME, leading to the maintenance of an exhausted state.[47,53] The loss of cytotoxic activity against tumor cells results in tumor expansion, clinical disease, and possible progression to metastasis.[20,21]

A



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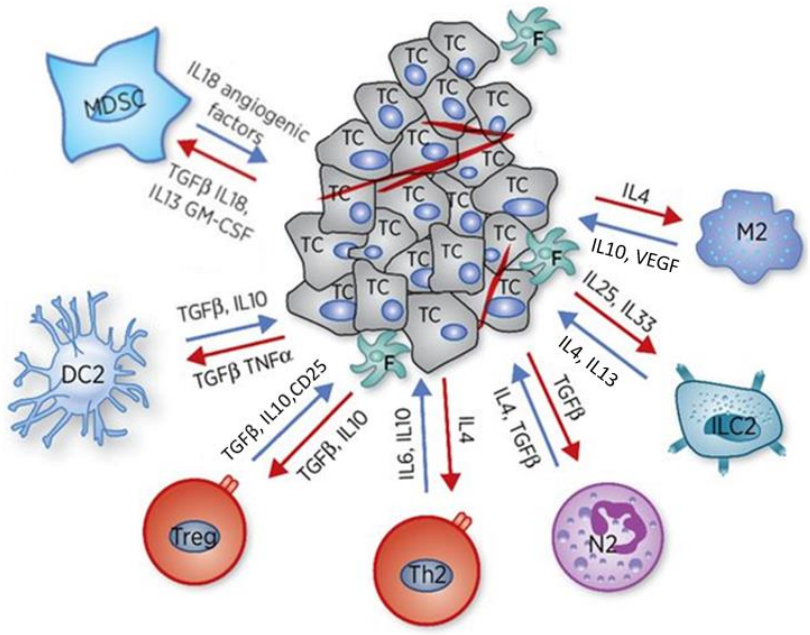

Figure 2: Key Immune Cell Interactions in the Tumor Microenvironment

(A) Summary of immune interactions that facilitate tumor killing.
(B) Immune interactions that promote an immunosuppressive tumor microenvironment. TC: tumor cell, M: macrophage, F: fibroblast, DC: dendritic cell, N: neutrophil, ILC: innate lymphoid cell, NK: natural killer cell, MDSC: myeloid derived suppressor cell, Th2: Th2 polarized T cell, Treg: regulatory T cell. (Adapted from Ref. 25)

1.3 Targeting CD8+ TIL Activity in Cancer Immunotherapy

As the understanding of the immune system and its regulation of tumor growth improved, researchers and clinicians sought to leverage a patient's immune system to eliminate neoplastic cells. Some of the first modern cancer immunotherapy treatments involved the administration of large doses of IL-2 to patients with metastatic cancers to facilitate proliferation of T cell populations.[54] Since these pioneering treatments, several types of cancer immunotherapy have been developed that augment the cytotoxic T cell response to treat clinically detectable cancers,

achieving response rates above 80% in some cases.[55-57] Immunotherapy-based treatments have been proven to hold several advantages over more traditional cancer treatments due to the systemic reach of the immune system and the specificity immune-mediated cancer destruction.[55,56,58] Cancer immunotherapy treatments are able to induce the destruction of undetected metastases in addition to clearance of the primary tumor, which is advantageous compared to surgical- or radiotherapy-based methods of cancer treatment, while also targeting cancer cells with much greater specificity and longevity that chemotherapy treatments.[55,58] However, there are still significant challenges facing the current iterations of cancer immunotherapies. As previously discussed, the mechanisms that cancer cells use to escape immune destruction are highly diverse and heterogeneously expressed in different types of cancers or even within the same tumor, resulting in inconsistent response rates and effective treatment in only limited subgroups of cancer patients.[56,58] Furthermore, the side effects of cancer immunotherapies, while generally not as severe as more traditional cancer treatments, can still cause life-threatening complications and deaths.[55,56,58,59]

Immune checkpoint therapies are antibody-based treatments that target inhibitory signalling pathways responsible for maintenance of peripheral immune tolerance in T cells under normal conditions.[55,56,60] These pathways are often utilised by cancer cells to escape destruction mediated by cytotoxic CD8+ T cells.[55,60] The two immune checkpoint pathways that are targeted by current therapies are cytotoxic T lymphocyte-associated protein 4 (CTLA4) and the PD-1/PD-L1 axis.[55,56,58-60] CTLA4 mediates suppression of CD8+ T cell activation and effector function through both cell intrinsic and extrinsic mechanisms.[55,60] In naïve CD8+ T cells, CTLA4 is

expressed at low levels but is sharply upregulated following TCR engagement.[55,60] CTLA4 possess higher affinity and avidity for the B7 ligands CD80 and CD86 that bind CD28 to deliver costimulatory signals.[55,60] By outcompeting CD28 for its ligands, CTLA4 regulates CD8+ T cell activation in secondary lymphoid organs and promotes an anergic state.[55,60] Notably, CTLA4 is expressed constitutively at high levels on Treg cells, and mediates suppression of CD8+ T cells by binding and degrading B7 ligands expressed on APCs.[55,60] Antibody blockade of CTLA4 is thought to mediate antibody-dependent cellular cytotoxicity (ADCC) of Treg cells, preventing Treg cells from sequestering and degrading B7 costimulatory ligands on APCs and improve the activation state of CD8+ T cells by blocking the inhibitory effect of CTLA4 expression on effector cells.[55] Expression of PD-1 is also upregulated by T cell activation, and upon binding its ligands PD-L1 and PD-L2, triggers the recruitment of phosphatases SH2 domain-containing phosphatase-1 (SHP1) and SH2 domain-containing phosphatase-2 (SHP2) that dephosphorylate and inactivate signal transduction for the CD3ζ chain, ZAP70, and CD28.[55,60,61] PD-L1 and to a lesser extent PD-L2 are expressed on APCs and on a wide range of non-lymphoid tissues, suggesting that while PD-1 may play a role in modulating activation in secondary lymphoid organs, PD-1 inhibitory signalling primarily occurs in the peripheral tissues.[55,60] The use of antibodies targeting either PD-1 or PD-L1 prevents inhibitory signalling from occurring in CD8+ T cells, preserving the activation status of CD8+ TILs.[60,62]

Clinically, the use of antibodies targeting CTLA4 and the PD-1/PD-L1 axis confer durable increases in survival when compared with traditional chemotherapeutic treatments.[55,60] CTLA4-blocking antibodies have demonstrated effectiveness in treating advanced melanomas, but were

less effective against other forms of cancer.[55] On the other hand, antibody treatments targeting PD-1 and PD-L1 have seen success in treating a wide range of malignancies, possibly because PD-L1 upregulation occurs frequently in a broad range of tumors.[55] Adverse effects have been reported for both forms of the therapy, the causes of which are mainly due to acute inflammation and the accumulation of T cell in the peripheral organs.[55,63] Patients that experienced immune-related adverse events after treatment with anti-CTLA4 blocking antibodies had increased TCR clonal diversity in circulating T cells, suggesting that elimination of autoreactive T cells can be compromised in immune checkpoint therapies.[63]

Adoptive cell therapy (ACT) is another type of immunotherapy that involves the infusion of T cells into the patient to facilitate the treatment of cancer. The first conventional forms of ACT were developed out of the discovery of tumour antigen-specific T cells that infiltrate solid tumors.[64,65] TILs were isolated from resected tumors, expanded *in vitro* using IL-2, and reinfused back into the patient.[64,65] Modern forms of TIL-based ACT have incorporated lymphodepletion regiments to delete endogenous Treg cells and reduce competition for cytokines that mediate T cell survival and proliferation.[59,66] Additional methods of improving TIL treatments have involved selectively transferring T cells that express activation markers such as PD-1 or CD137 and screening for neo-antigen specific T cells.[55,66] The efficacy of this form of ACT against metastatic melanomas has been established in several clinical trials and more modest successes have been reported in lung, breast, and head and neck squamous cell cancers.[67,68] However, tumor-infiltrating T cell therapy remains limited to a subset of malignancies that generate strong immune responses and have relatively high rates of mutations and neoantigens.[59] T cells within

many tumors exist in an exhausted state and may not proliferate strongly or efficiently mediate cytotoxic tumor clearance.[53,66]

To improve the efficacy and range of targetable cancers, infused T cells have been genetically engineered with different forms of tumor antigen-specific receptors.[59] The most successful of these strategies is known as chimeric antigen receptor (CAR) T cells. CAR constructs are synthetic receptors that are designed to target a tumor antigen and induce T cell activation.[69] Antigen specificity is generally mediated through the inclusion of a single-chain variable fragment (scFv) from an antibody specific for the tumor antigen of interest in the extracellular domain.[69,70] The intracellular domain of most CARs contain the signalling domains of the CD3ζ chain and one or more costimulatory domains derived from costimulatory molecules such as CD28 or CD137.[70,71] This results in the delivery of both TCR and costimulatory signalling when a CAR binds its cognate antigen, allowing for complete activation of the T cell. CAR T cells targeting CD19 have demonstrated remarkable response rates in clinical trials for various B cell malignancies and similar results have been achieved using B-cell maturation antigen (BCMA)-targeted CAR T cells to treat multiple myloma.[72,73] However, current CAR T cell therapies targeting solid tumors have only been able to achieve relatively moderate efficacy.[55,74] One of the major limitations in designing CARs for solid tumors is selecting an appropriate antigen. Most tumor-associated antigens are also expressed at low levels on healthy cells; this can cause on-target off-tumor toxicity when CAR T cells recognize and destroy healthy tissue.[55,59,74] This form of toxicity has been reported in many solid tumor CAR T cell clinical trials and in the most severe cases has led to fatalities or organ damage.[75,76] The effectiveness of CAR T cells against

tumors is also limited by the immunosuppressive nature of most TMEs.[74] The expression of high levels of PD-L1 by cells in the TME in combination with suppressive cytokines such as TGFβ and IL-10 and chronic signal 1 activation can result in the exhaustion of CAR T cells and failure to prevent tumor outgrowth.[55,59,74]

1.4 Cbl Protein Family and Regulation of T cell Function

The Cbl (Casitas B−lineage lymphoma) family of protein are a group of E3 ubiquitin ligases that mediate a wide range of receptor and transcription signals.[77] In mammals, the Cbl family contains 3 members: c-Cbl, Cbl-b and Cbl-3.[77,78] As illustrated in **Figure 3**, all three Cbl family members share the same N-terminal motifs. The proteins possess a tyrosine kinase binding domain that can bind phosphorylated tyrosine moieties, a linker, and a RING finger domain that mediates the E3 ubiquitin ligase activity by stably interacting with E2 ubiquitin conjugating enzymes facilitating the transfer of ubiquitin moieties to the target protein.[77,79] However, Cbl-3 has a truncated C-terminus and lacks the proline-rich domains, tyrosine residues, and UBA domain found in c-Cbl and Cbl-b.[77] The expression of Cbl-3 is also markedly different that the other members of the Cbl family; Cbl-3 is only detected in epithelial cells whereas c-Cbl and Cbl-b are expressed in a wide range of cell types including all hematopoietic cell lineages.[77,80-82] The similarities in conserved domains and the overlapping expression in hematopoietic cells, including T cells suggests that c-Cbl and Cbl-b have redundant functionality. This is further supported by germline knockouts of c-Cbl and Cbl-b in mice. c-Cbl- and Cbl-b-deficient mice undergo normal development while the loss of both genes results in embryonic lethality, suggesting that the loss of one protein is being compensated by the activity of the other.[83]

