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The Influence of Co-Signaling Proteins on Restimulation-Induced Cell Death in Effector
T Lymphocytes

By Camille Lake

Dissertation submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2021



UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES

SCHOOL OF MEDICINE GRADUATE PROGRAMS

Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814



APPROVAL OF THE DOCTORAL DISSERTATION IN THE EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

Title of Dissertation: "The Influence of Co-Signaling Proteins on Restimulation-Induced Cell Death in Effector T Lymphocytes"

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Doctor of Philosophy Degree
March 26, 2021

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Acknowledgements

My principal investigator: Andrew Snow. Thank you for teaching me how to be a strong scientist and pushing through when times were rough. I'm grateful that our relationship evolved from mentor/mentee to collaborators.

My lab members: Dr. Kelsey Voss, Dr. Swadhinya Arjunaraja, Dr. Bat Djorbal, Christopher Luthers, Bradly Bauman, Gina Dabbah, Katherine Pohida, and Dr. Henry Lu. Thank you for putting up with my questions and being such a fun, strong group. You made coming into the lab every day the most fun I've had with any group.

My committee members: Dr. Brian Schaefer, Dr. Rob Kortum, Dr. Edward Mitre, Dr. Gabriela Dveksler, Dr. Pamela Schwartzberg. Your continued scientific support and ideas made this project happen.

My mentee: Timothy Jiang. Thank you for your patience as I learned how to mentor others.

My friends at USUHS: lab members, friends in my program and department Rama Atitkar, Mary Brockett, Shannon Rhodes, Guzal Khayrullina, and Nate Lott. You all kept me sane during this process. I love you all.

My lifetime friends: Bethany Lettiere, for getting me through college physics and helping me fall asleep after watching scary movies; Jeffrey Felker, for having the most (in)appropriately timed jokes UwU; Jackson Kunz, for your steadfast friendship over the years which has brought me a lot of joy; Ashley Simpton, for being the best friend a girl could ask for; Clare Callahan, for challenging me in Jiu Jitsu and being a ray of sunshine;

Christina and Veronica Spandler, for all the trouble we've caused and will continue to cause; and Laura Sartori, for our many adventures abroad both past and future.

My family: Mom and Dad, and siblings Michaela, Sophie, Kellen, and Bella. You all supported me throughout this process and always showed up to everything I've done while at USU. Thank you for being the best family a girl could ask for.

My husband: Zachary Monahan. You've been a constant source of love, support, and sounding board for ideas. I hope I can be half the scientist, thinker, and researcher that you are.

Dedication

I dedicate this work to my life partner and other half – Zachary Monahan.

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Abstract

The Influence of Co-Signaling Proteins on Restimulation-Induced Cell Death in Effector T Lymphocytes

Camille Lake, Doctor of Philosophy, Emerging Infectious Diseases 2021

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The immune system consists of a tightly self-regulated system that balances appropriate expansion and contraction of cell populations in order to control infections while preventing autoimmunity. One such regulatory mechanism, called restimulation-induced cell death (RICD), is a propiociidal mechanism of apoptosis in which a proportion of activated T cells succumb to death following restimulation through the T cell receptor (TCR) as a means to limit rampant T cell expansion. Mutations in key signaling proteins which mediate RICD sensitivity cause autoimmunity and associated immunopathology, highlighting the significance of RICD in maintaining a healthy immune system. A significant contributing factor for facilitating RICD sensitivity in T cells is the strength of the TCR signal during restimulation: the more intense the signal, the higher proportion of the population will commit to apoptosis. We and others have discovered that proteins that regulate TCR signal strength during restimulation play immediate, critical roles in directing T cell populations towards or away from apoptosis commitment. *We hypothesize,*

therefore, that co-signaling proteins TIM-3 and PD-1 – which directly fine-tune TCR signal strength – are important contributors to RICD sensitivity during the course of expansion and/or contraction of T cells. In this study, we found that both co-signaling proteins influence RICD sensitivity of effector T lymphocytes through distinct mechanisms. PD-1 protects expanding populations (day 4 post-activation) through SHP2 activity and BATF expression, with larger implications for changing the transcriptional landscape to allow them to survive in the face of strong restimulation; its absence in late-stage effectors likely contributes to stronger TCR signal strength through the absence of SHP2 activity and BATF, thereby increasing RICD indirectly during T cell contraction. TIM-3 localizes to the cell surface and binds to its ligand CEACAM1 in clonally expanding cells, which protects cells from premature RICD. In contrast, TIM-3 remains predominantly intracellular in terminally differentiated effector T cells (day 14 post-activation), and facilitates increased proximal TCR signaling and pro-apoptotic protein activity, thus helping to set the upper limit of T cell expansion. The findings from this dissertation have important implications for currently used immunotherapies, as well as for our understanding of paradigms related to T cell signaling, differentiation, and expansion.

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Chapter 1: Introduction

General Introduction: Orchestration of Immunity

A properly functioning immune system relies on functional cross-talk between multiple cell types, orchestrated by two major arms: the innate and adaptive branches. One crucial difference between the innate and the adaptive immune systems are their relative specificities for different antigens, and the timing of the ensuing response. The innate immune system works as the body's first response to a pathogen; cells in the innate arm (e.g. myeloid cells and granulocytes) rely largely on broadly conserved pathogen-associated molecular patterns (PAMPs) to distinguish a foreign microbe from self. Germline-encoded receptors known as pathogen-recognition receptors (PRRs) are the basis by which innate immune cells are able to recognize PAMPs on invading pathogens and begin the process of pathogen clearance (206). A rapid innate immune response also primes the subsequent adaptive immune response by inducing inflammation and equipping professional antigen presenting cells (APCs, e.g. dendritic cells) to effectively prime naïve T lymphocytes via foreign peptide presentation on major histocompatibility complex molecules and expression of co-stimulatory ligands (see below).

Although some infections may be effectively resolved through innate immunity, the adaptive immune compartment is usually engaged to provide a slower but more targeted antigen-specific response provided by clonal expansion and differentiation of T and B lymphocytes. The adaptive immune system consists of two diverging branches: cellular (T cell) and humoral (B cell) immunity. Although T and B cells provide distinct effector mechanisms that cooperate for effective pathogen clearance, both rely on the generation of highly specific antigen receptors which are created through somatic gene recombination

(116). The variable regions of each unique T cell receptor (TCR) and B cell receptor (BCR, a.k.a. surface immunoglobulin) are created through a process involving the recombination of specific gene segments – V, D, and J – during lymphocyte development (116). Rearrangement of V, D, and J segments requires the lymphoid-specific recombinases RAG1 and 2 and other proteins which promote homologous recombination. Mutations in RAG1/2 and other genes involved in V(D)J recombination often result in severe combined immunodeficiency (SCID); affected patients suffer from multiple recurrent life-threatening infections due to a loss of T and B cells, underscoring the importance of adaptive immunity (229). The huge diversity in receptors across both the T and B cell compartments forms the basis for tailored pathogen-specific immunity and long-term memory (116). Aided by CD4⁺ helper T cells, B cells secrete exquisitely specific antibodies that can recognize virtually any macromolecule and work to neutralize, opsonize and destroy many extracellular pathogens with the assistance of other immune cells. Conventional T cells, the focus of this dissertation, recognize antigen in a fundamentally different manner, involving close contact with APCs (i.e. dendritic cells, macrophages and B cells) (79). The expression of specific co-receptors CD4 and CD8 demarcate major subsets known as “helper” versus “killer/cytotoxic” T cells, respectively. Once activated to differentiate into “effector” T cells as a result of stimulation by specific antigen, CD4⁺ effector T cells serve to orchestrate and manage the broader adaptive immune response including antibody production, while CD8⁺ effector T cells can recognize and directly kill infected or transformed target cells. Despite their distinct roles, both subsets share highly similar requirements for activation and effector differentiation.

The process of T cell activation involves the recognition of cognate peptide-MHC (pMHC) by the T cell receptor (TCR). Naïve CD4⁺ and CD8⁺ T cells recognize antigenic peptides in the context of MHC class II and I, respectively. Whereas MHC class II expression is restricted to professional APCs, class I MHC is present on all nucleated cells. Additional “co-stimulation”, however, is equally as important as TCR-pMHC recognition in the initial steps of T cell activation. Specifically, a second signal is generated when the T cell-specific protein CD28 binds to its ligands (B7-1 and 2) on the surface of professional APCs presenting pMHC and rendered “mature” through PRR activation and cytokine signaling. These two signals through the TCR and CD28 combine to initiate the transcription of new genes including interleukin-2 (IL-2) and its high affinity receptor CD25, which drive clonal expansion through autocrine/paracrine signaling. Loss of CD28 co-stimulation and/or IL-2 signaling prevents proper T cell activation, which has been linked to enforcing inactivation of self-reactive T cells via processes referred to as anergy and peripheral tolerance (138, 224). During clonal expansion, additional signals provided by metabolic reprogramming and a multitude of cytokines and other co-signaling proteins govern differentiation of CD4⁺ T cells into various helper T cell subsets with specific effector functions: Th1 cells secrete IFN- γ and enable destruction of intracellular pathogens, Th2 cells secrete IL-4 and IL-13 to combat helminth infections, and Th17 cells secrete IL-17 and IL-22 to recruit neutrophils and eradicate extracellular bacteria and fungi (149). Activated CD8⁺ T cells differentiate into cytotoxic T cells (CTLs), equipped with pro-apoptotic effector molecules such as FASL, perforin and granzyme that can kill target cells, particularly those infected by an intracellular pathogen or transformed into an immortalized tumor cell (149). Inborn errors of Th subset or CTL function thus leave

patients susceptible to focused classes of pathogens. Additionally, the presence of persistent antigens (in the form of chronic intracellular infections or cancer) can lead to “exhaustion” of the CD8⁺ T cell compartment characterized by a greatly diminished, functionally inert effector T cell response that renders individuals incapable of clearing the infection or the tumor (256), as discussed in more detail throughout this dissertation. Remarkably, both CD4⁺ and CD8⁺ T cell activation share a complex cascade of signaling pathways triggered by TCR engagement that ultimately converge on the stimulation of key transcription factors that promote T cell growth, survival and differentiation, as reviewed below.

TCR Signaling

TCR Structure

The conventional TCR is a heterodimer of two polypeptide chains (alpha and beta), comprised of unique variable regions built from V(D)J recombination that recognize unique pMHC molecules, coupled to constant and transmembrane regions that tether the TCR to the T cell surface (149). The TCR works directly with co-receptors CD4 or CD8 to recognize antigen-presenting cells (APCs) and initiate a cascade of signaling. These signaling events trigger significant changes to T cells, including cytoskeletal reorganization – characterized by actin polymerization near the immunological synapse to facilitate increased duration and integrity of contact between the bound cells (80) – and metabolic changes, which occur downstream of successful TCR signaling. Naïve cells rely heavily on oxidative phosphorylation (OXPHOS); T cells switch to glycolysis following activation to meet the ATP requirements necessary for rapid proliferation and expansion,

even in the presence of oxygen (75, 136, 171, 177). These changes occur to allow the T cell to grow and clonally expand to respond appropriately to an invading pathogen; thus, the earliest signaling events are critical to initiating and maintaining a successful immune response. Abnormalities in T cell signaling can lead to a range of outcomes, from autoimmunity to severe combined immunodeficiency (SCID), indicating the necessity of highly regulated, balanced TCR signaling networks in maintaining immune homeostasis (92, 237).

Molecular mechanisms of TCR Signaling

The successful binding of the TCR with pMHC complex first leads to a conformational rearrangement of the CD3 chains in the TCR; this restructuring of the proteins within the plasma membrane facilitates the appropriate formation of an immune synapse, which promotes increased proximity and prolonged T:APC contact and the accumulation of associated signaling proteins downstream of this synapse formation (59, 60, 90). CD3 reorganization and modification facilitates downstream phosphorylation and activity of signaling proteins (7) (Fig. 1).