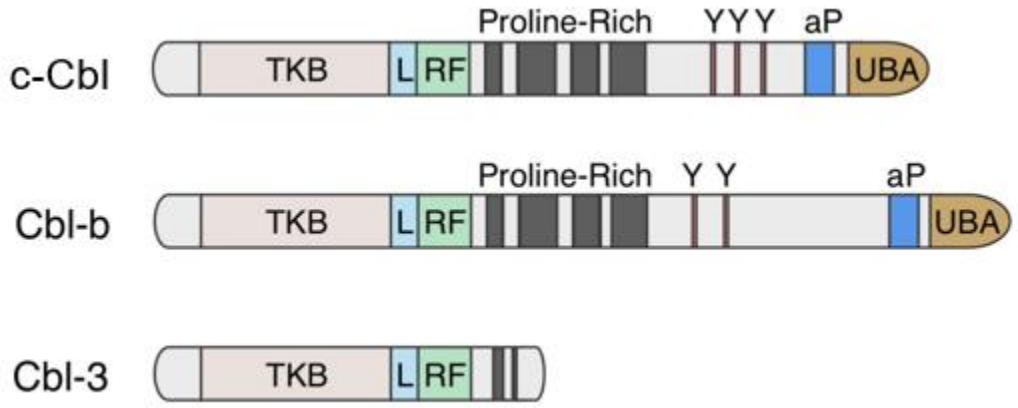

Figure 3: Structure of the Cbl family of proteins

All three members have similar N-terminal structures containing a tyrosine kinase binding domain (TKB), a linker (L), and a RING figure domain (RF). The C-terminal regions of c-Cbl and Cbl-b contain several proline-rich motifs, tyrosine residues (Y), and a UBA domain. The TKB domain interacts with proteins containing phosphorylated tyrosine residues. The RING domain interacts with E2 ubiquitin conjugating enzymes. Proline-rich domains can interact with proteins containing Src homology region 3 (SH3) domains. Tyrosine residues after being phosphorylated can interact with proteins containing Src homology region 2 (SH2) domains. (Adapted from Ref. 77)

Although c-Cbl and Cbl-b do display redundancy and are both expressed in T cells, their expression patterns differ in both developing thymocytes and mature T cells. c-Cbl is more highly expressed in CD4+CD8+ DP thymocytes while Cbl-b is more abundant in mature CD4+ and CD8+ T cells.[77,83] In line with these expression patterns, depletion of c-Cbl in mice results in an increased number of CD4+ single positive thymocytes due to elevated positive selection signals while Cbl-b-deficient mice possess normal thymocyte development.[77,84] Consequently, peripheral CD4+ T cells in c-Cbl$^{-/-}$ mice respond more poorly to TCR stimulation than cells isolated from wildtype (WT) or Cbl-b-deficient mice.[77,84] c-Cbl is thought to regulate TCR signalling through ubiquitination of CD3ζ following ZAP70 recruitment and phosphorylation.[77,85] c-Cbl can interact with phosphorylated ZAP70 or other scaffold proteins

through its TKB domain to form a stable complex with the CD3ζ chain, allowing for the transfer of ubiquitin moieties.[77,85] This results in the internalization and downmodulation of the TCR-CD3 complex after ligand engagement, limiting the duration of TCR signalling; the mechanism by which this occurs is not totally clear but is thought to involve lysosomal-dependent degradation.[83,85] Thus, c-Cbl-deficient CD4+CD8+ DP thymocytes will receive prolonged TCR signalling after binding MHC molecules, increasing the survival of thymocytes with lower affinity TCRs.[83] c-Cbl has also been reported to ubiquitinate and mediate the degradation of two critical members of the TCR signalling pathway, Lck and linker for activation of T cell (LAT), in both human T cell lines and murine primary T cells, further suggesting that c-Cbl plays a role in regulating TCR signalling.[82] However, the effects of c-Cbl on TCR expression and signalling in peripheral T cells are not as well-defined as in T cell development. c-Cbl-deficient peripheral T cells produced similar or reduced levels of cytokines compared to WT peripheral T cells after *in vitro* stimulation with either anti-CD3 antibody alone or in combination with anti-CD28 antibody.[83] Furthermore, TCR downmodulation was identical in WT and c-Cbl knockout peripheral T cells after anti-CD3 stimulation and TCR internalization was only slightly reduced in c-Cbl-deficient cells shortly after TCR crosslinking.[83] The lack of major phenotypic changes in c-Cbl knockout peripheral T cells is thought to be due to the relatively low expression of c-Cbl in mature T cells.[77,83,85]

Cbl-b has also been shown to mediate ubiquitination of the CD3ζ chain following TCR-MHC interaction in mature T cells.[77,82,83,86] In accordance with the higher expression level of Cbl-b in mature T cells, Cbl-b knockout resulted in a greater impact on TCR expression. Peripheral T

cells isolated from Cbl-b-deficient mice had moderately reduced TCR downmodulation after anti-CD3 stimulation and a slightly greater reduction in TCR internalization following TCR cross-linking compared to c-Cbl-deficient cells.[83] Interestingly, deficiency in both c-Cbl and Cbl-b resulted in a substantial decrease in TCR downmodulation and internalization when compared to WT, c-Cbl knockout, and Cbl-b knockout T cells.[83,85] Additionally, colocalization of internalized TCRs with lysosomes was impaired in c-Cbl and Cbl-b double knockout T cells.[83] The abrogation of TCR downmodulation and lysosomal colocalization in c-Cbl and Cbl-b double knockout T cells suggest that c-Cbl and Cbl-b regulate TCR signaling in a redundant manner by inducing TCR internalization after TCR-MHC engagement and TCR degradation via lysosomal trafficking.[77,82,83]

Studies analysing Cbl-b-deficient murine T cells have revealed that Cbl-b also acts as a key regulator of T cell activation and dysfunction independently of its capacity to induce TCR degradation. Notably, Cbl-b knockout T cells do not require CD28 costimulatory signalling to become activated while WT T cells that only receive TCR signalling enter a state of anergy.[53,77,81,82] Anergic T cells are hyporesponsive to antigen stimulation and do not strongly proliferate or produce IL-2, ensuring that peripheral T cells that recognize self-antigens do not mount an autoreactive immune response.[45,53] However, the induction of anergy in CD8+ T cells also frequently occurs within tumors due to the lack of costimulatory molecules expressed on tumor cells, contributing to the failure to eliminate tumor cells and limiting the efficacy of cancer immunotherapies.[47,77,82] Mechanistically, Cbl-b-mediated regulation of costimulatory signaling in T cell activation occurs through suppression of TCR-induced PI3K and Vav activation.[77,82,85]

Cbl-b has been shown to ubiquitinate the p85 subunit of PI3K, which does not result in proteasomal degradation but prevents its association with the TCR and CD28.[77,82,87] In WT T cells, CD28 costimulatory signalling results in both the activation of PI3K and the phosphorylation of Cbl-b, which is thought to target Cbl-b for ubiquitination and proteasomal degradation.[38,82,88] In the absence of CD28 costimulation, WT T cells cannot become activated due to inadequate PI3K and Vav activity, whereas Cbl-b-deficient T cells are able to elicit sufficient Vav activity through TCR signalling via the recruitment of PI3K to the TCR.[77,82,87] Cbl-b has also been identified as an important regulator of PD-1 and TGFβ receptor signalling in murine T cells, which frequently suppress CD8+ T cell activity against cancer cells.[77,82,89,90] T cells isolated from Cbl-b knockout mice are resistant to *in vitro* PD-L1 suppression of proliferation and IFN-γ production and Cbl-b knockout mice were able to reject PD-L1 expressing tumors that normally escape immune clearance in WT mice.[82,89] Additionally, TILs derived from Cbl-b-deficient mice also had fewer PD-1+Tim+ exhausted T cells than their WT counterparts.[82] Cbl-b was found to positively regulate TGFβ receptor signalling via ubiquitination and degradation of the inhibitory protein SMAD7.[90] This allows for the efficient phosphorylation and nuclear translocation of SMAD2 and SMAD3, driving a wide range of suppressive transcriptional changes in CD8+ effector T cells.[90,91] Consequently, Cbl-b knockout murine T cells were also found to be resistant to suppression by Treg cells and high levels of exogenous TGFβ.[90,91,92] Altogether, there is substantial evidence that Cbl-b is a key regulator of T cell activation and suppression in the context of the T cell response to cancers.

1.5 Rational and Hypothesis

Since the recognition of CD8+ cytotoxic T cells as the most prominent effectors of the anti-cancer immune response, extensive research has been dedicated to understanding and combating the suppression of their function. The advent of immune checkpoint therapies against CTLA4 and PD-1/PD-L1 demonstrated that targeting suppressive signalling pathways in T cells is a viable method of augmenting the immune response against established tumors. CAR T cell therapies have also established the importance of costimulation in T cell activation and cytotoxic activity through the inclusion of intracellular costimulatory signalling domains. Furthermore, cancer vaccines strategies and inhibitors have been developed to target other pathways involved in T cell activation and TGFβ signalling, some of which are currently being evaluated clinically.[55,93,94] Synergistic activity between forms of immunotherapy that target distinct pathways such as anti-CTLA4/anti-PD-1 combination therapy has also been reported in advanced forms of certain cancers.[55,56,58] Thus, it is likely that novel cancer immunotherapies that simultaneously enhance T cell activation and blockade suppressive signalling pathways will possess broader activity against malignancies that are highly immunosuppressive.

Cbl-b has been identified as a key regulator of T cell activation through TCR downmodulation after ligand binding and maintenance of CD28-dependence in addition to mediating PD-1 suppression of T cell function and positively regulating TGFβ receptor signalling in peripheral murine T cells. Given that Cbl-b suppresses CD8+ cytotoxic T cells activity through a wide range of mechanisms, it has been suggested that Cbl-b-deficient CD8+ T cells would be able to generate a strong anti-tumor immune response. Studies performed by our lab and other groups

have confirmed that Cbl-b knockout mice are capable of rejecting transplanted and spontaneous tumors and that adoptive transfer of Cbl-b-/- CD8+ T cells is sufficient to eliminate established tumors.[92,95] However, the effect of Cbl-b-deficiency in human CD8+ T cells has not been investigated. It is not yet certain whether Cbl-b knockout in human CD8+ T cells can potentiate the same strong anti-tumor response seen in murine tumor models. On the other hand, the effect of c-Cbl in peripheral mouse T cells is limited to regulation of TCR downmodulation, controlling the duration of TCR signalling during activation in a redundant manner with Cbl-b. Strong activation of CD8+ cytotoxic T cells is critical for mounting an effective anti-tumor immune response and defective T cell activation often occurs with established tumors due to the impairment of cDC1 function and the induction of Treg cells. Therefore, it is conceivable that the knockout of both c-Cbl and Cbl-b in CD8+ T cells may further improve antitumor cytotoxic activity. Based on this evidence, we hypothesize that c-Cbl and Cbl-b double knockout human CD8+ T cells will mount a stronger anti-tumor immune response than WT CD8+ T cells or CD8+ T cells deficient in either c-Cbl or Cbl-b. This project will bring additional insights into the role c-Cbl and Cbl-b play in regulating the cytotoxic T cell response against tumors. Additionally, it may also highlight new targets for novel cancer immunotherapies or possible methods of augmenting current immunotherapy treatments.

Chapter 2: Materials and Methods

Human CD8+ T cell Isolation and Activation

Fresh whole blood from healthy volunteer donors was obtained from the Montreal Clinical Research Institute clinic. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and CD8+ T lymphocytes were then purified using the EasySep™ Human CD8+ T Cell Enrichment Kit (Stemcell Technologies) according the to manufacturer's protocol. Isolated cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1 mM Sodium Pyruvate (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), and 55 µM 2-Mercaptoethanol. CD8+ T cells were activated with Human T-Activator CD3/CD28 Dynabeads (ThermoFisher) in a 1:1 cell:bead ratio with 50 IU/ml recombinant human IL-2 (rhIL-2) at a density of 10^6 cells per ml of media for 48 hours. After activation, CD8+ T cells were separated from the Dynabeads magnetically.

crRNA Design

3 CRISPR RNAs (crRNAs) were designed to target exon 1 of the *CBL* gene and exon 3 of the *CBLB* gene. The target regions were selected using Integrated DNA Technologies' crRNA design tool and were verified in the CHOPCHOP web toolbox to ensure crRNAs had high on-target and low off-target scores.[96] crRNAs were ordered from IDT in their proprietary Alt-R format. The sequences of the crRNAs are as follows:

CBL crRNA 1: CGUCCUUCAUGAGCCCAAUC
CBL crRNA 2: AUCUUCUUGUCCACCGUCCC

CBL crRNA 3: AGAGCUCUUCUUCACGUUGC

CBLB crRNA 1: AAAUAUCAAGUAUAUAUGGU
CBLB crRNA 2: CGUAAAUGCUGAUAUGUAUC
CBLB crRNA 3: AGUACUCAUUCUCACUGAGU

Genetic Editing of Human CD8+ T Cells

To prepare the gRNA duplexes, each Alt-R crRNA, Alt-R tracer RNA (tracrRNA), and ATTO550-labeled Alt-R tracrRNA (IDT) were reconstituted to 100 µM in Nuclease-Free Duplex Buffer (IDT). Each crRNA and tracrRNA were mixed at equimolar concentrations in sterile PCR tubes and were annealed by heating at 95°C for 5 min in a PCR thermocycler. The gRNA duplexes were then slowly cooled to room temperature over 15 min. The Cas9 ribonucleoprotein (RNP) complex was prepared by combining 2 µL (100 pmol) of each of the 3 gRNAs targeting *CBL* or *CBLB* with 2 µL (124 pmol) of Alt-R S.p. Cas9 Nuclease V3 (IDT). The solution was gently mixed and incubated at room temperature for at least 15 min. Human CD8+ T cells were electroporated with a Neon transfection system (Invitrogen). 2.5×10^5 T cells per electroporation were washed twice with phosphate-buffered saline (PBS) before resuspension in 9 µL of buffer R (Invitrogen). The resuspended cells were gently mixed with 1 µL of Cas9 RNP complex to generate single knockout T cells or 1 µL of both Cas9 RNP complexes to produce Cbl dKO CD8+ T cells. Cas9 RNP complex was not added to WT control T cells. 10 µL of the T cells suspension was then electroporated with a Neon transfection device using the following protocol: 1600 V, 10 ms/pulse, 3 pulses. Electroporated cells were immediately

transferred to pre-warmed complete RPMI 1640 supplemented with 50 IU/mL rhIL-2 for about 2 days.

Western Blot

Western blot was performed according to standard protocol. In brief, human CD8+ T cells were lysed in TNE buffer (50mM Tris, 140mM NaCl, 5mM EDTA, 0.5% SDS) on ice for 30 minutes. Cell lysates were then collected by centrifugation at 15 000 RPM for 10 minutes at 4°C. To denature the samples, 2X SDS loading buffer with 20% 2-mercaptaethanol was added to the supernatant and incubated at 95°C for 10 minutes. 20 µL of each protein sample was loaded into the wells of an SDS-PAGE gel, along with a molecular weight marker. After running the gel for 4.5 hrs at 90 V, the proteins were transferred from the SDS-PAGE gel to a PVDF membrane at a voltage of 30 V for 2 hrs. The membrane was incubated with blocking buffer (5% milk in TBST) for 1 hr at room temperature. After blocking, the membrane was incubated with different primary antibodies at 4°C overnight, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 hr. Primary antibodies that were used were anti-Cbl (Santa Cruz), anti-Cbl-b (Abcam), and anti-β-Actin (Abcam). HRP-conjugated anti-mouse IgG (Cell Signaling) was used as a secondary antibody. Membranes were developed with the Amersham enhanced chemiluminescence detection reagent (Cytiva Life Sciences) and imaged using a Bio-Rad ChemiDoc XRS+ system.

Flow Cytometry

For flow cytometry analyses, cells in culture were centrifuged and washed twice in FACS buffer (1% bovine serum antigen, 0.1% Sodium Azide in PBS). To stain for the surface markers, cells were incubated with the corresponding antibodies at dilutions between 1:30 and 1:200 on ice for 30 minutes in dark condition. For intracellular staining, cells were first fixed and permeabilized with the transcription factor staining kit (Invitrogen) for 40 min at room temperature in the dark. Cells were then washed and stained with the corresponding antibodies at a 1:200 dilution. Afterwards, cells were washed with FACS buffer twice and were analyzed on a BD Fortessa. The following antibodies were used for staining: anti-B220, anti-human CD3, anti-human CD8 and anti-human IFN-γ (Biolegend), anti-human TCR α/β (BD Bioscience). Flow cytometry data were analyzed with FlowJo v10 software (BD Bioscience).

Cell Lines

MDA-MB-231 cell line was obtained from ATCC and were cultured in high glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco).

Cytokine Production and Proliferation Assays

For intracellular IFN-γ staining, 1×10^5 WT and knockout CD8+ T cells were plated in 96 well flat-bottom plates and stimulated for 16 hours with 3 µg/mL plate-bound anti-human CD3 and 2 µg/mL soluble anti-human CD28 antibodies (Biolegend) in the presence or absence of 5 ng/mL recombinant human TGFβ (Peprotech). GolgiPlug (BD Bioscience) was then added to media

according to the manufacturer's instructions and the cells were incubated for an additional 6 hours. After incubation, cells were harvested and intracellularly stained with anti-human IFN-γ antibody.

For proliferation assays, WT and knockout CD8+ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (Biolegend) according to the manufacturer's protocol. 8×10^4 labeled WT and knockout CD8+ T cells were cultured in the same initial conditions as in the IFN-γ experiment for 72 hours. Then, cells were harvested and CFSE dilution was used to determine proliferation. Proliferation assays involving CD8+ T cell and MDA-MB-231 co-culture were conducted in 48 well flat-bottom plates. WT and knockout CD8+ T cells were stained with CFSE (Biolegend) and MDA-MB-231 cells were stained with CellTrace™ violet (CTV) (Biolegend). 5×10^4 T cells and 5×10^4 MDA-MB-231 cells were cultured together for 72 hours with 3 μg/mL soluble anti-human CD3 and 2 μg/mL soluble anti-CD28 (Biolegend). Cells were then harvested and analyzed via flow cytometry.

To calculate the average number of cell divisions, the following formulae were used:

Calculated Input T cells: $T_i = \sum_{d=0}^{n}(2^{-d})E_d$

Average Cell Division: $D = \frac{\sum_{d=1}^{n}(2^{-d})E_d}{T_i}$

T cells were assigned to a particular cell division group d where $d = 0$ to n cell divisions based on the peaks of CFSE fluorescence in the population, and the number of T cells observed within each cell division group (E_d) was determined in Flowjo. Average cell division (D) was calculated by using these equations that correct for the 2-fold increase in cell number that is associated with each successive division.[97-99]

Cytotoxicity Assays

The cytotoxicity of the CD8+ human T cells was determined by a flow cytometry-based assay. Prior to the experiment, WT and knockout CD8+ T cells were activated for 48 hours using 3 μg/mL plate-bound anti-human CD3 and 2 μg/mL soluble anti-human CD28 antibodies (Biolegend). Following activation, 1×10^4 CTV-labeled MDA-MB-231 target cells were cultured with CFSE-labeled WT and knockout CD8+ T cells in triplicates at the indicated effector:target ratios in 96 well round-bottom plates for 6 hrs. MDA-MB-231 target cells cultured without effector CD8+ T cells served as a negative control. Cells were collected and stained with propidium iodide (PI) (Biolegend) according to the manufacturer's instructions prior flow cytometry analysis to identify dead cells.

Quantification and Statistical Analysis

Statistical analyses were performed with a two−tailed, unpaired Student's t test in GraphPad Prism V8 software. A P value < 0.05 was considered statistically significant.

Chapter 3: Results

3.1 Generation of Cbl Knockout Human CD8+ T Cells

To investigate the role of c-Cbl and Cbl-b in human CD8+ T cell function, we designed Cas9 RNP complexes that target the *CBL* and *CBLB* genes. These RNP complexes were electroporated into activated human CD8+ T cells isolated from healthy donors. The molar ratio of gRNA and Cas9 protein is predicted to be 1:1 within the RNP complex, however, experimental data has shown that increasing the ratio of gRNA to Cas9 protein can substantially increase the efficiency of gene knockout, thus we prepared our RNP complexes at a molar ratio of 2.42:1 gRNAs/Cas9.[100,101] To further ensure a high level of knockout efficiency, three different gRNAs were used to target each gene (**Figure 4A**), which has been shown to significantly increase knockout efficiency compared to the use of a single targeting RNA in cells that are difficult to transfect, including T lymphocytes, with no adverse impact on cell viability.[99] We also used ATTO550-labeled tracrRNA to ensure that electroporation conditions were appropriate and transfection efficiency of the RNP complexes was high (**Figure 4A**). Flow cytometry analysis revealed that the Cas9 RNP complexes were transfected into activated human CD8+ T cells at very high efficiency (**Figure 4B and C**). The knockout of *CBL* and *CBLB* was subsequently verified by Western blot analysis. c-Cbl protein was successfully depleted in T cells transfected with *CBL*-targeting Cas9 RNP complexes alone or combined with *CBLB*-specific Cas9 RNPs. Likewise, Cbl-b proteins were absent in human Cbl-b KO and c-Cbl/Cbl-b double KO (Cbl dKO) CD8+ T cells (**Figure 4D**). These results validate the knockout strategy that we developed and will allow for the analysis of T cell functions in c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells.