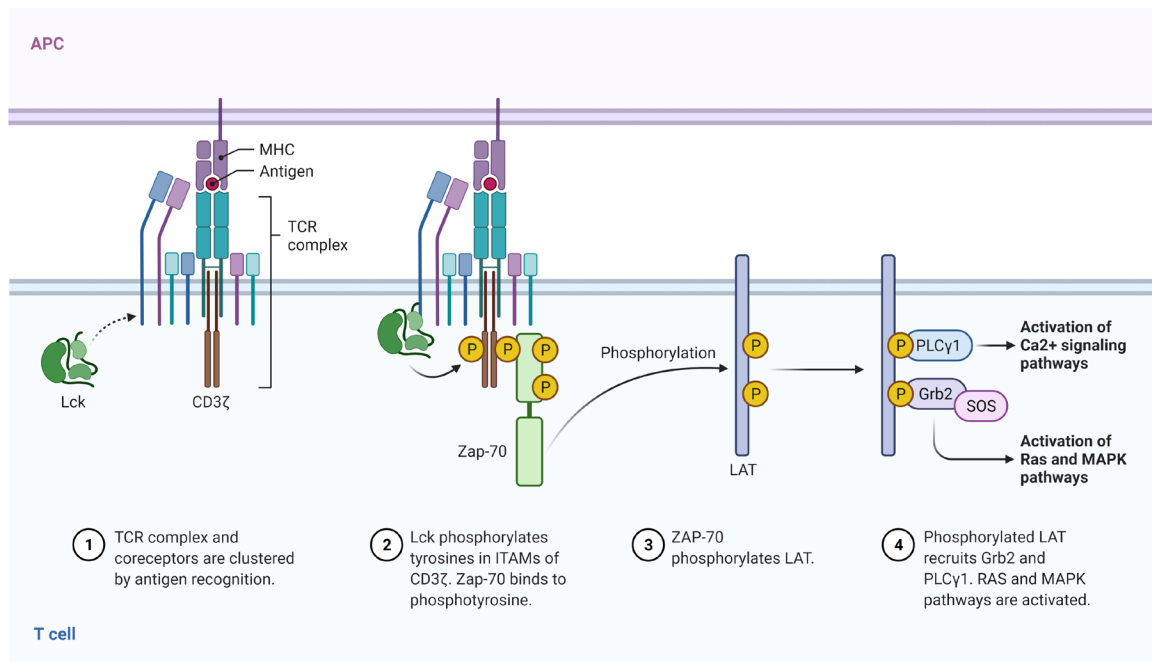


Figure 1: Overview of the TCR Signaling Cascade. (1-2) Upon binding of the TCR with its cognate pMHC, the ITAMs in the tail of the TCR are phosphorylated, which initiate a cascade of signaling. This involves the phosphorylation of proximal signaling partners ZAP-70 and LCK, which in turn go on to initiate phosphorylation of scaffold protein LAT **(3)**. Phosphorylation of LAT promotes the recruitment of other proximal signaling proteins, which lead to a branching network of signaling pathways, which are described in more detail in the text **(4)**. This image was taken from Biorender.com, and was created by Akiko Iwasaki, PhD and Ruslan Medzhitov, PhD.

Specific signaling motifs within the CD3 chains called immunoreceptor tyrosine-based activation motifs (ITAMs) become phosphorylated by the kinase LCK following successful T cell recognition of pMHC, as well as co-receptor recognition (109). LCK activity leads to the recruitment of the signaling protein ZAP-70, whose phosphorylation is proceeded by recruitment of the scaffold protein LAT. LAT recruits a second molecular scaffold, SLP76, whose binding to LAT is mediated by Gads (Grb2-related adapter protein 2, or GRAP2) (115, 202). These proteins work together to provide a signaling platform for the recruitment and interaction of other kinases and molecules. One of the most important

proteins to be recruited to this scaffold is phospholipase C- γ (PLC γ). PLC γ hydrolyzes phosphatidylinositol biphosphate (PIP₂), yielding the products diacylglycerol (DAG) and inositol triphosphate (IP₃). Multiple diverging steps follow this initial hydrolyzation step (92). The membrane-associated lipid DAG recruits protein kinase C- θ (PKC θ) and RAS guanyl nucleotide-releasing protein (RasGRP). RasGRP activates Ras, a GTPase and activator of mitogen-activated protein kinases (MAPK) Erk1 and Erk2. One output of MAPK signaling is increased transcriptional activity of Jun and Fos (comprising the AP-1 transcription factor) in the nucleus of T cells (92). Successful MAPK signaling pathways in T cells has been linked to cell proliferation, differentiation, and apoptosis, highlighting the importance of initial signaling events in promoting a healthy immune response (187, 195).

In addition to promoting MAPK signaling, DAG is critical for the formation of the CARD11-BCL10-MALT1 (CBM) complex through its activity in recruiting PDK1 and PKC θ . TCR and CD28 co-stimulation triggers PDK1 recruitment and phosphorylation at the plasma membrane, which in turn enables PKC θ activation and phosphorylation of the scaffold protein CARD11 (169). CARD11 phosphorylation induces a conformational change which facilitates the recruitment of BCL10 and MALT1 (92). We and others have shown that correct formation of this complex is crucial to functioning T and B cell responses, as patients with germline mutations in CARD11 have significant immune impairment with an associated spectrum of clinical phenotypes (135, 137, 228). Other regulatory proteins are recruited after the formation of the CBM complex, including TRAF6 and TAK1. Both BCL10 and MALT1 become polyubiquitinated through TRAF6, a change which then facilitates the recruitment of the IKK complex through the IKK γ

polyubiquitin binding motif; this event triggers the polyubiquitination of IKK γ and phosphorylation of IKK β (175). The IKK β complex phosphorylates the inhibitor of κ B (I κ B), releasing NF- κ B from inhibition and promoting its translocation into the nucleus (134). CBM complex formation is also required for c-Jun N-terminal kinase (JNK) activation, which enables AP-1 transcriptional function (134). While DAG recruits PKC θ , IP $_3$ triggers the release of calcium from the endoplasmic reticulum. This increase in intracellular calcium levels promotes the activation of the phosphatase calcineurin, which dephosphorylates NF-AT and leads to its translocation into the nucleus as well (46). Cumulatively, these signaling events lead to differential cellular transcription through their direct influence over the location of NF- κ B, AP-1, and NF-AT (135).

TCR Signal Regulation

The full coordination of these signaling events culminates in a multitude of changes, including changes in metabolism, cytoskeletal organization, cytokine release, intracellular signaling, cellular trafficking, morphology, plasticity, as well as effector function. These events are the direct result of the changes in the transcriptional program that T cells undergo following initial activation, which is largely governed by the coordinated actions of NF- κ B, NF-AT, and AP-1. Just as importantly, the TCR signaling cascade is subject to intensive regulation to ensure a calibrated T cell response that is eventually “turned off” (28). The TCR is partially regulated by Src-homology 2 domain (SH2)-containing protein tyrosine phosphatases (SHP): primarily SHP1 and SHP2 (133). Both proteins are composed of catalytic domains which dephosphorylate target proteins, two SH2 domains at their N-termini for protein recognition, and C-termini which become

phosphorylated and lead to their activation (133). These two proteins share similarity in structure and activity, and cumulatively dephosphorylate a number of targets, including Vav, LCK, ZAP-70, and SLP76, PLC γ and PKC θ (133).

In addition to these regulatory proteins, other mechanisms exist to “fine-tune” the TCR signal. LCK is regulated by the tyrosine kinase C-terminal Src kinase (Csk), which inhibits proximal TCR signaling by phosphorylating a regulatory tyrosine motif in the C-terminal tail of LCK (25). In addition, ubiquitin ligases present at the immunological synapse, such as CBL-B and ITCH, target key proteins in the signaling cascade for proteasomal degradation (58). These regulatory events are orchestrated in part by co-signaling proteins – predominantly co-inhibitory in nature – that play a significant role in limiting the strength and duration of an effector T cell response, as well as homeostatic processes which govern T cell expansion and contraction.

Co-signaling Proteins

As mentioned, the initial events of TCR signaling are heavily regulated (28). Not only is the full signal amplitude of the TCR dependent upon co-receptor proteins and their successful binding (such as CD4 and CD8), but the TCR is directly impacted by the presence and strength of co-signaling proteins as well. CD28, for example, is the most widely recognized (and perhaps most widely studied) co-signaling protein, and has a heavy influence on the overall signal strength of the TCR (194, 251). CD28 recruits additional LCK to the immunological synapse, facilitating increased phosphorylation among downstream partners; in addition, CD28 increases the activation PI3K and assists in recruitment of PDK1 and PKC θ . These functions work to collectively amplify the TCR

signal cascade (142). Despite this seemingly elementary function of this protein, we now recognize that functional CD28 signaling is required for T cell activation, cytoskeleton reorganization, and calcium signaling in the T cell, and that it works primarily as a “molecular amplifier” of quantitative and qualitative TCR signaling cascades (142, 183). Other members of the CD28 family, however, share structural and functional homology to CD28 but act to antagonize the actions of CD28 – including cytotoxic T lymphocyte antigen -4 (CTLA-4) (251). Since the discovery of co-signaling proteins – both co-stimulatory and co-inhibitory – we now appreciate the enormous influence that these proteins have over fine-tuning the signal strength of the TCR. Not only do these proteins directly influence the activation status of T cells, they also influence other processes which are critical for maintaining a healthy immune response, including T cell plasticity, tolerance, and exhaustion (16, 52, 220).

Most co-signaling proteins of importance belong either to the Immunoglobulin superfamily (IgSF) or the Tumor Necrosis Factor Receptor superfamily (TNFSR) (36, 251). Members of the IgSF are characterized by domains which are immunoglobulin-like, consisting of 70-100 amino acids with a unique structure and Ig fold. Among members of the IgSF, common domains have been binned into distinct types, termed V- and C-domains, because of their unique structural similarity to the V- and C- domains of Ig and T cell receptors (95). Although the members of this comprehensive group share structural similarity, their functions range widely – from co-stimulatory to co-inhibitory – leading to a broad range of effects on the cells on which they are expressed (Fig 2).

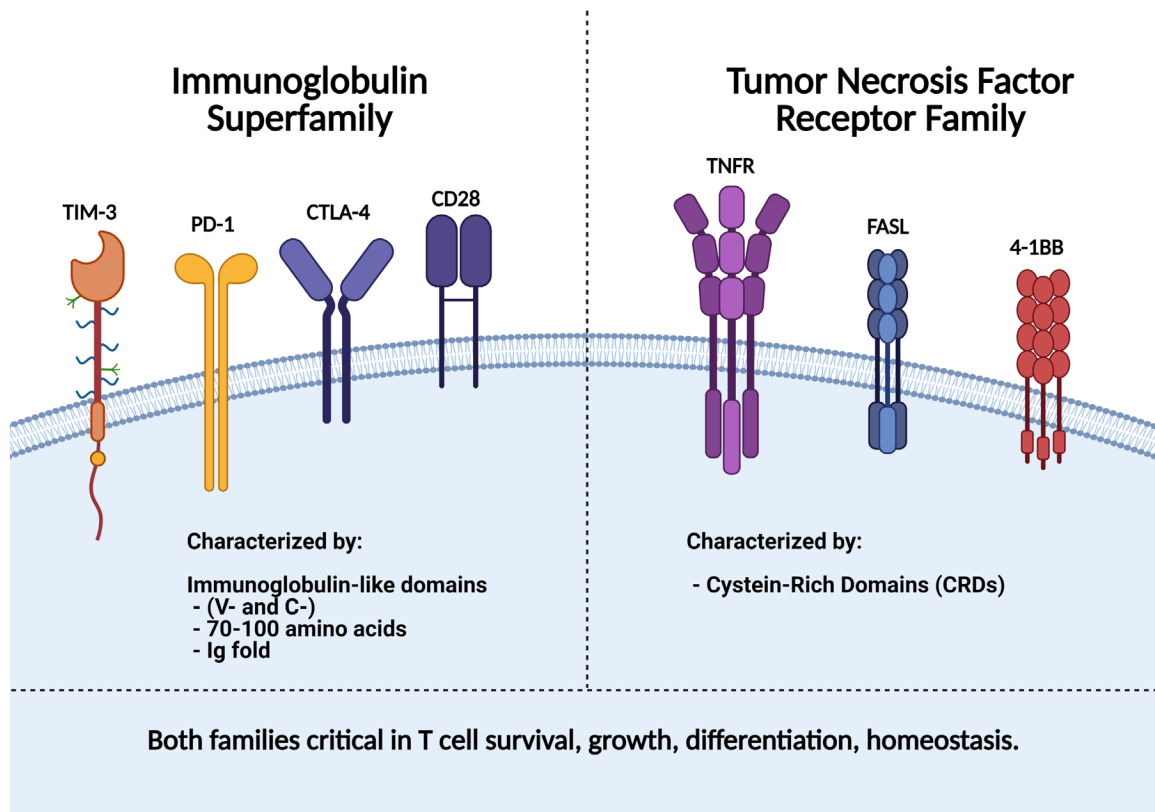


Figure 2: Examples of members of the Immunoglobulin Superfamily (IgSF) and Tumor Necrosis Factor Receptor Superfamily. Created using Biorender.com.

Co-inhibitory proteins, also appropriately termed immune checkpoint proteins, serve to prevent or inhibit the T cell signal and overall response following TCR activation and restimulation (36). Multiple mechanisms exist by which dampening of the TCR signal is achieved. CTLA-4, as mentioned above, competes with CD28 for its ligands, B7-1/B7-2, preventing CD28 from recruiting LCK and other proximal signaling proteins to the immunological synapse (36, 251). CTLA-4 and other co-inhibitory proteins also directly recruit phosphatases. Programmed Cell Death -1 (PD-1) is also induced shortly after T cell stimulation and recruits the phosphatase SHP2 following binding to either of its ligands PD-L1/2 (139). Phosphatases like SHP2 directly deactivate important proximal signaling

partners such as LCK, ZAP-70, PLC γ and PKC θ , leading to dampened proximal TCR signaling. Thus these two proteins work via both distinct and overlapping mechanisms to ultimately regulate the overall T cell response. An important and distinguishing factor between these two proteins is their differential temporal expression: while CTLA-4 is expressed immediately upon activation (it is expressed in naïve cells inside membrane-bound vesicles and traffics to the plasma membrane upon TCR-pMHC recognition), naïve cells do not initially express PD-1; PD-1 and many others co-signaling proteins reach peak expression over 1-4 days following T cell activation (238). These discoveries suggest that their expression is part of timed regulatory homeostatic network; this network has been termed the “tide model” of T cell signaling (267). This model suggests that co-signaling protein expression is temporally regulated to facilitate full expansion and contraction of the T cell response. Though this warrants further investigation, it is clear that co-signaling proteins – particularly co-inhibitory proteins which act to temper T cell responses – are key to maintaining a balanced immune response.

Of particular relevance is the expression of these proteins within the tumor microenvironment on tumor infiltrating lymphocytes (TILs). CD8⁺ effector T cells function to clear tumor cells through their recognition of pMHC (often tumor antigen) complexes, leading to their release of perforin and granzyme; ideally, these events culminate in tumor cell lysis. However, it has long been noted that many cells within the tumor microenvironment, which are being constantly “restimulated” through their interaction with tumor cells, become functionally anergic and “exhausted” (20, 254, 256). Exhausted cells are characterized by their lack of functionality (cytokine and effector molecule production), as well as the expression of a characteristic set of proteins on their

plasma membrane, including CTLA-4, PD-1 and T cell immunoglobulin domain-containing-3 (TIM-3) (103, 215, 236). Intriguingly, interfering with ligand binding of these co-signaling proteins can rescue the ability of these cells to promote tumor lysis. The above facts suggest that T cell exhaustion is a distinct state from senescence, (a unique, non-reversible T cell fate wherein cells are destined for apoptosis (47)), and that exhaustion is a specifically programmed state that may have evolved to preserve antigen-specific T cell populations despite heavy antigenic burden (121).

The discovery of co-inhibitory molecules that “check” TCR signaling and function prompted the recent development of “checkpoint blockade” therapy, interventional monoclonal antibody (mAb) immunotherapy designed to block co-inhibitory proteins on TILs and restore their function by boosting TCR/CD28 signaling (216). PD-1, which is expressed on exhausted TILs; and PD-L1, which is expressed by various human cancers, including gastrointestinal, urothelial, lung, breast, skin, and ovarian cancers were among the first targets of checkpoint blockade therapy (84). So far, the most successful use of checkpoint blockade therapy has been in patients with melanoma and non-small cell lung cancer, for which a number of monoclonal antibodies have been developed and approved for clinical use, including PD-1 (e.g. nivolumab), PD-L1 (e.g. pembrolizumab), and CTLA-4 (ipilimumab) (84). While these therapies have demonstrated clinical success, multiple factors influence the overall success of monoclonal antibody therapy. Mutational burden, for example, predicts the susceptibility of tumors to checkpoint blockade therapy to a high degree: while mutations in *TP53* and *KRAS* have been associated with increased responsiveness to PD-1/L1 blockade therapy in lung adenocarcinoma (57), biallelic loss of *PTEN* (phosphatase and tensin homolog – also associated with the initiation and

development of the cancer in question) is associated with resistance to PD-1 blockade in sarcoma (73). Loss-of-function DNA mismatch repair pathways have long been associated with multiple types of cancer, including solid tumors; it was recently demonstrated that a deficiency in mismatch repair predicts favorable outcomes in response to PD-1/L1 blockade (123). Because of these limitations to checkpoint blockade therapy, scientists and clinicians have begun combining various checkpoint blockade therapies – targeting a variety of co-signaling proteins – in hopes of boosting TIL activity and promoting tumor lysis. Specific therapies targeting PD-1 and TIM-3 together have been tested in the settings of some forms of cancer, with notable success in cancer remission (201). It is important to note, however, that monoclonal antibody therapies, directed to block the interaction of co-signaling proteins with their ligands, has a multitude of associated symptoms, including severe immunopathology (termed immune-related adverse events, or irAEs) (192). Such adverse clinical manifestations suggest that administration of these therapies also may be targeting bystander cell populations and disrupting homeostatic mechanisms facilitated by co-inhibitory proteins. The work described in this dissertation reveals novel roles for two such molecules, TIM-3 and PD-1, in tuning human T cell apoptosis sensitivity, with potential ramifications for our understanding of checkpoint blockade therapeutic application and irAEs.

T Cell Immunoglobulin and Mucin Domain Containing-3 (TIM-3)

T cell immunoglobulin and mucin-domain containing-3 (TIM-3) was originally discovered as a marker of Th1 CD4⁺ T cells, and is now commonly associated with severely

exhausted effector CD8⁺ T cells (66, 78, 101, 104, 147, 199, 203). Shortly following its discovery, TIM-3 was investigated in a murine model of multiple sclerosis (termed experimental autoimmune encephalomyelitis, or EAE); interventional blockade of TIM-3 was found to exacerbate symptomology of the disease, leading to the conclusion that TIM-3 played an important role in immune regulation and tolerance (262). This finding was corroborated by similar effects seen in mice with type 1 diabetes (114). A multitude of *in vitro* and *in vivo* studies have been published since its discovery in the early 1990s, and the general consensus among the scientific community for many years was that TIM-3 was a co-inhibitory protein, whose role in promoting exhaustion could be functionally exploited through monoclonal antibody therapy in the setting of certain types of cancer.

TIM-3 binds to a number of ligands which have been described in the literature, including Galectin-9 (266), phosphatidyl serine (PS) (54, 155), high mobility group box-1 (HMGB1) (56), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (91). The binding of TIM-3 with each of these ligands is thought to play various roles in the larger network of immune signaling, primarily because of the differential expression of both TIM-3 and these ligands across a wide variety of cell types. For examples, TIM-3 has been implicated in driving T cell apoptosis within the tumor microenvironment through the binding of its ligand, Galectin-9 (5, 56, 266); in contrast, TIM-3 on the surface of dendritic cells can also bind to PS on apoptotic cells and promote their clearance (54, 155). Our knowledge of TIM-3 and its functional capacity has expanded dramatically since its discovery, and we now appreciate that its functionality is uniquely tied to its *in vivo* context.

Co-inhibitory proteins modulate T cell signaling in several normal and disease contexts, including the promotion of exhaustion in response to chronic infection or cancer (254, 256). Most co-inhibitory receptors are thought to blunt T cell activation and function by directly or indirectly influencing TCR proximal signaling (145, 161). TIM-3 is a member of the IgSF, with a characteristic IgV-domain; however, the structure of TIM-3 is unique among this family in that it does not contain any canonical immunoreceptor tyrosine-based activation, switch, or inhibitory motifs (ITA/S/IMs) (51). Intriguingly, investigation into TIM-3 signaling mechanisms yielded mixed results: TIM-3, despite being linked to functional exhaustion and dampened immune responses, can actually boost T cell proximal signaling in certain settings (125). Tyrosines 265 and 272 within the tail of TIM-3 may play a role in recruiting proximal signaling partners (239, 245). One model suggests that TIM-3 is recruited to the immunological synapse following T cell activation and stabilizes the interactions of LCK and FYN through the intermediary protein Bat3, leading to increased phosphorylation and activation of ZAP-70; when TIM-3 binds to a ligand, these residues are phosphorylated, the interactions are destabilized, and the TCR signal strength is dampened (51). This hypothesis is supported by the aforementioned work from Kane et al.: when TIM-3 was overexpressed in Jurkat T cells, they saw an augmentation of NF- κ B and NF-AT translocation into the nucleus (125). Of note, the experiments in this study were performed in the absence of any known ligands, suggesting that the presence of TIM-3 ligands may serve to directly dampen proximal TCR signaling and promote exhaustion. Other models suggest that the phosphorylation of Y265 and Y272 allow TIM-3 to interact with proteins containing SH2 domains, including PI3K, PLC γ , and RasGAP1, eventually culminating in increased NF- κ B and NF-AT translocation into the

nucleus, thus facilitating T cell exhaustion over time through its presence (51). These theories, though diametrically opposed to one another, suggest the possible solution that the function of TIM-3 is uniquely tied to its environment, and potentially even to its cellular expression kinetics. Indeed, other proteins can “switch” functions depending on the kinetics of their expression on T cells – including FAS and FASL (26) - rendering credibility to this theory, though it has never been directly tested, to our knowledge. Further work is needed to delineate the exact contributions of expression kinetics, ligand-binding, and downstream signaling events to understand the contributions of TIM-3 to immune homeostasis.

Programmed Cell Death-1 (PD-1)

Programmed Cell Death -1 (PD-1) is a member of the immunoglobulin gene superfamily, first described as a protein which participated in bringing about functional programmed cell death in certain cell lines (99, 166). Original discoveries of PD-1 documented its expression on cells in the thymus and spleen, and its increased expression on cells following injection of anti-CD3 antibody in mice. This increase in PD-1 expression was correlated with increased thymocyte deletion, leading the authors to believe that the primary function of PD-1 is to facilitate apoptosis, leading to its current name (99).

The influence of PD-1 on multiple diverse physiological processes has led to the consideration of this protein as a paradigm for understanding the full relevance of co-signaling proteins on the immunological landscape. Similar to CD28, PD-1 signaling participates in events occurring at the beginning of a long signaling cascade following

TCR-pMHC recognition. Investigation of the PD-1 signaling pathway demonstrates that following ligation with either of its ligands – PD-L1 or PD-L2 – PD-1 recruits SHP2 to its cytoplasmic immunoreceptor tyrosine-based switch motif (ITSM), causing a dampening of the entire proximal signaling cascade following TCR engagement with MHC on neighboring cells (139). Even small changes in these initial signaling events have a strong influence on the TCR signaling cascade and downstream processes, highlighting the significant role of PD-1 (and other co-signaling proteins, as well) in broadly influencing T cell activation, including transcription factor expression and activity, effector function, and overall cell survival (217)(217).

Though PD-1 is expressed on a variety of cell types, it has most thoroughly been studied in the context of T cell exhaustion in the tumor microenvironment (20, 216, 217), inducing peripheral tolerance, and prevention of autoimmunity (64, 67, 113, 162). It is widely accepted that constant signal-dampening facilitated by PD-1 promotes exhaustion of T cells, with particular relevance to chronic antigen restimulation within the tumor microenvironment, in which PD-L1 is often expressed (241). Though cellular exhaustion was believed to be a singular state, we now recognize that T cell exhaustion encompasses a gradient of transcriptional states, ranging from progenitor exhausted cells to terminally exhausted cells (41). Exploratory analysis into these unique transcriptional states has found that only the cells in the “progenitor” exhaustive state are susceptible to checkpoint blockade, linked to expression of TCF7 and intermediate levels of PD-1 (144). More recently, studies have been published which have reported that these various exhausted states of T cells are completely distinct from effector and memory cells (214). Because of the influence of PD-1 over the expression patterns of transcription factors which play roles

in T cell differentiation and survival (including BATF (117, 242)) it is plausible that the presence of PD-1 on T cells plays a crucial role in promoting the survival of T cells even in the presence of strong antigenic restimulation through its role in promoting exhaustion. However, this theory has yet to be tested.