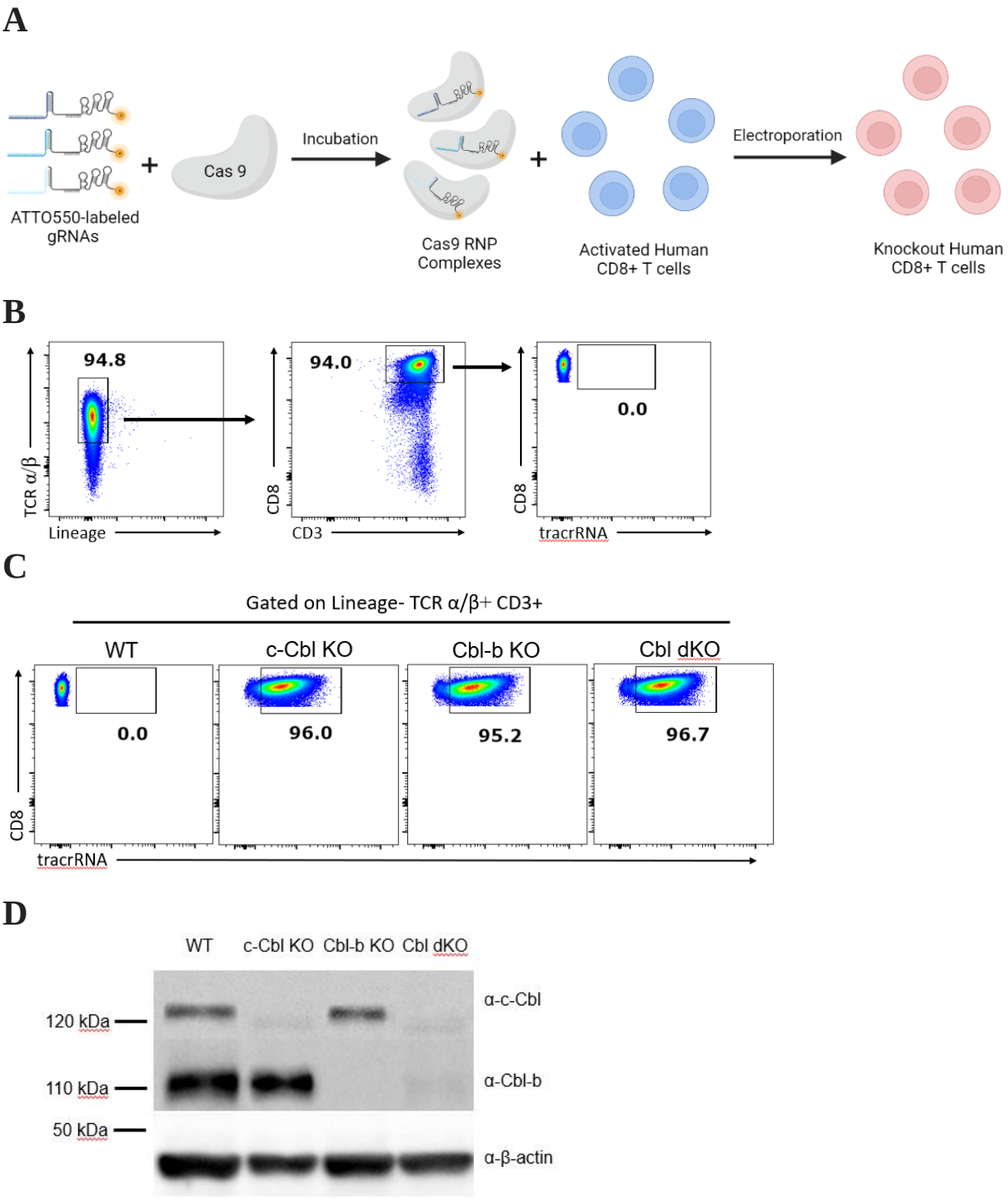

Figure 4: Efficient Knockout of *CBL* and *CBLB* in Human CD8+ T Cells via Cas9 RNPs

(A) Experimental scheme for generating Cas9 RNP complexes targeting *CBL* and *CBLB* to produce human c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells. Three different crRNAs

targeting *CBL* and *CBLB* were incubated with ATTO550-labeled tracrRNA to form ATTO550-labeled gRNAs. An equal amount of each gRNA was then incubated with Cas9 protein to produce *CBL*- and *CBLB*-targeted Cas9 RNP complexes that can be detected by flow cytometry after electroporation into human CD8+ T cells.
(B) Gating strategy for flow cytometry analysis of human CD8+ T cells. CD8+ T cells are gated as the Lineage- TCR α/β+ CD3+ CD8+ population. B220 was used as the negative lineage marker.
(C) Flow cytometry analysis of ATTO550 fluorescence in human CD8+ T cells 48 hours after electroporation with *CBL*- and/or *CBLB*-targeted Cas9 RNP complexes.
(D) Western blot analysis of c-Cbl and Cbl-b expression in human CD8+ T cells 72 hours after electroporation with *CBL*- and/or *CBLB*-targeted Cas9 RNP complexes. β-actin was used as a protein loading control.

3.2 Human Cbl dKO CD8+ T Cells Overexpress IFN-γ in a TGFβ Resistant Manner Following Activation

IFN-γ is a critical cytokine in the immune response against cancers that is produced by activated CD8+ T cells. IFN-γ can directly induce apoptosis in cancer cells in addition to activating innate immune cells to improve their ability to control tumor growth or activate naïve T cells through upregulation of human leukocyte antigens and costimulatory molecules.[16,17] IFN-γ receptor (IFNGR) signalling is also integral in maintaining Th1 polarization in CD4+ T helper cells and augmenting CD8+ cytotoxic T cell proliferation and production of cytotoxic molecules such as granzyme B and TRAIL.[44,48] Given that the simultaneous knockout of c-Cbl and Cbl-b in murine T cells led to a reduction in TCR downmodulation and increased activation, we investigated whether knockout of c-Cbl or Cbl-b would affect IFN-γ expression in activated human CD8+ T cells. WT, c-Cbl KO, Cbl-b KO, and human Cbl dKO CD8+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies to ensure strong activation. Following stimulation, the proportion of T cells expressing IFN-γ and quantity produced per cell were analyzed by flow cytometry.

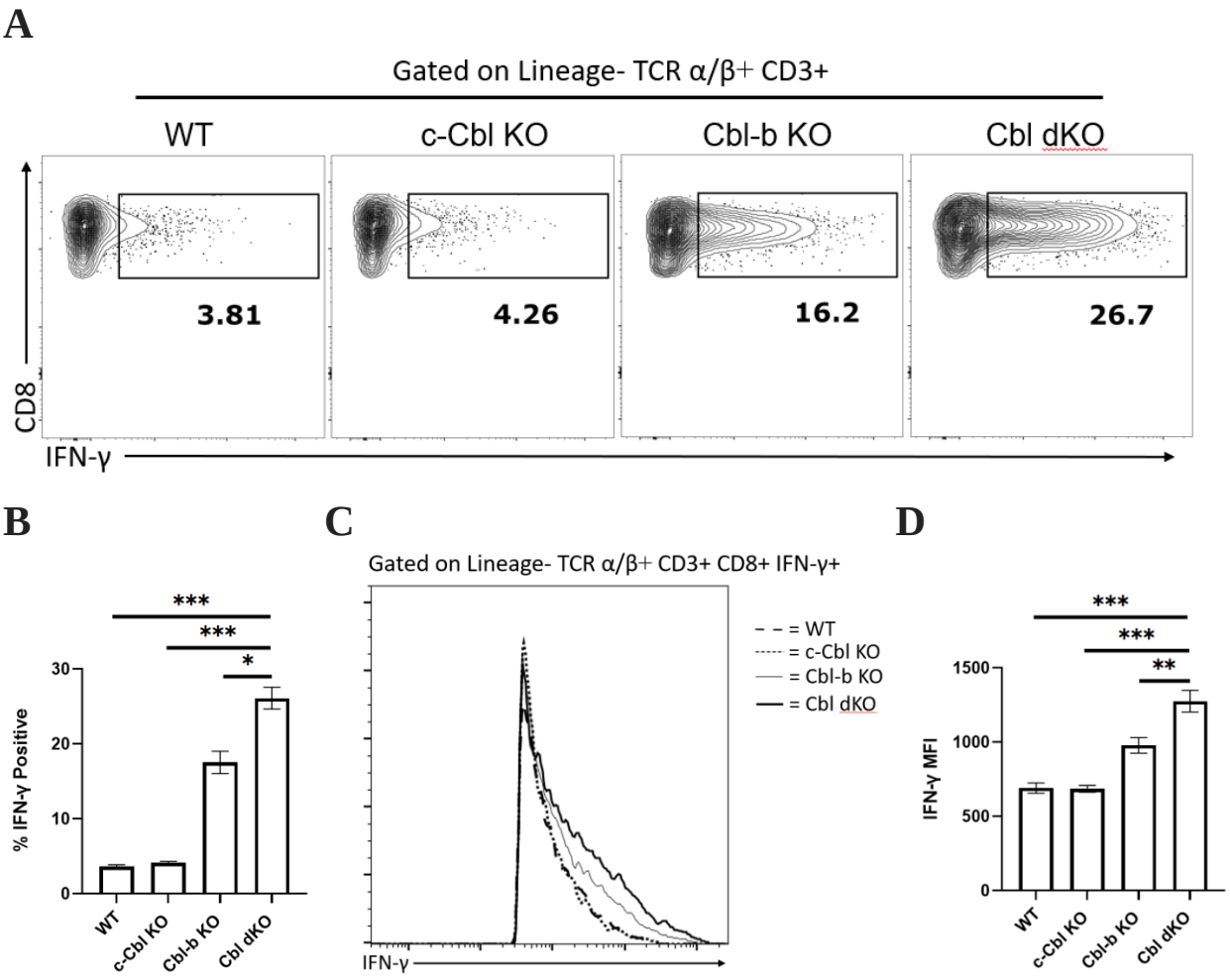

Figure 5: Human Cbl dKO CD8+ T Cells Produce Increased Levels of IFN-γ

(A) Representative contour plots of IFN-γ production in activated human WT, c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells. CD8+ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml) and soluble anti-CD28 (2 μg/ml) for a total of 22 hours. IFN-γ–producing cells were identified via intracellular staining and flow cytometry analysis.
(B) Statistical analysis of the percentages of IFN-γ producing cells among activated human WT, c-Cbl KO, Cbl-b KO and Cbl dKO CD8+ T cells.
(C) Representative histogram comparing IFN-γ expression of the IFN-γ+ population of activated WT (long dashed line), c-Cbl KO (short dashed line), Cbl-b (thin solid line), and Cbl dKO (thick solid line) human CD8+ T cells.
(D) Statistical analysis of IFN-γ MFI of the IFN-γ+ population of activated human WT, c-Cbl KO, Cbl-b KO and Cbl dKO CD8+ T cells.
Data are reported as mean ± SEM and are representative of three independent experiments.
$*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (unpaired Student's T test)