Lending credibility to this theory is the discovery that PD-1 functions not only to promote exhaustion, but also to serve as a crucial regulator of T cell activity following activation. PD-1 has been implicated in shaping the overall magnitude of T cell responses, not only in terms of population numbers but also in terms of T cell differentiation (89). Finally, PD-1 has also been shown to play a critical role in the formation of immunological memory. Conflicting reports exist in this regard: some have determined that PD-1 drives decreased memory responses (9, 70), while others have shown that PD-1 can be a critical component of initiating commitment to memory lineages following activation (186). The formation of memory cells is inextricably linked to IL-2 responsiveness and relative CD25 expression, as well as the presence of antigen, which collectively regulates susceptibility to proapoptotic forms of cell death, including restimulation-induced cell death (RICD) and cytokine withdrawal-induced death (CWID) (107, 227). The genetic absence of PD-1 in mice leading to proportionally increased memory pools following viral infection suggests that PD-1 is an important regulator of the effector differentiation and their relative survival in comparison to memory cells in the population: more specifically, genetic lack of PD-1 may be leading to increased ratios of effector:memory phenotypes by promoting increased susceptibility to IL-2 and apoptosis in the form of RICD (see next section), allowing memory phenotypes (CD25^{low}) to persist in the population. Though this theory remains to

be verified, it is clear that PD-1 is a central regulator of multiple crucial processes not only during exhaustion, but also soon after T cell activation.

PD-1 has also been associated with preventing autoimmunity while promoting immune tolerance. PD-1 employs the same signaling pattern as mentioned above – primarily, through the recruitment of SHP2 to the immunological synapse – to prevent autoimmunity and promote peripheral tolerance, findings which were first noted in *Pdcd1*^{-/-} mice (156, 157). We now appreciate that PD-1 enforces tolerance through the promotion of Treg development and by inhibiting the survival and activity of autoreactive T cells (67). All of these findings clearly demonstrate the involvement of PD-1 in enforcing crucial elements of immune homeostasis, principally through ligation with either one of its ligands and the resulting influence over TCR signal strength.

RICD

RICD: A Critical Homeostatic Process in Cellular Immunity

Proximal signaling events following TCR-pMHC binding are heavily regulated in part because of their direct influence on the magnitude of a T cell response during infection. TCR signaling also directly influences homeostasis in the form of propiociidal cell death mechanisms. (The term propiociidal has been used in the context for cellular commitment to apoptosis since the early 1990s (21), being directly translated to “self-death”.) One such process, termed restimulation-induced cell death (RICD) is critical for preventing excessive effector T cell responses. During clonal expansion, T cells must rapidly expand to meet the needs of a strong immune response against a pathogen; following pathogen clearance, mechanisms must take over to allow the cell populations to contract to regain

balance in T cell numbers. RICD, alongside other preprogrammed death mechanisms such as cytokine-withdrawal induced death (CWID), cooperate to shape the magnitude and duration of the effector T cell response. More specifically, RICD sets the upper limit boundary of T cell population expansion, while CWID is initiated following RICD to enable contraction of most of the effector T cell pool. The collaboration of these two mechanisms working in tandem in the setting of a natural infection fine-tunes the magnitude of the T cell response while promoting the formation of the memory compartment (Fig. 3 (191, 227)).

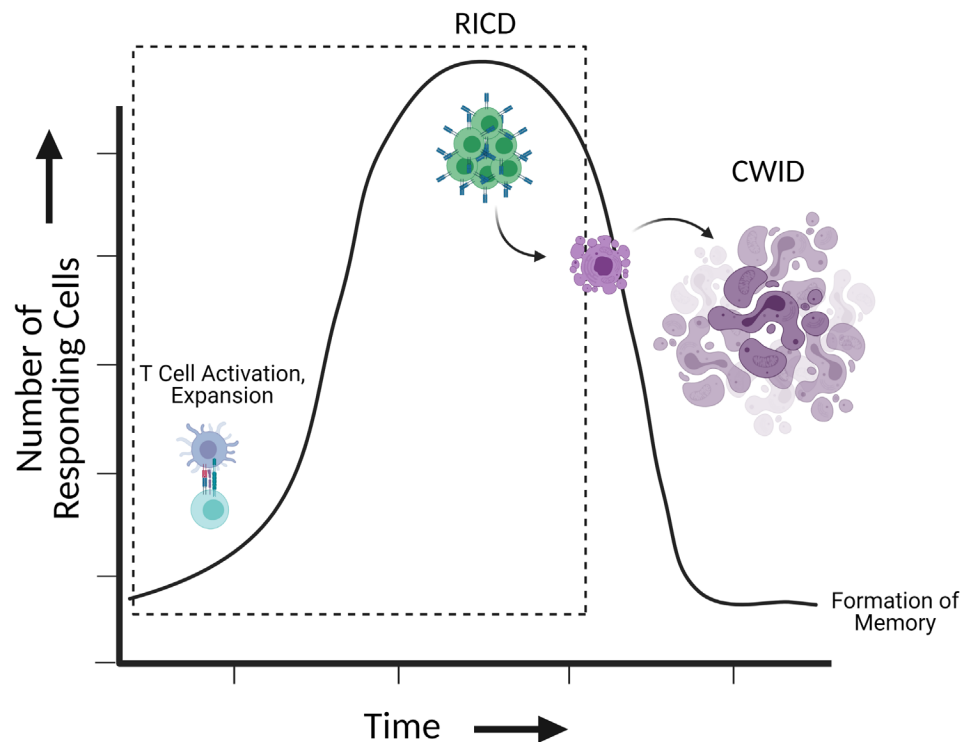


Figure 3: Overview of Key Homeostatic, Propriocidal Mechanisms of Death following T Cell Activation. During an infection, T cell populations expand in order to clear pathogens while preventing immunopathology. RICD sets the upper limit boundary of expansion following T cell activation, while CWID subsequently culls the population specifically through IL-2 withdrawal. The culmination of these two processes brings about the return of T cell numbers to steady-state.

These two critical mechanisms of programmed cell death ensure a balanced effector response following T cell activation. As one physiologically relevant example, upon infection with Epstein-Barr virus (EBV), patients with a mutation in SLAM-associated protein (SAP) – a critical mediator of RICD sensitivity in T lymphocytes – often suffer morbidity and mortality as a result of an overwhelming CD8⁺ effector T cell response, due to an acute lack of RICD sensitivity (153, 160, 225). A cycle in which CD8⁺ T lymphocytes, with poor ability to clear EBV-infected B cells, allows these B cells to perpetuate in the body, facilitating increased contact and restimulation of CD8⁺ T cells. SAP-deficient CD8⁺ T cells that never achieve a critical TCR signal threshold for RICD induction consequently accumulate in these individuals, eventually leading to severe immunopathology and even death.

History & Discovery

The phenomenon of “activation-induced cell death (AICD)” was first documented in T cell hybridomas undergoing apoptosis following exposure to antigen presenting cells (APCs) with loaded pMHC complexes (13, 14). Following this seminal discovery, multiple papers emerged detailing mechanisms which sensitize activated T cells to what we know more accurately refer to as RICD. The canonical description attributes RICD to increased

FASL expression in primary T lymphocytes following TCR restimulation, which induces apoptosis through the death receptor FAS (27, 55, 105, 127). Indeed, mutations in FASL or FAS in murine T cells lead to a mild autoimmune phenotype; upon further investigation, it was found that CD4⁺ T cells showed markedly reduced sensitivity to RICD, demonstrating the necessity of the extrinsic mechanism of apoptosis for maintaining homeostasis (76, 198). This phenotype is mirrored in humans with similar mutations in FAS or FASL who suffer from Autoimmune Lymphoproliferative Disorder (ALPS) (65). It was originally believed that extrinsic apoptosis involving FASL-FAS was exclusively associated with the RICD, while CWID was driven by intrinsic mechanisms of apoptosis (including upregulation of the pro-apoptotic BCL-2 family member BIM). However, more recent data show that FAS and BIM may compensate for each other *in vivo* to facilitate apoptosis if either mechanism is defective (22). It is now recognized that RICD can be facilitated by both types of apoptosis, particularly in CD8⁺ T cells (227). Other “executioner” molecules also play distinct roles in mediating RICD, including tumor necrosis factor receptor (244), perforin and granzyme (232), and others.

Mechanisms of RICD Sensitivity

Upstream of pro-apoptotic protein induction, many mechanisms and cellular processes contribute to RICD sensitivity in T cells, including: progression through S phase of the cell cycle (13, 21); IL-2 signaling (with associated CD25 expression) (107, 128); activity of proximal signaling proteins, including SLAM-associated protein (SAP), SLAM family member 6 expressed on Natural Killer, T and B lymphocytes (NTBA), and diacylglycerol kinase alpha (DGK α) (110, 197, 259); metabolic state, including glycolysis and autophagy engagement (122, 250); T cell receptor (TCR) signal strength (44); and

specific transcription factors, including FOXP3 (248). Intriguingly, many of these mechanisms work at distinct points following T cell activation to allow expanding cells to be protected from RICD and terminally differentiated cells to be highly sensitive to RICD; this demonstrates that an orchestrated, timed response of these mechanisms is key to maintaining homeostasis. For example, soon after T cell activation – when cellular expansion is necessary - protective mechanisms are employed to ensure that cells do not die prematurely. We have found that transient FOXP3 expression increases protective autophagy and suppresses p53 induction to protect newly activated cells from premature RICD (248). In late-stage effector T cells (days 14-20 in culture), increased glycolysis is associated with increased expression of FASL, facilitating higher levels of RICD in the population (122) (Fig. 4).

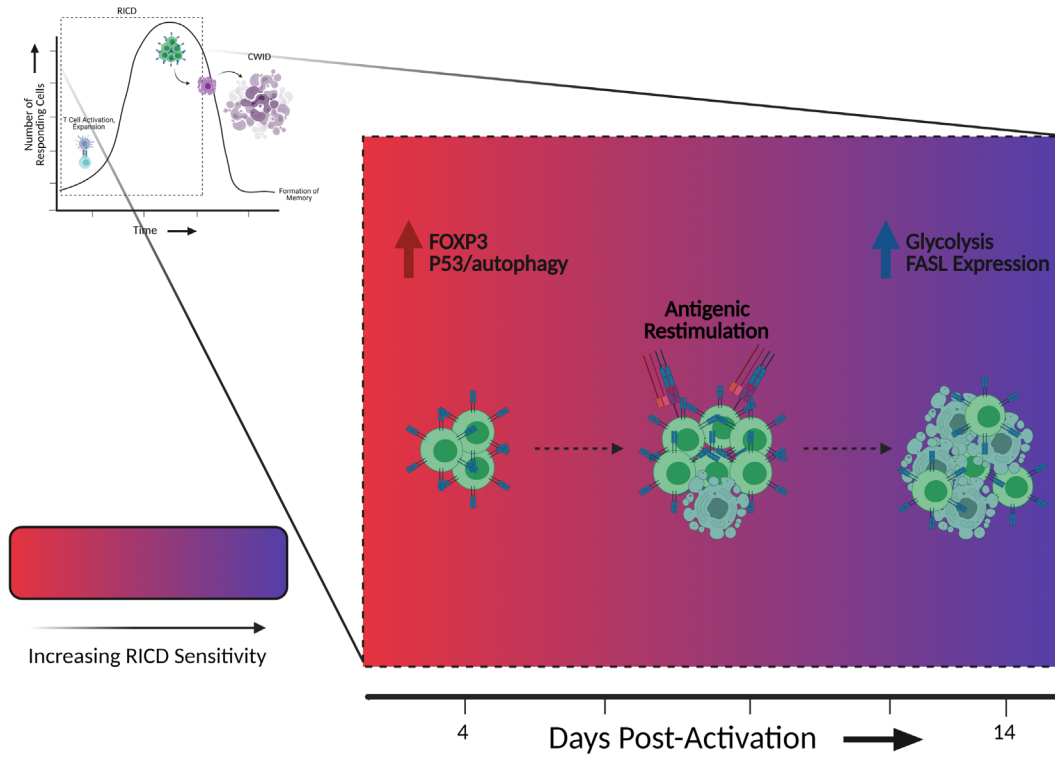


Figure 4: Activated T cells exhibit a distinct increase in RICD sensitivity over time. In the presence of antigen restimulation, T cells become increasingly sensitive to RICD, a process which is mediated by several mechanisms, as noted in the text. In newly-activated T cells, our group discovered that transient FOXP3 induction sustains protective autophagy and relative RICD resistance. By contrast, increased glycolysis in late-stage, terminally-differentiated CD8⁺ T cells facilitates TCR-induced FASL induction and enhanced RICD susceptibility. A full biochemical understanding of these temporal differences in RICD sensitivity over the course of the effector T cell response remains to be elucidated.

Research Question

One crucial element of RICD sensitivity that remains unexplained is the influence of TCR signal strength over the sensitivity of T cells to RICD at different time points post-activation. We and others have shown that treatment of cells with increased amounts of anti-CD3 antibody leads to increased RICD susceptibility in T cell populations, demonstrating the unique contribution of overall TCR signal strength on providing a “threshold” for signal amplitude that must be reached in order to sensitize cells to RICD (44, 219, 225, 248). However, treatment of effector T cells *in vitro* with similar amounts of anti-CD3 antibody over time reveals a puzzling conundrum: newly activated “early stage” T cells die much less readily from RICD compared to their “late-stage”, terminally differentiated counterparts (248). While this is explained in part by aforementioned mechanisms such as transient FOXP3 expression, this temporal difference suggests that RICD sensitivity may be fine-tuned at the level of the initial TCR signaling events. This theory is supported in part by evidence suggesting that CTLA-4, the direct antagonist of the co-stimulatory protein CD28, prevents RICD in CD4⁺ T lymphocytes through a mechanism directly related to dampening of the TCR signal (48, 168). There remains a paucity of knowledge regarding the influence of other co-signaling proteins on overall RICD sensitivity; however, its importance is of substantial clinical significance, as illustrated by cancer immunotherapies that target these co-signaling proteins in order to exploit their role in regulation of T cell effector function.

Hypothesis & Aims

In this dissertation, I investigated the influence of the two prominent co-signaling proteins, TIM-3 and PD-1, in order to define their mechanistic contributions to RICD sensitivity in CD8⁺ T lymphocytes, and to understand more fully this critical form of homeostasis. *My hypothesis is that the co-inhibitory proteins TIM-3 and PD-1 protect cells from RICD through their modulation of TCR signal strength.*

Specific Aim 1: Determine if and how TIM-3 provides protection from RICD in effector T lymphocytes.

Specific Aim 2: Determine if and how PD-1 provides protection from RICD in effector T lymphocytes.

My overarching goal is to understand the role that these two co-inhibitory proteins play in this important form of immune homeostasis. My hope moving forward is that researchers and clinicians will be able to use these findings to refine the clinical use of immunotherapies which target PD-1 and TIM-3 to improve patient outcomes and mitigate harmful side effects of these therapies.

Abstracts

TIM-3

Immune homeostasis depends upon effective clearance of pathogens while simultaneously preventing autoimmunity and immunopathology in the host. Restimulation-induced cell death (RICD) is one such mechanism where by activated T cells receive subsequent antigenic stimulation, reach a critical signal threshold through the T cell receptor (TCR), and commit to apoptosis. Many details of this process remain unclear, including the role of co-stimulatory and co-inhibitory proteins that influence the TCR signaling cascade. Here we characterize the role of T cell immunoglobulin and mucin domain containing 3 (TIM-3) in RICD regulation. TIM-3 protected newly activated CD8⁺ effector T cells from premature RICD during clonal expansion. Surprisingly, however, we found that TIM-3 potentiated RICD in late-stage effector T cells. The presence of TIM-3 increased proximal TCR signaling and pro-apoptotic protein expression in late-stage effector T cells, with no consistent signaling effects noted in newly activated cells with or without TIM-3. To better explain these differences in TIM-3 function as T cells aged, we characterized the temporal pattern of TIM-3 expression in effector T cells. We found that TIM-3 was expressed on the surface of newly activated effector T cells, but remained largely intracellular in late-stage effector cells. Consistent with this, TIM-3 required a ligand to prevent early RICD, whereas ligand manipulation had no effects at later stages. Of the known TIM-3 ligands, CEACAM1 showed the greatest difference in surface expression over time and also protected newly activated cells from premature RICD, with no measurable effects in late-stage effectors. Indeed, CEACAM1 enabled TIM-3 surface expression on T cells, implying a co-dependency for these proteins in protecting expanding T cells from premature RICD.

Our findings suggest that co-signaling proteins like TIM-3 and CEACAM1 can alter RICD sensitivity at different stages of the effector T cell response, with important implications for checkpoint blockade therapy.

PD-1

The immune system utilizes tightly regulated homeostatic processes to clear pathogens while avoiding host immunopathology. Restimulation-Induced Cell Death (RICD) is one such critical process in which activated T cells that receive subsequent antigenic stimulation reach a critical T cell receptor (TCR) signal threshold and commit to apoptosis, facilitating appropriate contraction of the T cell pool following pathogen clearance. Programmed cell death -1 (PD-1) is a co-inhibitory protein expressed in newly activated effector T cells, which dampens TCR signal strength through recruitment of the phosphatase SHP2 to the immunological synapse. Though its role in regulating exhaustion has been investigated thoroughly and blockade of this protein is being utilized as a cancer therapeutic, its role in regulating the RICD remains unknown. We hypothesized that PD-1 would protect activated, expanding T cells from premature RICD. We found that PD-1 is expressed immediately following T cell activation, and that during this critical expansion phase, PD-1 acts to protect early effector T cells from premature RICD. In line with our hypothesis, pharmacological inhibition of SHP2 provides similar protection from RICD, suggesting that PD-1 protects newly activated cells from RICD directly via recruitment of SHP2. Downstream of this pathway, we have discovered that the transcription factor BATF also plays a role in protecting newly activated cells from premature RICD. Analysis of previously published murine microarray and CHIP-seq data suggests multiple pathways by

which BATF might drive large-scale transcriptional changes, resulting in protection from RICD at this critical expansion stage. Future studies should expound upon these findings to fully extrapolate the involvement of this pathway in RICD.

Chapter 2: Materials & Methodology

Blood collection and effector T cell selection

Peripheral blood mononuclear cells (PBMC) were acquired from healthy human volunteers at the National Institutes of Health Blood Bank (access kindly provided by Dr. Michael Lenardo). PBMC were separated using a Ficoll separation gradient. Following centrifugation, the PBMC layer was extracted and washed twice using phosphate-buffered saline (PBS). CD8⁺ T cells were isolated via negative selection using Stem Cell Magnetic Bead Separation Kit (17953, Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's instructions.

Cell culture and activation

Cells were activated using Immunocult Human CD3/CD28/CD2 T Cell Activator (Stem Cell Technologies, #10970) and cultured in RPMI 1640 media (RPMI 1640 ThermoFisher Scientific, Waltham, MA) supplemented with 2 mM L-glutamine, 10% Fetal Bovine Serum (Millipore Sigma, Burlington, MA) and an antibiotic cocktail of 1% penicillin and streptomycin (Lonza, Basel, Switzerland). On day three post activation, cells were washed in PBS and cultured in complete RPMI media supplemented with 200 U/mL recombinant human IL-2 (# 100-02, Peprotech, Rocky Hill, NJ). Primary cells were cultured for up to 20 days post activation, checked daily for cell count, and kept at a concentration between $1-2 \times 10^6$ cells/mL. Jurkat T cells (ATCC clone E6.1) were maintained in the above media conditions as well without supplemental IL-2.

siRNA transfection

Cells were transfected with 250-500 pmol TIM-3 siRNA (ThermoFisher Scientific, 4392420), 250-500 pmol PD-1 siRNA (ThermoFisher Scientific, 37421419), 500 pmol BATF siRNA (Ambion/ThermoFisher Scientific, 4392420 (ID: s20666, s223128), CEACAM1 siRNA (Integrated DNA Technologies (IDT), Coralville, IA) or nonspecific siRNA scramble control (Thermo Fisher, 12935300) (see Supplemental Table 1 for siRNA sequences) using a P3 kit for the Amaxa 4D Nucleofector (Lonza). All cells were assessed for knockdown efficiency by quantitative RT-PCR, flow cytometry or western blotting on days 3-4 post-transfection. Freshly isolated T cells were transfected immediately post negative selection, allowed to rest for 30 minutes, and activated with Immunocult (Stem Cell Technologies) according to the manufacturer's instructions. Late-stage cells (days 10-14 post-activation) were transfected on day 10 and assessed for knockdown efficiency on day 13 post-activation.

Pharmacological Inhibitors

Cells were treated with 1 μ M AKT inhibitor AZK5363 (S8019), 1 μ M PI3K inhibitor BKM120 (S2247), Selleck Chemicals, Houston, TX), 20 nM SHP2 inhibitor RMC-4550 or appropriate solvent control for 1 hour prior to restimulation with 100 ng/mL OKT3. Cells were assessed for death 24 hours later via propidium iodide staining and flow cytometry as detailed below.

Cloning and cell transfections

TIM-3 and CEACAM1 DNA constructs were purchased from Addgene (49212, Watertown, MA) and Origene (RC221096, Rockville, MD), respectively. Open reading frames were cloned into the pUNO expression vector using NEBuilder HiFi DNA

Assembly kit (New England Biolabs, Ipswich, MA), including a C-terminal FLAG tag. Purified plasmids (10 ug per cuvette) were transfected into Jurkat T cells using a ECM630 electroporator (Harvard Apparatus BTX, Holliston, MA) at 260V, 950 mF as previously described (233). Expression of proteins was assessed by flow cytometry or western blot.

RICD assays

Primary effector T cells in RPMI were aliquoted into 96 well plates at 1×10^6 cells/mL and restimulated with anti-CD3 antibody OKT3 (#05121-05, Bio-gems, Westlake Village, CA). Unless otherwise stated, all experiments performed per independent blood sample were completed with 3 technical replicates. All restimulation experiments were performed using 100 ng/mL of OKT3 unless otherwise stated. For death assessment, cells were cultured for 24 hours in OKT3 and assessed for apoptosis 24 hours later using propidium iodide staining and flow cytometry (see below). For newly activated cells, cells were restimulated on day 4 post-activation; late-stage cells were restimulated on day 13 post-activation.

Calculation of Restimulation-Induced Cell Death

RICD sensitivity was calculated as previously described (111). Propidium iodide-negative cells (viable) were counted for each sample under a fixed rate of fluidics and time of collection on the Accuri C6 Flow Cytometer. The following formula was used to calculate percentage cell loss: $[\% \text{ Cell Loss} = ((\text{Control} - \text{Restim}) / \text{Control}) \times 100]$, for which the Control equaled the total number of untreated viable cells, and Restim equaled the total number of viable cells following restimulation with OKT3. Triplicate wells were averaged prior to assessment.

Flow cytometry

General flow cytometry protocol: T cells were spun in polypropylene round-bottom tubes and placed in 100 μ L of FACS buffer (PBS supplemented with 0.1% sodium azide, 1% FBS). Tubes were stained with antibodies, vortexed briefly and placed on ice in the dark for 30 min. Following antibody binding, tubes were washed twice with 2 mL FACS buffer and spun down. Prior to flow cytometric analysis, cells were resuspended in 300 μ L FACS buffer and immediately analyzed on an Accuri C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ). TIM-3 surface staining was performed using PE-conjugated anti-TIM-3 antibody (clone F38-2E2, Biolegend, San Diego, CA) at 5 μ L/test; CEACAM1 surface staining was performed using unconjugated anti-CEACAM1 monoclonal antibody (clone 283340, ThermoFisher Scientific) with secondary antibody (Biolegend, 406715), both at 2 μ L/test. APC-conjugated anti-CD3 (100236) and PE-conjugated CD25 anti-CD25 antibodies (356104, Biolegend) were used at 5 μ L/test; PD-1 surface staining was performed using APC-conjugated anti-PD-1 antibody (329908, Biolegend) at μ L/test; PD-L1 surface staining was performed using APC-conjugated anti-PD-L1 antibody (329707, Biolegend) at μ L/test; PD-L2 surface staining was performed using PE-conjugated anti-PD-L2 antibody (329605, Biolegend) at 5 μ L/test. The following antibodies were used to stain for surface co-signaling proteins according to manufacturer's instructions: AF647-conjugated anti-BTLA (Biolegend, 344519), FITC-conjugated anti-LAG-3 (Biolegend, 369307), PE-conjugated anti-TIGIT (Biolegend, 344519)

Intracellular Staining: intracellular staining of BATF was performed using the FOXP3 / Transcription Factor Staining Set (eBioscience, 00-5523) according to manufacturer's

instructions, and PE-conjugated anti-BATF clone MBM7C7 (Invitrogen, 12-9860-42) at 5 μ L/test.