In line with previous results in murine T cells, we found that ablation of c-Cbl and Cbl-b in human CD8+ T cells resulted in a significant increase in the percentage of IFN-γ producing cells compared to WT, c-Cbl KO, and Cbl-b KO CD8+ T cells (**Figure 5A and B**). Following activation, the proportion of Cbl dKO cells that expressed IFN-γ was approximately 7 times greater compared to WT cells and almost 50% greater than Cbl-b KO cells (**Figure 5B**). Further analysis of the IFN-γ+ populations of WT, c-Cbl KO, Cbl-b KO, and Cbl dKO cells revealed that c-Cbl and Cbl-b deficiency not only increased the proportion of IFN-γ producing cells but also led to greater expression of IFN-γ within IFN-γ+ T cells (**Figure 5C and D**). IFN-γ expression was increased approximately twofold in IFN-γ+ Cbl dKO cells in comparison to IFN-γ+ WT and c-Cbl KO cells and exceeded the IFN-γ expression of Cbl-b KO cells by about 30% (**Figure 5D**). Interestingly, c-Cbl single KO CD8+ T cells did not differ significantly from WT CD8+ T cells in either the proportion of activated cells that produced IFN-γ or in the quantity of IFN-γ that was expressed. The absence of phenotypic differences in c-Cbl KO T cells could be explained by the relatively low expression of c-Cbl in mature human CD8+ T cells (**Figure 4D**). Conversely, ablation of Cbl-b alone led a marked increase in the percentage of IFN-γ+ T cells among activated cells and the expression levels of IFN-γ (**Figure 5C and D**), indicating that Cbl-b may regulate the activation of human CD8+ T cells similarly to the mechanisms that have been described in murine T cells. The further increase in the proportion of IFN-γ+ T cells and level of IFN-γ expression in Cbl dKO T cells over Cbl-b single KO T cells suggests that c-Cbl and Cbl-b play a redundant role in regulating human CD8+ T cell activation. Functional redundancy between c-Cbl and Cbl-b could also contribute to the lack of observable differences in IFN-γ

production between WT and c-Cbl KO T cells. The effect of depleting the less abundant c-Cbl could be compensated for by the activity of Cbl-b, which is expressed at much higher levels (**Figure 4D**), resulting in the lack of IFN-γ overexpression in activated c-Cbl KO T cells. Thus, human CD8+ deficient in both c-Cbl and Cbl-b may possess greater cytotoxic activity against cancer cells due to the overexpression of IFN-γ.

The tumor killing activity of cytotoxic CD8+ T cells is often suppressed in established tumors through the recruitment of immunosuppressive cells such as Treg cells and MDSCs and the production of immunosuppressive cytokines. TGFβ is one such immunosuppressive cytokine that is often produced by both the tumor cells and recruited suppressive immune cells and is present in excessive amounts in the TME of established tumors.[18,25,93] TGFβ receptor signalling directly inhibits the production of IFN-γ and cytolytic granules in cytotoxic CD8+ T cells, impairing their ability to destroy tumor cells.[44,93] Since TGFβ-mediated suppression is nearly ubiquitous in CD8+ TILs, we decided to investigate if knockout of either c-Cbl, Cbl-b or both proteins would confer resistance to TGFβ inhibition in human CD8+ T cells. We activated human WT, c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells in the presence or absence of high concentrations of human TGFβ and analyzed IFN-γ expression of the activated cells. The proportion of both WT and c-Cbl KO T cells that expressed IFN-γ was markedly reduced in the presence of TGFβ (**Figure 6**). A significant decrease in the percentage of IFN-γ+ cells was also observed in Cbl-b single KO T cells that were exposed to high levels of TGFβ, although the relative reduction in IFN-γ+ cells was much smaller compared to WT and c-Cbl KO T cells (**Figure 6**). This indicates that Cbl-b positively regulates TGFβ receptor signalling and thus

contributes to the suppression of cytotoxic activity in human CD8+ TILs. In contrast, human CD8+ T cells that were both c-Cbl- and Cbl-b-deficient had elevated percentages of IFN-γ+ cells when cultured in the presence of human TGFβ, although the results were not statistically significant compared to control cells activated in the absence of human TGFβ (**Figure 6**). These results suggest that c-Cbl and Cbl-b also share functional redundancy in the regulation of TGFβ receptor signalling in human CD8+ T cells, as the depletion of c-Cbl alone did not significantly impact the inhibition of IFN-γ production by TGFβ while Cbl-b knockout failed to completely abolish the inhibitory effects of TGFβ signalling. The IFN-γ production of Cbl dKO T cells was totally uninhibited in the presence of TGFβ, indicating that c-Cbl also positively regulates TGFβ receptor signalling. As with the previous IFN-γ expression results, the lack of phenotypic changes in c-Cbl single KO T cells can be attributed to the low expression of c-Cbl in mature CD8+ T cells and possible redundant activity from Cbl-b. Together, these data demonstrate that human CD8+ deficient in both c-Cbl and Cbl-b express the greatest amounts of IFN-γ upon activation. They also maintain elevated expression levels of IFN-γ in the presence of high concentrations of immunosuppressive cytokines, and thus should better maintain their cytotoxic activity against established tumors with immunosuppressive TMEs.

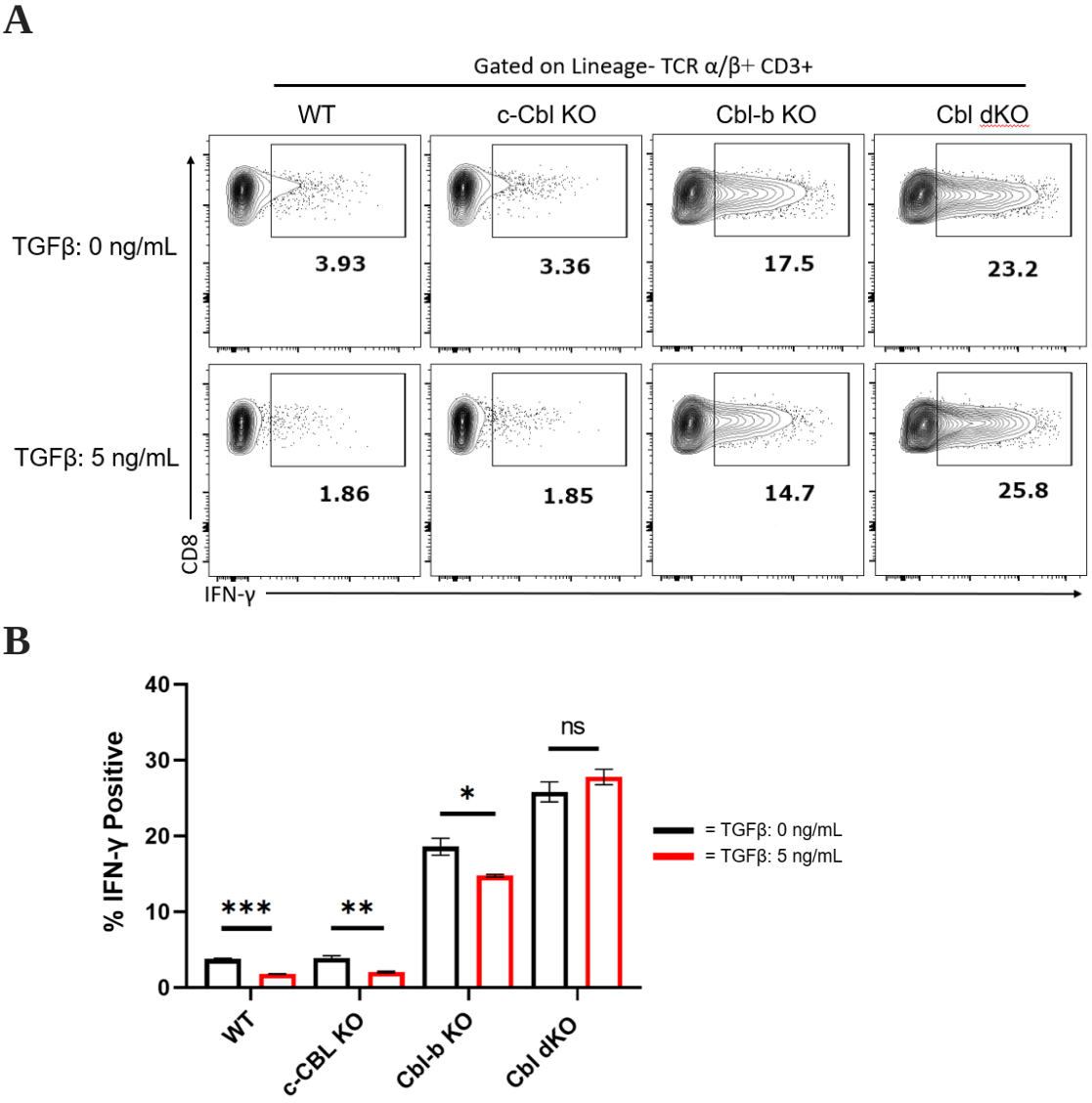

Figure 6: IFN-γ Expression is Uninhibited by TGFβ in Human Cbl dKO CD8+ T cells

(A) Representative contour plots of IFN-γ production in activated human WT, c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells. CD8+ T cells were cultured in the presence or absence of 5 ng/mL of recombinant human TGFβ and stimulated with plate-bound anti-CD3 (3 μg/ml) and soluble anti-CD28 (2 μg/ml) for a total of 22 hours.
(B) Statistical analysis of the percentages of IFN-γ producing cells among activated human WT, c-Cbl KO, Cbl-b KO and Cbl dKO CD8+ T cells in the presence or absence of human TGFβ. Data are reported as mean ± SEM and are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: not significant (unpaired Student's T test)

3.3 Activated Human Cbl dKO CD8+ T Cells are Hyperproliferative and Uninhibited by TGFβ and PD-1 Signaling

Another integral aspect of the cytotoxic CD8+ T cells response against tumors is the capacity of tumor-specific CD8+ T cells to proliferate after encountering their cognate antigen. CD8+ T cells that target tumor antigens undergo clonal expansion in secondary lymphoid organs after activation to reach sufficient numbers to mount an effective anti-tumor response. Continual proliferation of CD8+ T cell within the tumors and higher densities of CD8+ TILs are also associated with improved survival in a wide range of tumor-forming malignancies.[16,41,44] High levels of CD8+ T cell infiltration results in greater cell-cell contact between tumor cell and cytotoxic T cells that are necessary for tumor cell lysis by CD8+ TILs.[16,41] However, various immunosuppressive factors are frequently present in both tumor-draining lymph nodes and the TME that hinder the proliferation of CD8+ T cells. Treg cells present in lymph nodes and immunosuppressive cells in the TME produce large amounts of TGFβ that suppress CD8+ T cell proliferation by inhibiting the production of IL-2 and transcriptional regulation of key cycle proteins including cyclin-dependent kinase inhibitors.[90,93,94] Therefore, we examined whether c-Cbl, Cbl-b or both proteins positively regulate TGFβ inhibition of human CD8+ T cell proliferation.