Propidium iodide staining: Following overnight restimulation, cells were collected from the 96 well plate and placed into 0.3 ml polypropylene tubes. 10 μ L of propidium iodide (PI) (Millipore Sigma, P4864) at 1 μ g/mL stock solution was added to each tube. Cells were immediately assessed for apoptosis by flow cytometric analysis on the BD Accuri C6.

Flow Cytometric Software: All flow cytometric analyses were performed using Accuri C6 Flow Cytometer built-in software and FlowJo (Becton Dickinson).

Addition of Blocking Antibodies and Recombinant Proteins

TIM-3 Blockade: To disrupt TIM-3 ligand association, primary effector T cells were cultured with 10 μ g/mL TIM-3 blocking monoclonal antibody (clone F38-2E2, Biolegend, 345004), Recombinant Human TIM-3-FC Chimera (R&D Systems, 2365-TM-050), or 10 μ g/mL Mouse IgG1 Isotype Control SAFIRE (Bio-gems, 44212-25) for 1 hour prior to restimulation with OKT3. CEACAM1 and CEACAM3 Fc-chimera proteins were generously provided by Dr. Gabriela Dveksler (108), and were used at a concentration of 10-100 μ g/mL (as indicated in the text) each in primary cell solution for 1 hour prior to restimulation with OKT3.

PD-1/PD-L1 Blockade: To disrupt PD-1 ligand association, primary effector T cells were cultured with 10 μ g/mL anti-PD-1 blocking monoclonal antibody (Biolegend, 329926) or

10 µg/mL anti-PD-L1 blocking monoclonal antibody (Biolegend, 329702) for 1 hour prior to restimulation with OKT3.

Immunoblotting and Densitometry Analysis

Immunoblotting: Cells in each condition were lysed in 1% Nonidet P-40 (NP-40) lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1mM Na₃VO₄, and 1 mM NaF), supplemented with protease inhibitors (Sigma, 11836153001) and PhosStop Phosphatase Inhibitors (Sigma, 4906837001). Cell lysates were mixed with in a 1:1 ratio of Laemmli buffer supplemented with 50 µM βME and heated at 95°C for five minutes for denaturation. Proteins were separated on a 4-20% gradient acrylamide gel (456-1095, Bio-Rad, Hercules, CA) at 110 volts for 90 minutes for optimal band separation. Gels were transferred to a nitrocellulose membrane via semi-wet transfer (TurboBlot transfer system, Bio-Rad). The following primary antibodies were used at dilutions in Odyssey blocking buffer TBS (927-50000, LiCOR, Lincoln, NE) according to manufacturer's protocol: TIM-3 XP monoclonal antibody (clone D5D5R, Cell Signaling Technology, 45208S), 4G10 Platinum Anti-Phosphotyrosine Antibody (Millipore Sigma, 2859168), BIM/BOD polyclonal antibody (ADI-AAP-330E, Enzo, Farmingdale, NY), FASL monoclonal antibody (clone: Ab3, EMD Biosciences, Millipore Sigma), anti-FLAG monoclonal antibody (Sigma, F1804-200UG), β-actin monoclonal antibody (Sigma, A1978), anti-PD-L1 monoclonal antibody (Cell Signaling Technology, 13684T), anti-BATF monoclonal antibody (Cell Signaling Technology, 8638S), anti-total-SRC (Cell Signaling Technology, 2109), anti-phospho-SRC-Y416 (Cell Signaling Technology, 2101S). Licor IRDye secondary antibodies used at 1:10,000 dilution. All immunoblot images were visualized using an Odyssey CLx instrument (LiCOR) and assessed for

densitometry using Image Studio Lite Software. To calculate densitometry, total band signal density was assessed in comparison to total β -actin signal density for normalization.

LCK Restimulation Time Course: To assess phosphorylation of LCK over time, restimulated cells were allowed to incubate for 0, 2, 5, 10, 30, and 60 minutes post-restimulation at 37C. To stop restimulation, cell suspension was placed in ice-cold PBS and spun at 4C for 5 minutes, with subsequent cell lysis as above.

Quantitative real-time PCR

Cells in each condition were prepared for RNA isolation according to manufacturer's instructions (kit #11-331, Zymo Research, Irvine, CA). RNA was resuspended in nuclease free water at a concentration of approximately 50-100 ng/ μ L per sample. RNA samples were reverse transcribed into cDNA using 50-100 ng RNA total, using the iScript Advanced cDNA Synthesis Kit (Bio-Rad, 1725038). qRT-PCR was performed using the SSO Advanced SYBR Green qPCR Master Mix and manufacturer's protocol (Bio-Rad, 1725271); qRT-PCR was performed on Bio-Rad CFX96. Primer sequences are listed in Supplemental Table 1. Ct values of all samples were normalized to 18S Ribosomal RNA.

Restimulation Beads

Restimulation beads were created as previously described (173). Briefly, magnetic Dynabeads M450 Epoxy (protein conjugation beads) (Thermofisher Scientific, 14011) were coupled to anti-CD3 monoclonal antibody (BD Biosciences, 555336), anti-CD28 monoclonal antibody (BD Biosciences, 555725) and either Recombinant Human PD-

L1/B7-H1 Fc Chimera Protein (R&D Systems, 156-B7-100) or IgG1 Isotype Control SAFIRE (Bio-gems, 44212-25).

Confocal Microscopy

Slide Preparation: Approximately $30\text{-}100 \times 10^3$ cells were placed onto poly-L-lysine-coated slides (Electron Microscopy Sciences (EMS), Hatfield, PA) at a concentration of 1×10^6 cells/mL in PBS. All cells were fixed in 2% paraformaldehyde (EMS). Cells were washed with PBS and permeabilized using 0.1% Triton in PBS for 5 min at room temperature and subsequently washed with PBS. Cells were blocked using a 1% FBS/PBS mix for 30 minutes, followed by another PBS wash. Primary TIM-3 antibody (clone F38.2E2, Biolegend, 345004) was prepared in 1% PBS/FBS at a final concentration of 1 $\mu\text{g/mL}$ and allowed to bind to cells for 30 min. Cells were washed 3 times with PBS/FBS solution. AlexaFluor-647 anti-IgG2b secondary antibody (Biolegend, 406715) was prepared in 1% PBS/FBS at a final concentration of 1 $\mu\text{g/mL}$ and allowed to bind for 30 minutes. Cells were washed again with PBS/FBS 3 times. 10 μL DAPI Fluoromount-G mounting medium (0100-20, Southern Biotech, Birmingham, AL) was applied to the slides for DAPI staining and coverslip adhesion. All images were obtained on a Zeiss 700 confocal scanning microscope.

Image Processing / Code Availability: Microscopy analyses were conducted using ImageJ (Fiji) software version 1.53c. Image processing code can be found on Github using the following link: [mad-scientist-in-training/Microscopy-code: A repository for the code used in ImageJ to process the images present in the publication "TIM-3 drives temporal](https://github.com/mad-scientist-in-training/Microscopy-code)

[differences in Restimulation-Induced Cell Death Sensitivity in conjunction with CEACAM1 in effector T lymphocytes" \(github.com\)](#)

Murine Microarray Data Analysis

The publicly available dataset utilized in this dissertation was first published on March 2, 2014 to the Gene Expression Omnibus and is available at the following site: [GEO Accession viewer \(nih.gov\)](#) (117). The dataset was analyzed using the GEO2R, a built-in function by the Gene Expression Omnibus. In this analysis, the following groups were assessed for microarray analysis: WT_Effector and BATF_KO_Effector. Code for deploying the analysis, including statistical analysis and creating graphs, can be found at the following site: [GEO2R - GEO - NCBI \(nih.gov\)](#).

Statistical analysis

All statistical analyses were performed and visualized using Prism software version 8.4.3 (GraphPad, San Diego, CA). Bar graph heights represent sample means, and all error bars indicate the standard deviation within the samples. Specific statistical tests are indicated in the figure legends. All tests in which data was transformed significantly (including qPCR results and fold change values) were analyzed using the non-parametric equivalent of the appropriate statistical test to minimize type I error. All statistical tests were two-sided. All p values < 0.05 were considered significant.

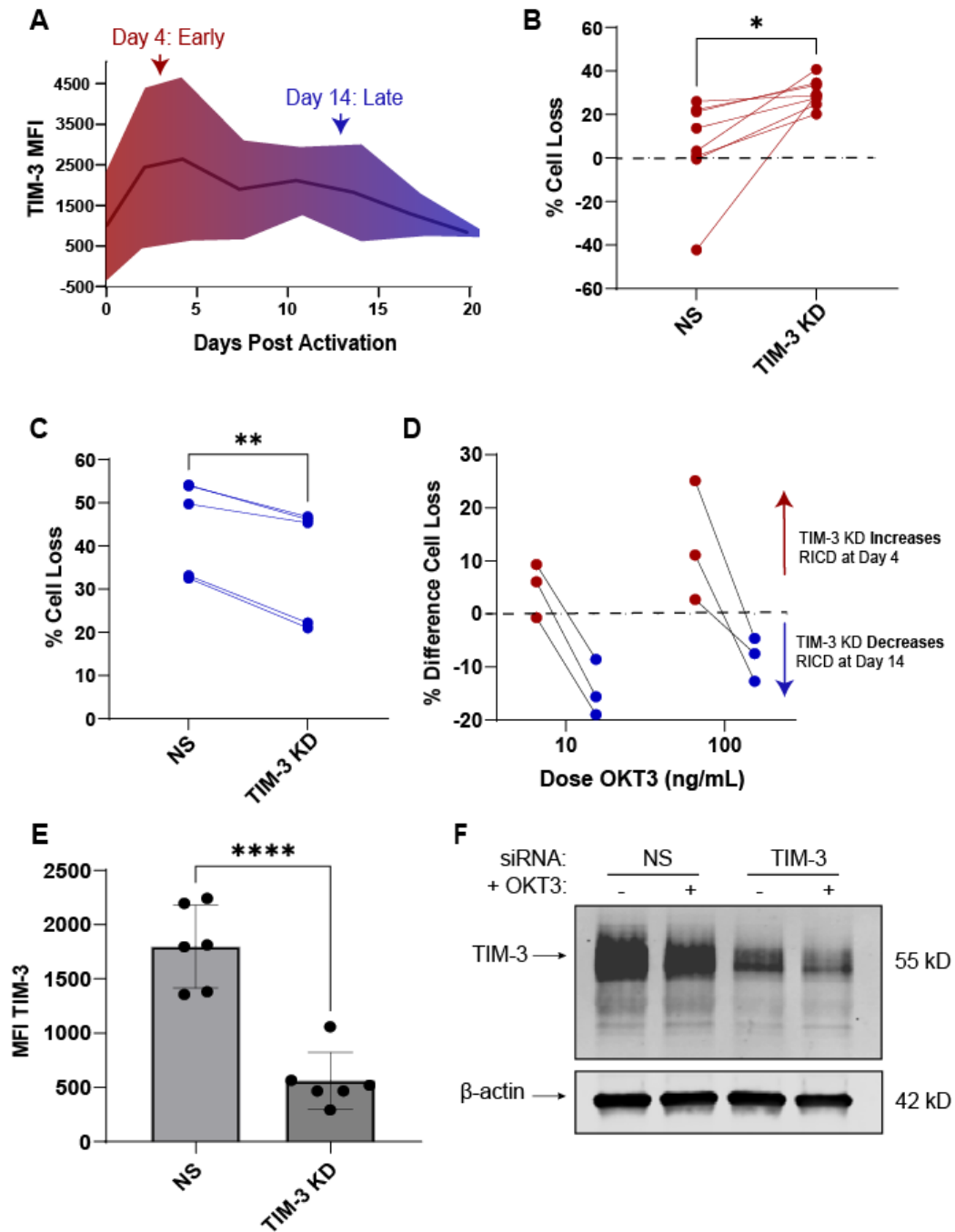
Molecular Modeling

Biochemical modeling and analyses of TIM-3/CEACAM1 interactions were performed with UCSF ChimeraX, developed by the Resource for Biocomputing, Visualization, and

Informatics at the University of California, San Francisco, with support from National Institutes of Health R01-GM129325 and the Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases.

Chapter 3: Results & Figures

Figure 5: TIM-3 Protects Newly Activated Cells from RICD, but Exacerbates RICD Late-Stage.

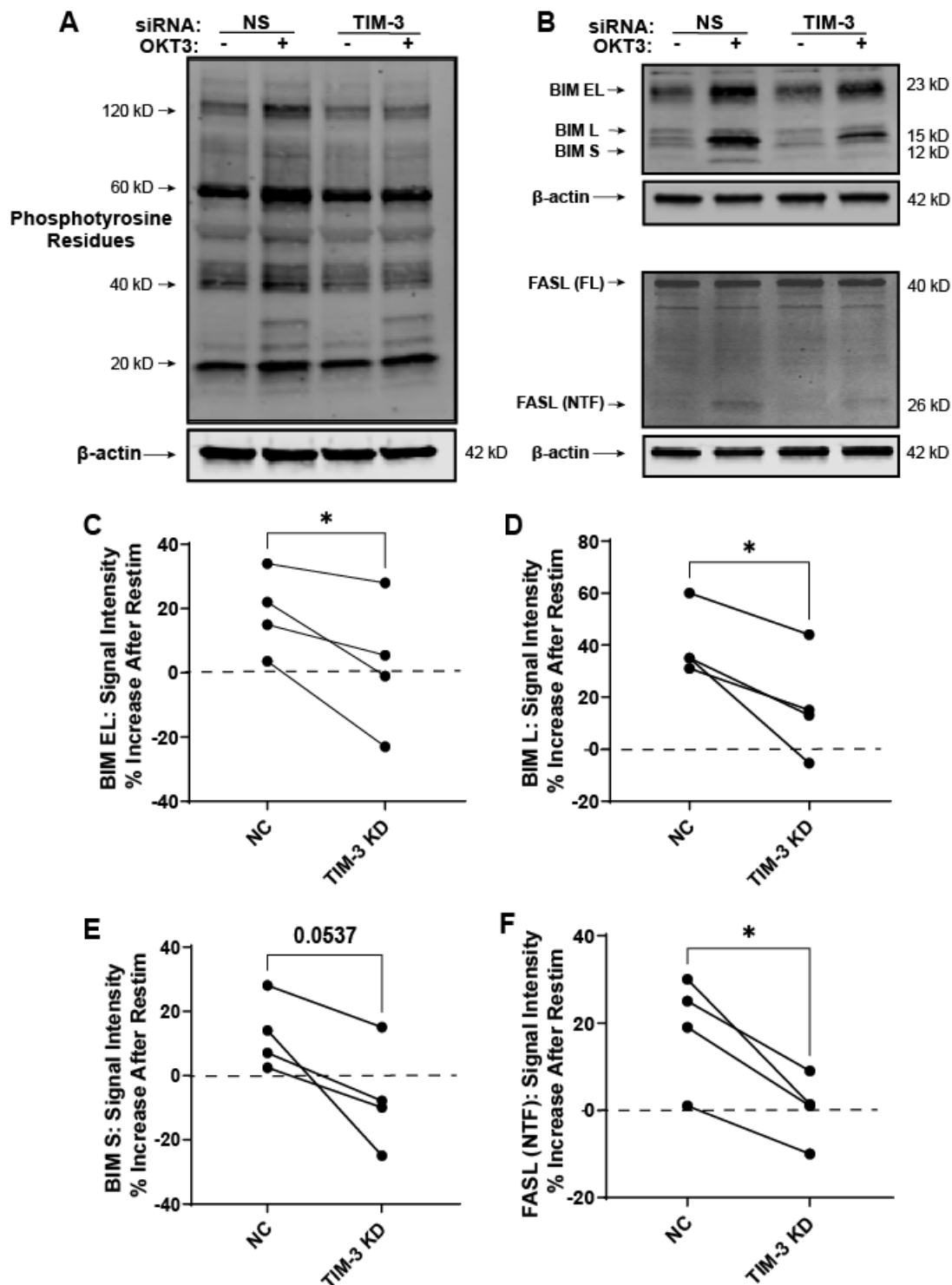


(A) Isolated human CD8⁺ effector T cells were activated and cultured with exogenous IL-2 for up to 20 days post-activation. Representative image of 3 donors' TIM-3 surface expression as assessed by flow cytometry; mean fluorescence intensity (MFI) is represented by the black line, outer edges represent standard deviation. **(B)** Purified CD8⁺ T cells were electroporated on day 0 with either TIM-3-specific siRNA (TIM-3 KD) or nonspecific siRNA (NS) and tested on day 4 for RICD sensitivity by restimulating with 100 ng/mL OKT3 antibody overnight. Cell loss was assessed by propidium iodide staining and flow cytometry. Connecting lines represent individual donors. **(C)** Cells were assessed as in (B) for death sensitivity at day 14 post-activation. **(D)** Differences in % cell loss, calculated per donor by subtracting NS % cell loss from TIM-3 KD % cell loss, were plotted for each donor at various concentrations of restimulating antibody OKT3. **(E)** Surface TIM-3 knockdown was assessed by flow cytometry; TIM-3 MFI of 6 representative donors are shown. Asterisks denote statistical significance using the following tests: (B) paired *t*-test, *p*=0.0156; (C) paired *t*-test, *p*=0.031; (E) paired *t*-test, *p*<0.0001.

TIM-3 has long been considered a marker of terminally differentiated, exhausted effector T cells (19, 254, 256). However, some reports have shown that TIM-3 is expressed immediately post-activation of resting T cells, and can even enhance activation during both acute and chronic viral infections (15). To first investigate the kinetics of TIM-3 expression on TCR-stimulated primary human CD8⁺ T cells, we assessed TIM-3 surface expression over a time course of 20 days post-activation. TIM-3 was expressed immediately post-activation of primary CD8⁺ T cells; variable expression across multiple donors waned over time in culture (Fig. 5A). Because TIM-3 was expressed at both early (red: days 0-6 post-activation) and late (blue: days 10-14 post-activation) time points in primary CD8⁺ T cells, we investigated the effect of TIM-3 siRNA-mediated silencing on RICD when cells at both stages are clonally expanding (day 4) or nearing contraction (day 14). Remarkably, for each donor tested, RICD increased following TIM-3 knockdown in newly activated effectors, and decreased following TIM-3 knockdown in late-stage effectors (Fig. 5B and C, respectively). This trend was apparent across individual donors at both early and late

stages using various doses of restimulating antibody OKT3 (Fig. 5D), with a reliable TIM-3 knockdown efficiency of >50% (Fig. 5E). These data suggest that TIM-3 protects newly activated cells from premature RICD during clonal expansion, but helps facilitate RICD-mediated contraction of effector T cells after 14 days in culture.

Figure 6: TIM-3 drives increased proximal TCR signaling and pro-apoptotic protein expression in late-stage effector CD8⁺ T cells.

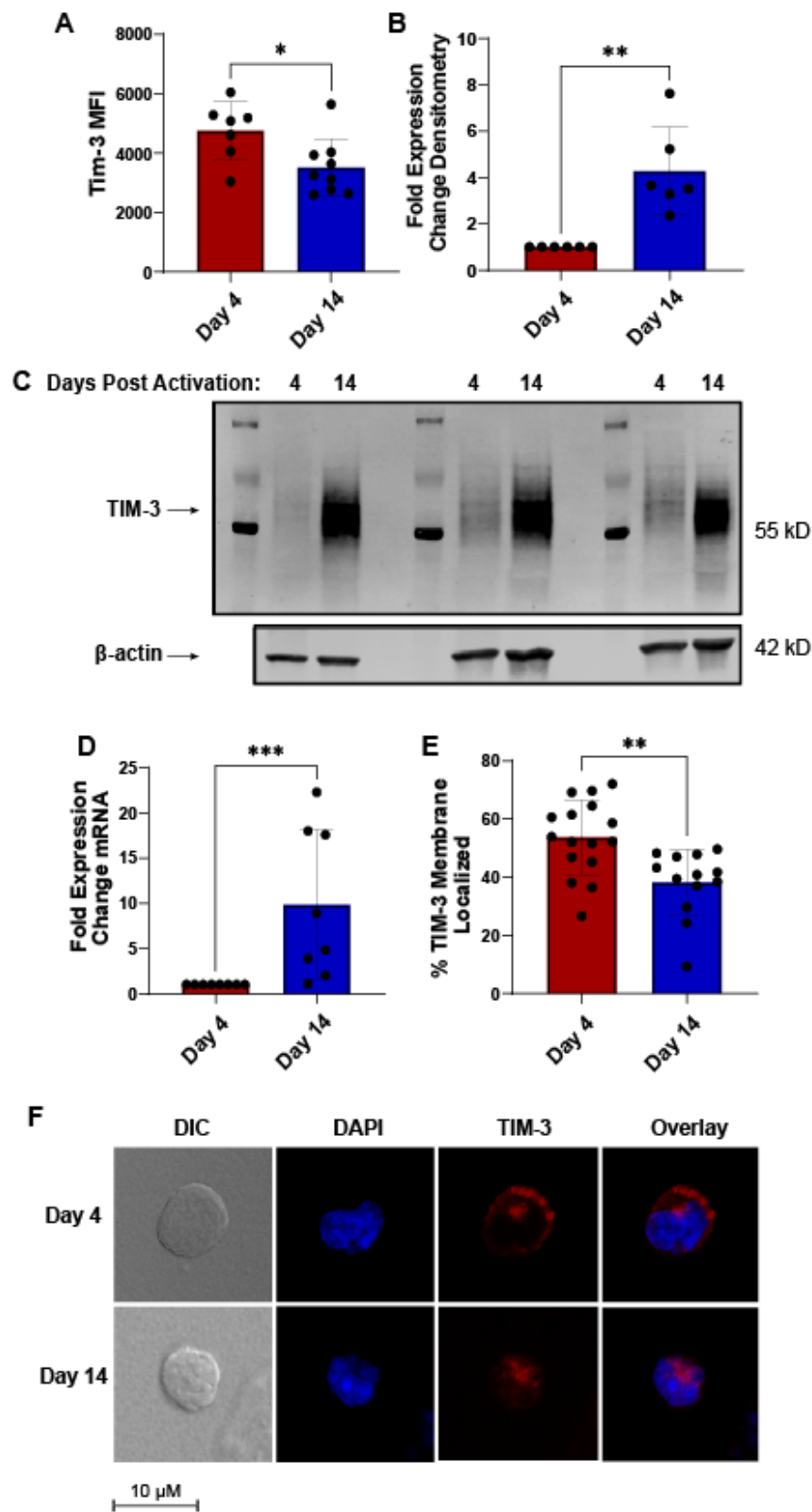


(A) Representative phosphotyrosine western blot of late-stage lysates following TIM-3 knockdown and restimulation with 100 ng/mL of OKT3. The blot represents 1 of 3 that were run from individual donors. **(B)** Western blots for BIM isoforms (EL=extra-long isoform, L= long isoform, S= short isoform) and Fas Ligand (FASL, FL = full-length, NTF = N-terminal fragment). Beta-actin serves as a loading control. **(C-F)** Differences in protein expression were assessed for statistical significance using spot densitometry, normalized to actin. Each connecting line represents a single donor. Asterisks denote statistical significance using the following paired t-tests: (C) $p=0.0480$, (D) $p=0.0265$, (E) $p=0.0537$, (F) $p=0.0157$.