Human WT, c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells were labeled with CFSE and proliferation was induced using plate-bound anti-CD3 and anti-CD28 antibodies in the presence or absence of high levels of human TGFβ. The T cells were allowed to proliferate for 72 hours and were then analyzed via flow cytometry. When looking at the total proportion of cells that

proliferated at least once, a greater percentage of Cbl-b KO and Cbl dKO T cells proliferated compared to WT and c-Cbl T cells under standard culture conditions. A small but significant decrease in the proportions of proliferating cells was observed in the WT and c-Cbl KO T cells when they were cultured with TGFβ, whereas Cbl-b Ko and Cbl dKO T cells did not show a reduction in the ratios of cells that proliferated (**Figure 7A and B)**. Further analysis of the proliferative capacity of these different mutant T cells was performed by calculating the average number of cell divisions within each population. WT and c-Cbl KO T cells on average underwent a low number of cell divisions under standard conditions, which decreased considerably in the presence of TGFβ (**Figure 7C)**. By contrast, Cbl-b KO T cells exhibited a twofold increase in the mean number of cell divisions over their WT counterpart under normal culture conditions and a much smaller but still significant decrease when cultured with TGFβ; Cbl dKO T cells showed an even greater number of average cell divisions than the Cbl-b single KO T cells in the absence of TGFβ and their proliferation was completely uninhibited by the presence of TGFβ (**Figure 7C)**. These results further highlight the compensatory nature of the regulatory activity of c-Cbl and Cbl-b in human CD8+ T cells and indicate that Cbl dKO CD8+ T cells may have increased tumor killing capacity compared to WT or Cbl-b single KO CD8+ T cells.

Another key source of negative regulation that limits CD8+ T cell proliferation within many solid tumor malignancies is the signalling axis involving PD-1 and its ligands. Many tumor cells have mutated to either constitutively express high levels of PD-L1 or highly upregulate the expression of PD-L1 in response to Th1 cytokines such as IFN-γ.[60,61] When activated CD8+ T

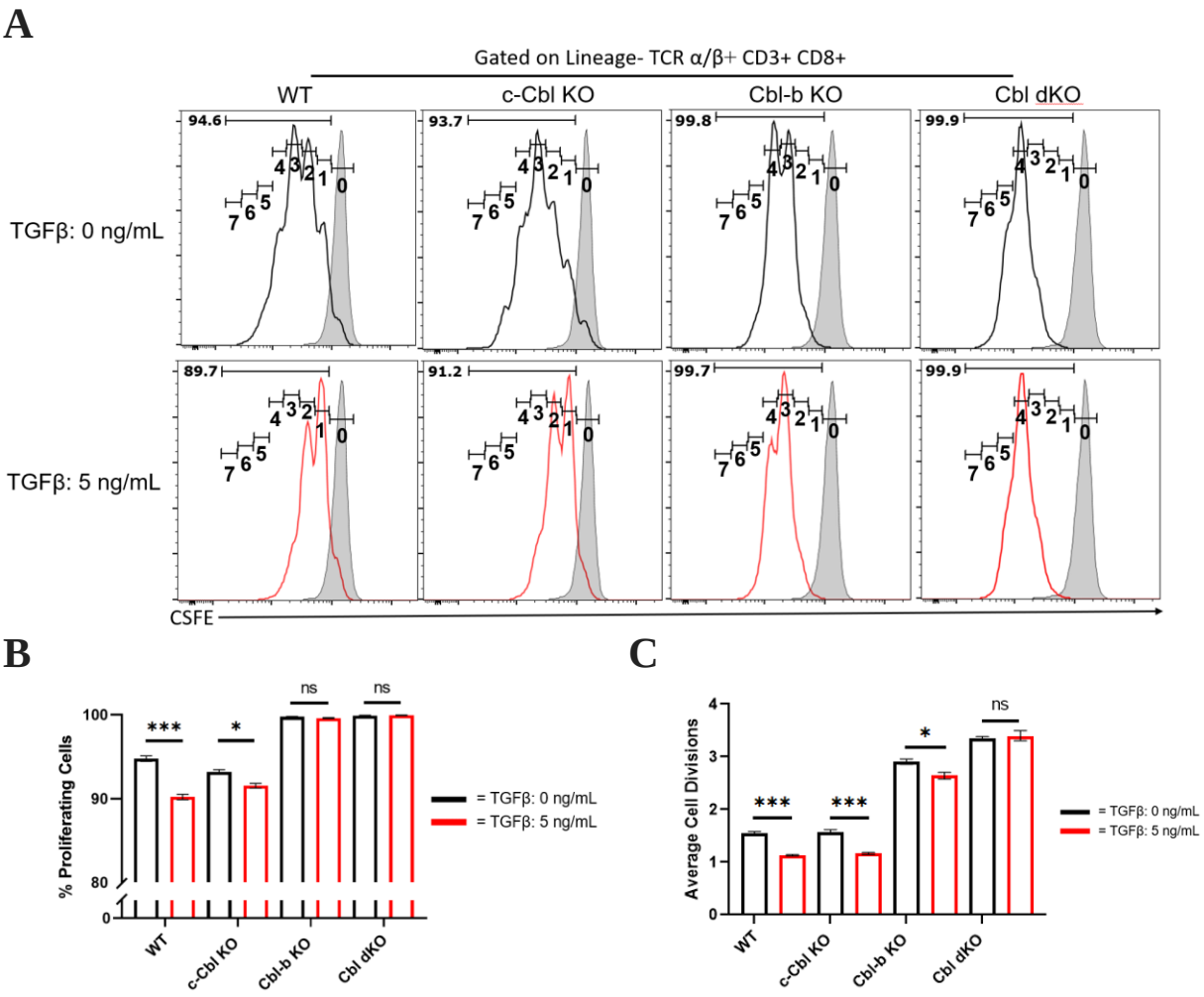

Figure 7: Human Cbl dKO CD8+ T cells Remain Highly Proliferative in the Presence of TGFβ

(A) Representative histogram plots of CFSE dilutions in activated human WT, c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells. CD8+ T cells were cultured in the presence or absence of 5 ng/mL of recombinant human TGFβ and stimulated with plate-bound anti-CD3 (3 μg/ml) and soluble anti-CD28 (2 μg/ml) for a total of 72 hours. Cells with a non-diluted CFSE signal were identified as non-proliferative and labeled as gate 0. Each subsequent peak was labeled with its corresponding division number.
(B) Statistical analysis of the percentages of proliferating cells among activated human WT, c-Cbl KO, Cbl-b KO and Cbl dKO CD8+ T cells in the presence or absence of human TGFβ.
(C) Statistical analysis of the average number of cell divisions of activated WT, c-Cbl KO, Cbl-b KO and Cbl dKO human CD8+ T cells in the presence or absence of human TGFβ.
Data are reported as mean ± SEM and are representative of three independent experiments.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: not significant (unpaired Student's T test)

cells come into contact with these tumor cells, PD-1 will be bound by these ligands, resulting in attenuation of T cell activation and changes in cell metabolism.[55,60] Persistent signalling through the PD-1 axis leads to T cell exhaustion and the loss of effector functions including proliferative capacity.[53] These negative costimulatory signals are transmitted through the recruitment and activation of the tyrosine phosphatases SHP1 and SHP2 and the upregulation of various suppressive factors including c-Cbl and Cbl-b.[31,62] In murine CD8+ T cells, ablation of Cbl-b alone resulted in resistance to PD-L1-mediated suppression of proliferation.[89] Based on the previous data that suggests c-Cbl and Cbl-b regulate human CD8+ T cell activation and proliferation, we investigated whether PD-1 inhibition of proliferation was mediated by c-Cbl and Cbl-b in human CD8+ T cells. In order to measure the proliferative capacity of CD8+ T cells under this different form of inhibition, WT, c-CBL KO, Cbl-b KO, and Cbl dKO cells were labeled with CFSE and co-cultured with MDA-MD-231 cells that express high levels of PD-L1 in a 1:1 ratio. Soluble anti-CD3 and CD28 were added to ensure the T cell remained activated throughout the co-culture period. After 72 hours, the proliferation of the CD8+ T cells were analyzed by flow cytometry.

Consistently with the proliferation results with TGFβ inhibition, the data shows that the Cbl dKO CD8+ T cells have an increased proliferative capacity compared to WT, c-Cbl, and Cbl-b single KO CD8+ T cells when cultured under conditions that would result in high levels of PD-1 signalling (**Figure 8a)**. When looking specifically at the proportions of cells that proliferated, all WT and KO T cells were able to undergo a high degree of proliferation, though a significantly higher percentage of Cbl dKO T cells underwent at least one round of division than WT CD8+ T

cells and CD8+ T cells deficient for a single Cbl protein (**Figure 8B)**. Moreover, Cbl dKO CD8+ T cells also had a significantly higher mean number of cell divisions compared to WT, c-Cbl and Cbl-b KO CD8+ T cells, demonstrating that depletion of both c-Cbl and Cbl-b result in the greatest increase in proliferation capacity in CD8+ T cells under PD-1 inhibitory signalling. (**Figure 8C)**. Interestingly, this data also suggests that c-Cbl KO CD8+ T cells experienced a reduction in proliferation compared to WT CD8+ T cells. The proportion of c-Cbl KO CD8+ T cells that divided at least once was lower than in WT CD8+ T cells, but the difference was not statistically significant (**Figure 8B)**, however a significant decrease in the mean number of cell divisions was observed in c-Cbl KO cells compared to their WT counterparts (**Figure 8C)**. This is in contrast with both the previous experimental results where WT and c-Cbl CD8+ T cells exhibited very similar phenotypes and the substantial increase in proliferative capacity of Cbl dKO CD8+ T cells compared to CD8+ T cells that are deficient in only Cbl-b. Altogether, these findings indicate that the combined activities of c-Cbl and Cbl-b play an important role in the negative regulation of activated human CD8+ T cell proliferation and in mediating the inhibitory effects of TGFβ and PD-1 signalling on proliferation. These findings suggest that ablation of both c-Cbl and Cbl-b in CD8+ T cells could lead to better tumor killing, especially in the context of solid tumors with immunosuppressive tumor microenvironments, as Cbl dKO cells are more likely to maintain sufficient numbers of cytotoxic cell necessary for the elimination of the cancerous tissue.

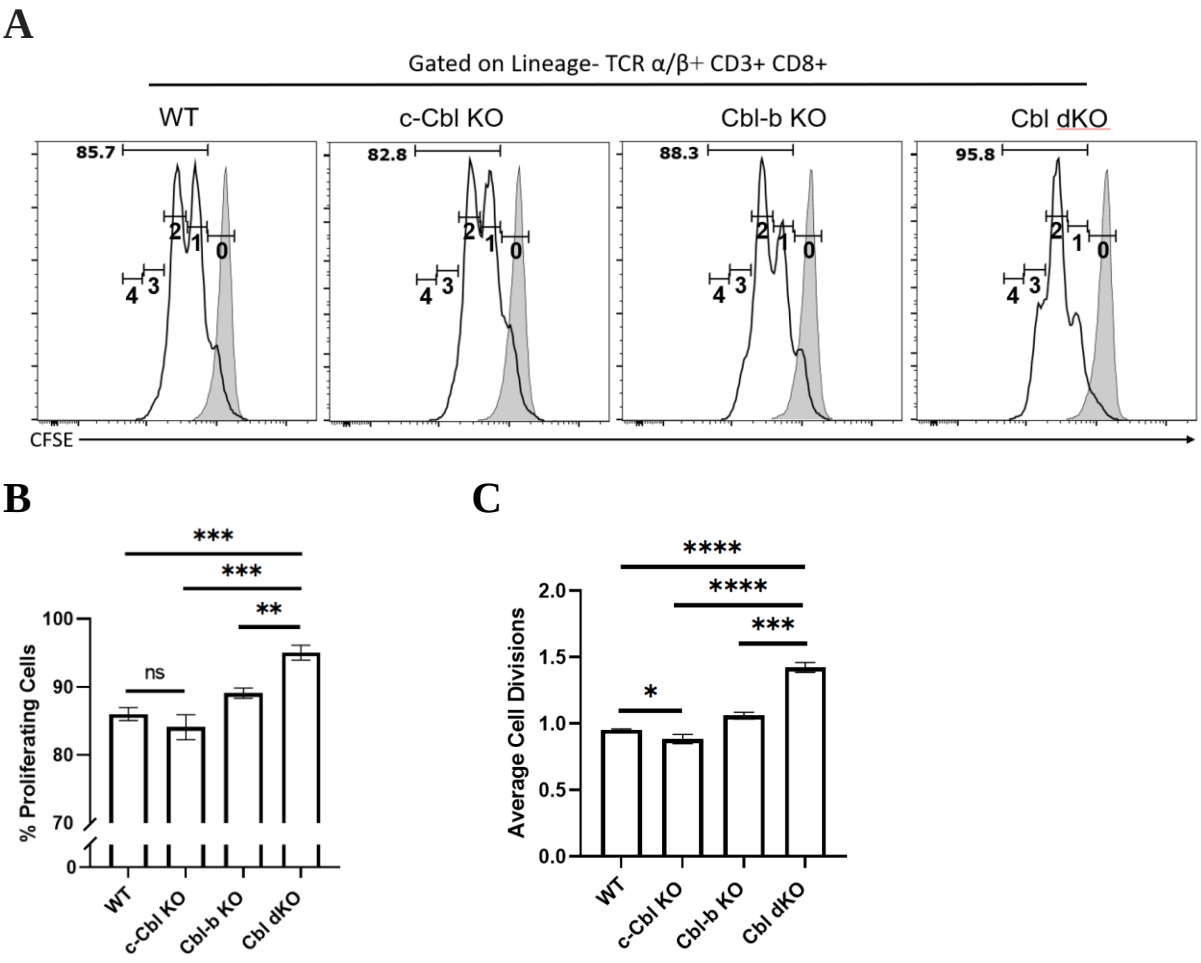

Figure 8: Human Cbl dKO CD8+ T cells Possess Elevated Proliferative Capacity when Cultured with PD-L1+ Tumor Cells

(A) Representative histogram plots of CFSE dilutions in activated human WT, c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells. 5×10^4 CD8+ T cells were cultured with 5×10^4 MDA-MB-231 cells and stimulated with soluble anti-CD3 (3 μg/ml) and soluble anti-CD28 (2 μg/ml) for a total of 72 hours. Cells with a non-diluted CFSE signal were identified as non-proliferative and labeled as gate 0. Each subsequent peak was labeled with its corresponding division number.
(B) Statistical analysis of the percentages of proliferating cells among activated human WT, c-Cbl KO, Cbl-b KO and Cbl dKO CD8+ T cells co-cultured with MDA-MB-231 cells.
(C) Statistical analysis of the average number of cell divisions of activated human WT, c-Cbl KO, Cbl-b KO and Cbl dKO CD8+ T cells co-cultured with MDA-MB-231 cells.
Data are reported as mean ± SEM and are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ ns: not significant (unpaired Student's T test)

3.4 Human Cbl dKO CD8+ T Cells Possess Increased Killing Capacity of Tumor Cells

Previous research in murine tumor models have demonstrated that germline knockout of the *Cblb* gene cells allow mice to reject implanted tumors and substantially reduce the onset of spontaneous cancers.[89,92] Furthermore, the adoptive transfer of murine Cbl-b KO CD8+ T cells allowed for the rejection of established tumors, while transfer of WT CD8+ T cells failed to control tumor growth.[92] However, relatively few studies have been conducted on the effects of c-Cbl alone or in conjunction with Cbl-b in mature CD8+ T cells with regard to cancer immunotherapy, possibly due to the relatively low expression of c-Cbl once T cells are fully developed. Additionally, it is still unknown whether Cbl-b limits the cytotoxic function of human CD8+ T cells in a similar manner to murine T cells. Based on the previous results examining proliferation and cytokine production, *in* vitro tumor cell killing assays were performed using human WT, c-Cbl KO, Cbl-b KO, and Cbl dKO CD8+ T cells. Following activation, human CD8+ T cells were co-cultured with CTV-labeled MDA-MB-231 cells, which are derived from a highly aggressive triple-negative breast cancer, at varying ratios of effector T cells to tumor cells for 6 hours. The cells were then harvested and analyzed via flow cytometry.

Across all effector cell concentrations, WT and c-Cbl KO CD8+ T cells were phenotypically similar and exhibited relatively low tumor killing activity (**Figure 9)**. The percentage of eliminated neoplastic cells did gradually increase as more CD8+ T cells were added, but even at extremely high concentrations of 25 T cells per cancerous cell, on average only 10% of all the cancer cells were killed. In contrast to this, Cbl-b KO CD8+ T cells eliminated two- to threefold greater amounts of tumor cells at every concentration compared to WT and c-Cbl cells,

indicating that the heightened activation state previously seen in Cbl-b KO cells does indeed translate into greater tumor killing efficiency (**Figure 9)**. Cbl dKO CD8+ T cells displayed greatly enhanced tumor killing capacity compared to WT, c-Cbl KO, and even Cbl-b cells at high concentrations of effector cells (**Figure 9)**. However, the magnitude of this difference in tumor killing between Cbl dKO cells and Cbl-b KO cells decreased as the concentration of effector cells decreased, to the point where there was no significant difference at the 5:1 effector-target cell ratio. When considered as a whole, these results demonstrate that targeting both c-Cbl and Cbl-b in human CD8+ T cells is an effective method of augmenting their *in vitro* tumor cell killing capacity, which raises the possibility of the use of Cbl dKO CD8+ T cells in cellular therapies targeting cancers.

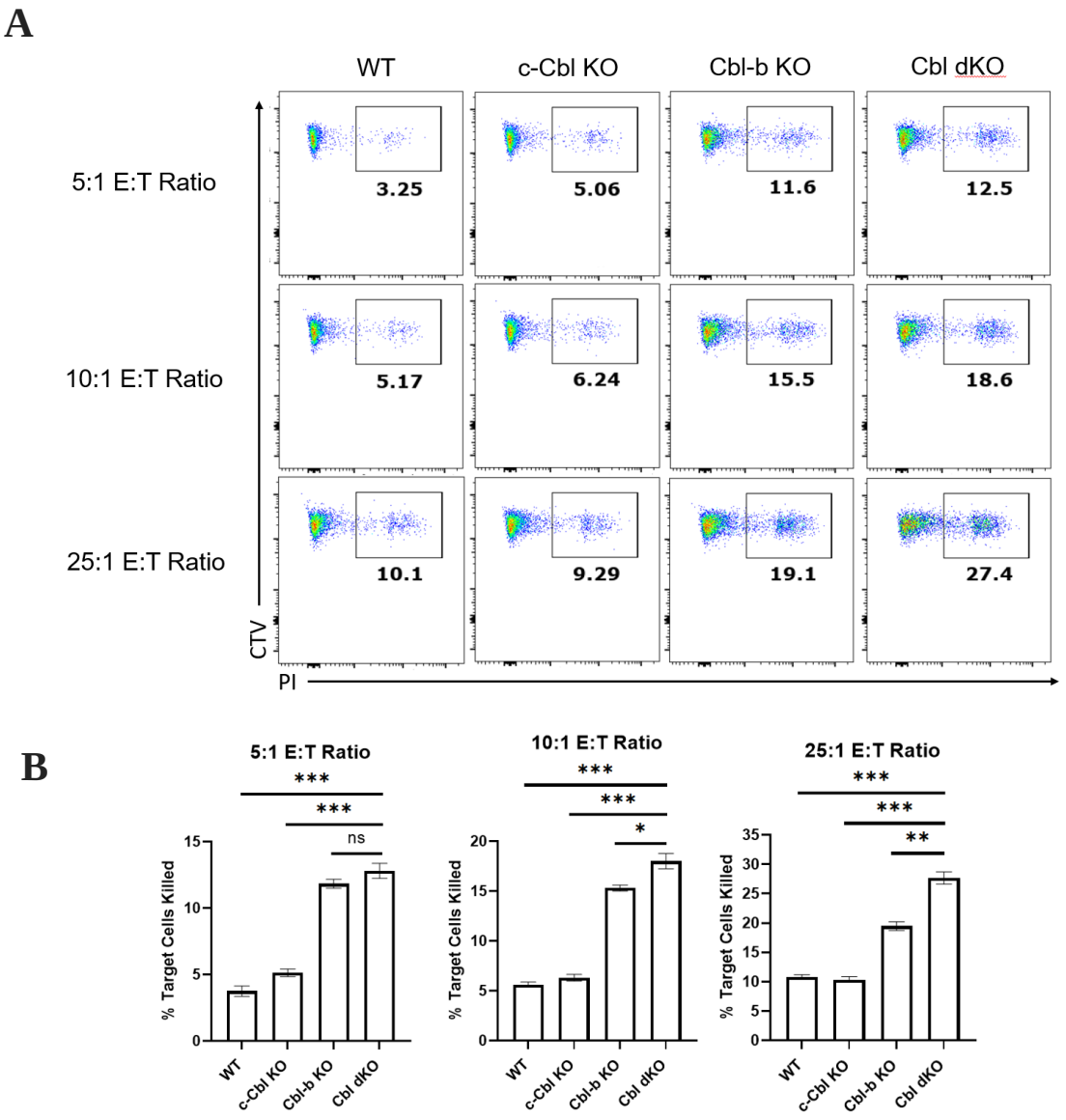

Figure 9: Human Cbl dKO CD8+ T Cells are Hyperresponsive to Allogenic Tumor Cells Expressing High Levels of PD-L1

(A) Representative dot plots of MDA-MB-231 cells cocultured with activated WT, c-Cbl KO, Cbl-b KO, and Cbl dKO human CD8+ T cells. T cells were cultured in 5:1, 10:1 and 25:1 ratios with the target MDA-MB-231 cells for 6 hours and propidium iodide staining was used to detect dead target cells.
(B) Statistical analysis of the percentages of MDA-MB-231 cells killed by WT, c-Cbl KO, Cbl-b KO and Cbl dKO human CD8+ T cells
Data are reported as mean ± SEM and are representative of three independent experiments.
$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns: not significant (unpaired Student's T test)

Chapter 4: Discussion and Future Perspectives

CD8+ T cells play a critical role in the immune system's ability to eliminate cancerous cells and control tumor growth. After tumor antigen-specific T cells are activated and differentiate, they eliminate cancer cells in an antigen-dependent manner through direct cell-to-cell killing and secretion of cytotoxic cytokines.[16,29] However, most established tumor cells evade destruction by limiting the effectiveness of CD8+ TILs through mutations that result in the expression of less immunogenic antigens, downregulating the expression of MHC class I molecules or acquiring ligands for immune checkpoint receptors such as PD-L1.[21,25,47] Tumor cells can also mediate immunosuppression of cytotoxic cells by producing inhibitory cytokines such as TGFβ, or by recruiting immune cells such as Tregs and myeloid-derived suppressor cells that also secrete inhibitory cytokines and pro-tumorigenic signals, further inhibiting the activity of CD8+ TILs.[10,18,25] This multifaceted suppression of CD8+ T cell activity in solid tumor malignancies demands solutions that can target multiple suppressive pathways; hence why ablation of the E3-ubiquitin ligases c-Cbl and Cbl-b presents an attractive opportunity to augment cellular immunotherapy as it could allow for complete T cell activation in the absence of costimulation in combination with attenuation of inhibitory signalling through both the PD-1 and TGFβ signalling pathways.

c-Cbl and Cbl-b Regulation of Human CD8+ T cell Activation and Anti-Tumor Immunity

In this study, we have identified that c-Cbl and Cbl-b play a critical role in the regulation of the activation state and tumor killing capacity of human CD8+ T cells. Ablation of Cbl-b alone led to a marked increase in the percentage of IFN-γ+ cells among activated cells and the expression

levels of IFN-γ compared to WT CD8+ T cells. Knockout of Cbl-b in conjunction with c-Cbl yielded further increases in the percentage of IFN-γ producing cells and IFN-γ expression levels. Cbl dKO CD8+ T cells also exhibited greatly enhanced proliferative capacity compared to WT cells and those deficient for a single Cbl protein. High concentrations of TGFβ also failed to suppress IFN-γ production or proliferation in Cbl dKO cells, while a significant reduction in both markers of CD8+ T cell activation was observed in WT and the single KO cells. Similarly, under high levels of PD-1 inhibitory signalling, Cbl dKO cells had the greatest proportion of cells that proliferated and the highest average number of divisions. The stronger activation state of the Cbl dKO cells after stimulation led to significantly improved allogenic tumor killing at nearly all effector cell concentrations, suggesting that Cbl-b retains its role as a regulator of CD8+ T cell activation, PD-1 signalling, and TGFβ signalling in human CD8+ T cells. These results also demonstrate that human c-Cbl and Cbl-b also have some degree of redundant activity. Previous studies in murine T cells have identified redundant activity between c-Cbl and Cbl-b in both the internalization of the TCR complex through polyubiquitination and in the ubiquitination of downstream signalling proteins following T cell activation.[82,102] Whether these activities also display redundancy in human CD8+ T cells has not currently been elucidated. Furthermore, the molecular mechanisms by which Cbl-b mediates inhibition of human CD8+ T cells through PD-1 and TGFβ signalling have not been identified, nor has the effect of c-Cbl depletion on either of these inhibitory pathways been explored. Additional experiments using co-immunoprecipitation, chromatin immunoprecipitation, and ubiquitination assays could be performed to determine if Cbl-b interacts with CD3ζ, CD28, PD-1 and SMAD7 to regulate CD8+ T cell activation through the same signalling pathways as in murine T cells. Similar experiments could also be performed

in Cbl-b KO human CD8+ T cells to determine whether the redundant activity of c-Cbl in primary human T cells proceeds through these same signalling pathways.

Interactions between c-Cbl and PD-1 in CD8+ T cells

While both Cbl-b KO cell and Cbl dKO cells demonstrated significant phenotypic differences from WT cells, c-Cbl KO cells were very similar to WT cells in terms of IFN-γ production, TGFβ inhibition of activation, and tumor killing capacity. However, there is one set of conditions where the response of the c-Cbl cells diverged from that of WT cells. The proliferative capacity of c-Cbl KO cells seems to trend lower than WT cells under inhibition by PD-1 signalling, as the mean proportion of cells that divided at least once was lower for c-Cbl cells and the average number of cell divisions was significantly lower, even though the ablation of c-Cbl in Cbl-b KO cells resulted in an increase in proliferation. A relationship between c-Cbl and PD-1 expression in mouse CD8+ T cells has been identified in one publication, where the authors propose that c-Cbl polyubiquitinates the cytoplasmic tail of PD-1, targeting it for proteasomal degradation.[103] This proposed mechanism could explain why c-Cbl KO cells experienced a reduction in proliferative capacity when cultured in the presence of high levels of PD-L1. Furthermore, since Cbl-b is one of the primary downstream mediators of PD-1 signalling, it is conceivable that the inhibitory effect of increased expression of PD-1 on c-Cbl KO cells would be negated by the depletion of Cbl-b in Cbl dKO cells, allowing them to remain highly activated in the presence of PD-L1. These proposed mechanisms would have to verified by further studies analysing whether c-Cbl is responsible for targeting PD-1 to the proteasome through ubiquitination in human CD8+

T cells and whether depletion or mutation of Cbl-b is sufficient to abrogate the possible increased PD-1 signalling in c-Cbl KO cells.

Future Studies and Applications of c-Cbl and Cbl-b in Cancer Immunotherapy

These results provide a foundation for further investigation into the molecular mechanisms by which these E3 ubiquitin ligases regulate T cell activation and effector function, as well as their potential as targets for immunotherapy. One potential follow-up study could investigate the effects of knocking out c-Cbl and Cbl-b on the function of other T cell subsets, such as CD4+ helper T cells. While CD4+ T cells have limited cytotoxic activity compared to CD8+ T cells, they still play a critical role in the adaptive immune response against tumors, as Th1-polarized CD4+ T cells produce high levels of IL-2, IFN-γ, and TNF-α, key cytokines that enhance the anti-tumour activity of CD8+ T-cell and cause apoptosis in tumor cells.[16,18] CD4+ T cells also support cDC1s that provide important activation signals for CD8+ T cells, leading to sustained CD8+ T cell-mediated anti-tumor responses.[29] If depletion of c-Cbl and Cbl-b in human CD4+ T cells elicits a similarly heightened state of activation as we have observed in CD8+ T cells, the use of a mixed population of Cbl dKO CD4+ and CD8+ T cells could produce an even stronger anti-tumor response. It would also be valuable to explore the effects of c-Cbl and Cbl-b knockout on CD8+ T cell memory formation and how these altered memory T cells interact with tumors. The differentiation of antigen-experienced CD8+ T cells into memory cells is another integral component of the adaptive immune response to cancer, as the chronic nature of the disease necessitates a durable, self-renewing T-cell response. The CD8+ T cell memory response to tumors is both complex and heterogeneous, involving central-, effector- and stem cell like-

memory T cells that can migrate to tumors, exhibiting varying degrees of effector molecule production and proliferation in response to TCR stimulation.[43,45,47] Additionally, tumor resident memory T cells have also been identified as an important component of the CD8+ T cell anti-tumor response.[45,47] If the depletion of c-Cbl and Cbl-b interferes with formation of memory CD8+ T cells or alters the composition of the CD8+ memory response, the augmented tumor-killing capacity of human Cbl dKO CD8+ T cells could be outweighed by their inability to mount a durable response against established tumors. Another important direction for future studies would be to investigate the tumor killing capacity of Cbl dKO T cells in a humanized mouse model. Immunodeficient mice engrafted with human CD34+ hematopoietic stem cells would allow for the formation of an immunosuppressive tumor microenvironment producing human cytokines like TGFβ and IL-10. A tumor xenograft model using these mice would allow us to determine whether the enhanced activation state of Cbl dKO CD8+ T cells would be maintained and mediate effective tumor rejection over a much longer time course. Furthermore, these experiments would provide greater insights into the resistance these Cbl dKO cells have against TGFβ inhibition and exhaustion.

As mentioned in this study, the hyperresponsiveness of human Cbl dKO CD8+ T cells against allogenic tumors suggests that targeting these E3 ubiquitin ligases could serve as a novel form of cellular cancer immunotherapy. While our results indicate that knockout of both c-Cbl and Cbl-b confers greater activation and resistance to two of the most common inhibitory signalling pathways, Cbl dKO cells could also be combined with other forms of cancer immunotherapy to produce highly effective combination therapies. For example, c-Cbl and Cbl-b do not regulate

the expression of other inhibitory molecules such as CTLA4 and TIM-3 in CD8+ T cells, nor are they the sole mediators of their inhibitory effects.[39,40] Thus, combining the adoptive transfer of Cbl dKO CD8+ T cells with antibodies that target these immune checkpoint molecules should allow the transferred T cells to resist inhibition and exhaustion to an even greater degree, increasing the likelihood that the tumors are successfully eliminated. c-Cbl and Cbl-b knockout could also augment established forms of adoptive T cell therapies. One of the major challenges in TIL-based cellular therapies is that these isolated T cells often have limited cytotoxic activity due to inhibition and exhaustion.[67,68] During the *in vitro* expansion of the isolated TILs, c-Cbl and Cbl-b could be knocked out, resulting in an augmented TIL population that could have increased tumor-killing capacity while also being much more resistant to the immunosuppressive tumor microenvironment. However, it is likely that depletion of c-Cbl and Cbl-b from the entire TIL population would also increase the severity of the adverse effects experienced by patients, as the activity of any autoreactive T cells would also be enhanced. This drawback could be mitigated through high-throughput screening and selection of TILs bearing TCRs that target tumor antigens. There is also strong evidence to suggest that knockout of c-Cbl and Cbl-b could improve CAR T cell therapies, as these engineered T cells are still vulnerable to the same forms of suppression as unmodified T cells.[16,74] By eliminating the Cbl proteins during CAR T cell production, it would likely increase their proliferative capacity and the durability of their cytotoxic activity in the tumor microenvironment, leading to a persistent T cell response against the target tumor cells. Overall, the findings from this study suggest that c-Cbl and Cbl-b could serve as promising targets for cancer immunotherapy, and further investigations into their effects on other T cell subsets, memory formation, and the activity of Cbl dKO CD8+ T cell *in vivo* are

necessary to determine the possible clinical significance of these proteins in adoptive T cell therapies.

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