To investigate whether TIM-3 was influencing differential RICD sensitivity by changing proximal TCR signaling, we first assessed larger signaling changes following TCR restimulation. In late-stage effector T cells, TIM-3 knockdown decreased the magnitude of proximal TCR signaling as assessed by total phospho-tyrosine signal (Fig. 6A). Downstream, TIM-3 knockdown also decreased TCR restimulation-induced expression of pro-apoptotic proteins connected with RICD execution, including BIM and FAS ligand (FASL) (32, 226) (Fig. 6B-F). Specifically, we noted a reduction in the cytoplasmic N-terminal fragment (NTF) of FASL produced upon cell surface exposure and cleavage by matrix metalloproteinases, indicative of enhanced FASL surface expression as noted previously (110). This was corroborated by reduced expression of FASL in TIM-3 KD cells by flow cytometry following restimulation (Supp. Fig. 1C). Other proteins which have also been documented to affect RICD, such as BCL-XL (82), were not assessed in their study; however, their contribution to the apoptotic landscape of the cells could determine fundamentally the overall commitment to death that cells experience, and therefore, future studies should focus on the full breadth of changes to pro- and anti-apoptotic proteins in CD8⁺ T cells that co-signaling proteins influence. Collectively, these results suggest that TIM-3 boosts proximal TCR signaling following restimulation in late-

stage effectors, which facilitates the increased expression of pro-apoptotic proteins required for RICD, similar to the role played by SAP and NTB-A signaling. (110). In contrast, we observed no consistent differences in proximal signaling or pro-apoptotic protein expression following TIM-3 knockdown in newly activated cells (data not shown), suggesting that TIM-3 influences RICD protection during T cell expansion via other mechanisms.

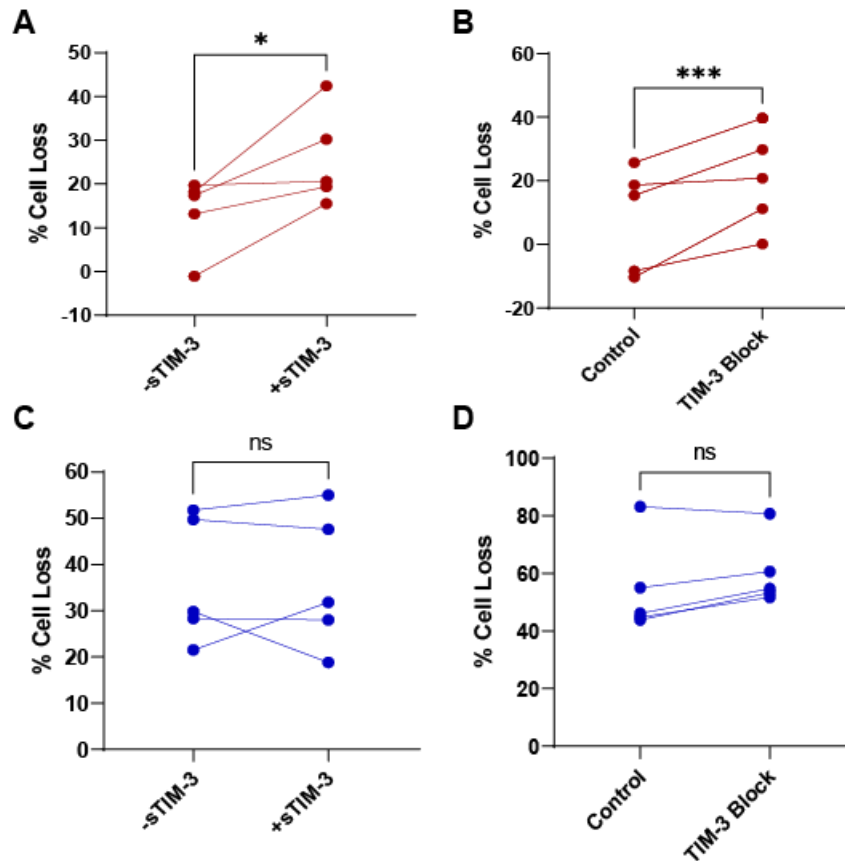
Figure 7: TIM-3 localization in effector CD8⁺ T cells changes over time in culture.



(A) Surface TIM-3 expression (MFI) was measured by flow cytometry for early-stage (day 4) and late-stage (day 14) effector T cells. **(B)** Total TIM-3 protein expression was quantified by spot densitometry of western blots, normalized against β -actin, plotted for each donor from early versus late-stage T cells. **(C)** Representative western blot of 3 donors tested for total TIM-3 expression, as quantified in (B). **(D)** mRNA was assessed by qPCR on day 4 and day 14 post-activation, with normalization against 18S rRNA. **(E)** Protein localization was quantified by confocal microscopy using ImageJ (see Methods section for code), in which single cells from individual donors (each point represents a representative cell from a different donor) on day 4 and day 14 were assessed for % TIM-3 distribution around the cell. 6 total donors were assessed, 2-4 single cells were assessed per donor. **(F)** Representative images of single cells from days 4 and 14 post activation, as quantified in (E). Asterisks denote statistical significance using the following tests: (A) t-test, $p=0.0229$; (B) Mann-Whitney test, $p=0.0002$; (D) Mann-Whitney test, $p=0.0022$; (E) paired t -test, $p=0.0125$.

Based on the recent study of patients harboring germline mutations in *HAVCR2* (encoding TIM-3), differential localization of TIM-3 within the cell can influence function and pathology in patients with subcutaneous panniculitis-like T cell lymphoma (SPTCL) (72). We therefore examined TIM-3 localization in newly activated versus late-stage effector T cells to potentially shed light on the mechanism underlying differential RICD sensitivity. We found that while TIM-3 surface expression is higher on day 4 than day 14 for each donor tested (Fig. 7A), TIM-3 total protein and mRNA expression was markedly higher in late-stage effectors (Fig. 7B-D). These data suggested that TIM-3 is primarily expressed on the plasma membrane immediately following activation, but remains predominantly intracellular in late-stage effector T cells. This finding was corroborated by microscopy analysis, which revealed more cell surface localization of TIM-3 in early-stage T cells, often in clusters (Fig. 7E-F). Collectively, these results suggest that differential RICD sensitivity could be driven by changes in subcellular localization of TIM-3.

Figure 8: TIM-3 ligand binding is required for mitigating RICD sensitivity in early-stage effectors.

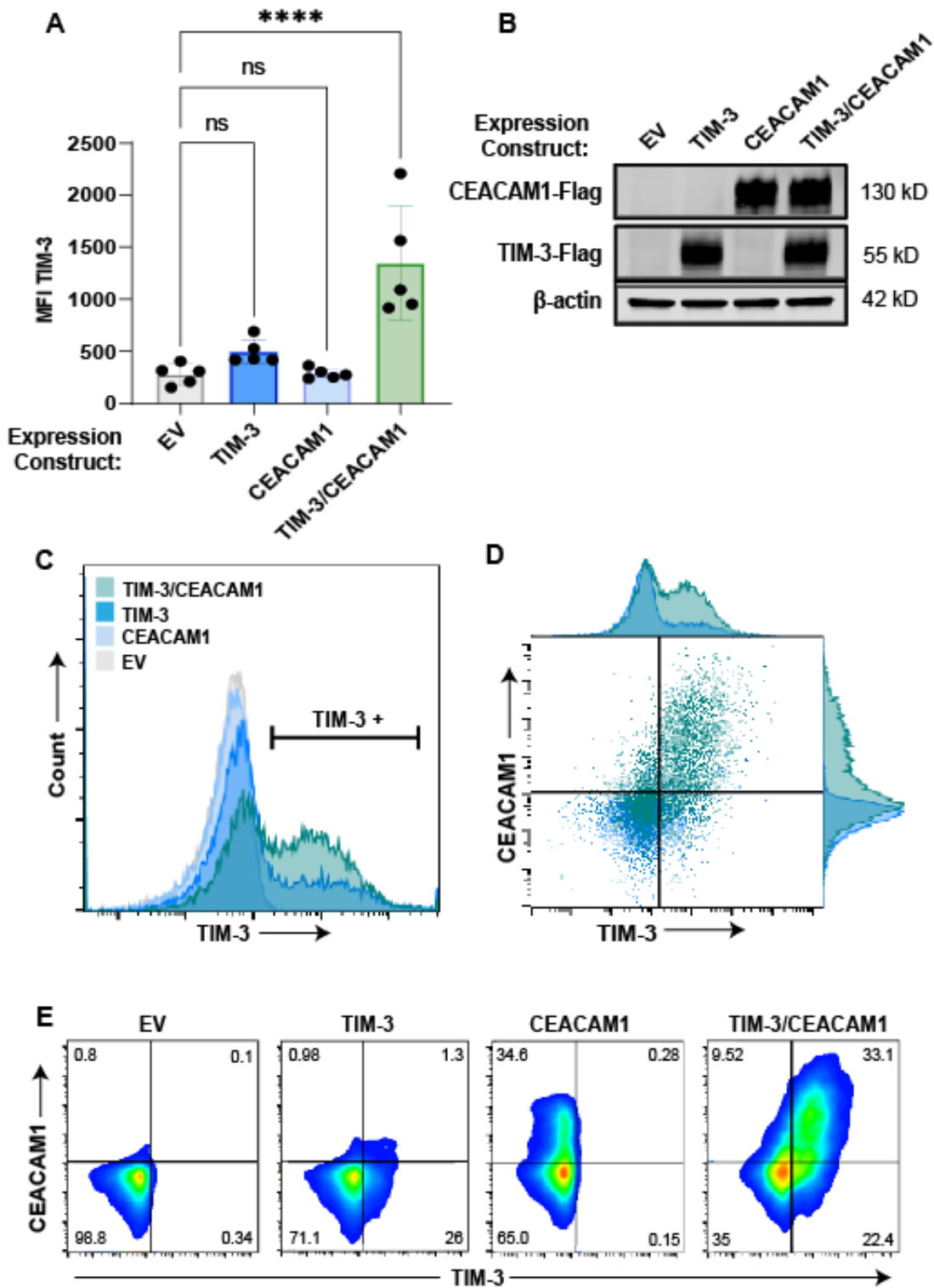


Effector T cells at day 4 (**A**) or day 14 (**C**) were treated with soluble TIM-3 (+sTIM-3) or isotype control (-sTIM-3) for 1 hour prior to restimulation with 100 ng/mL of OKT3 and death assessment 24 hours later. Cells were treated day 4 (**B**) or day 14 (**D**) with TIM-3 blocking antibody or an isotype antibody control for 1 hour prior to restimulation with 100 ng/mL of OKT3 and death assessment 24 hours later. Asterisks denote statistical significance using paired t-tests: (A) $p=0.0399$; (B) $p=0.0003$; (C) $p=0.9957$; (D) $p=0.0563$.

Because TIM-3 is preferentially localized to the cell surface immediately following activation, we next asked whether a ligand was required for TIM-3-dependent protection from premature RICD at this stage. To investigate this, we added either soluble TIM-3 (Fig. 8A, C) or a TIM-3 blocking antibody (Fig. 8B, D) to newly activated (Fig. 8A-B) and

late-stage (Fig. 8C-D) effector cells prior to restimulation and apoptosis assessment. Newly activated CD8⁺ T cells showed increased RICD sensitivity following the addition of soluble TIM-3 or TIM-3 blocking antibody, mirroring the effects of knocking down TIM-3 via siRNA at this stage. In contrast, these reagents had no effect on RICD sensitivity in late-stage effectors, a result consistent with TIM-3 intracellular localization. Collectively, these data imply that TIM-3 is dependent on a ligand to drive differential RICD sensitivity during clonal effector expansion. TIM-3 has a number of reported ligands (40, 54, 91, 205, 266). Of the four identified ligands of TIM-3, three exhibit plasma membrane expression: phosphatidylserine (PS), Galectin-9 (Gal-9), and Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Of these ligands, CEACAM1 expressed the highest difference in surface expression by flow cytometry over time, mirroring the pattern of TIM-3 expression in effector T cells (data not shown). CEACAM1-4L has been documented to be the major isoform expressed; we independently verified this in our system using previously established isoform recognition primers (222) (Supp. Table 1). Intriguingly, the blocking antibody used in Figs. 8B and 8D has been shown to block the interaction of TIM-3 with CEACAM1, suggesting that CEACAM1 may be responsible for driving differential changes in RICD in conjunction with TIM-3 during clonal expansion (200).

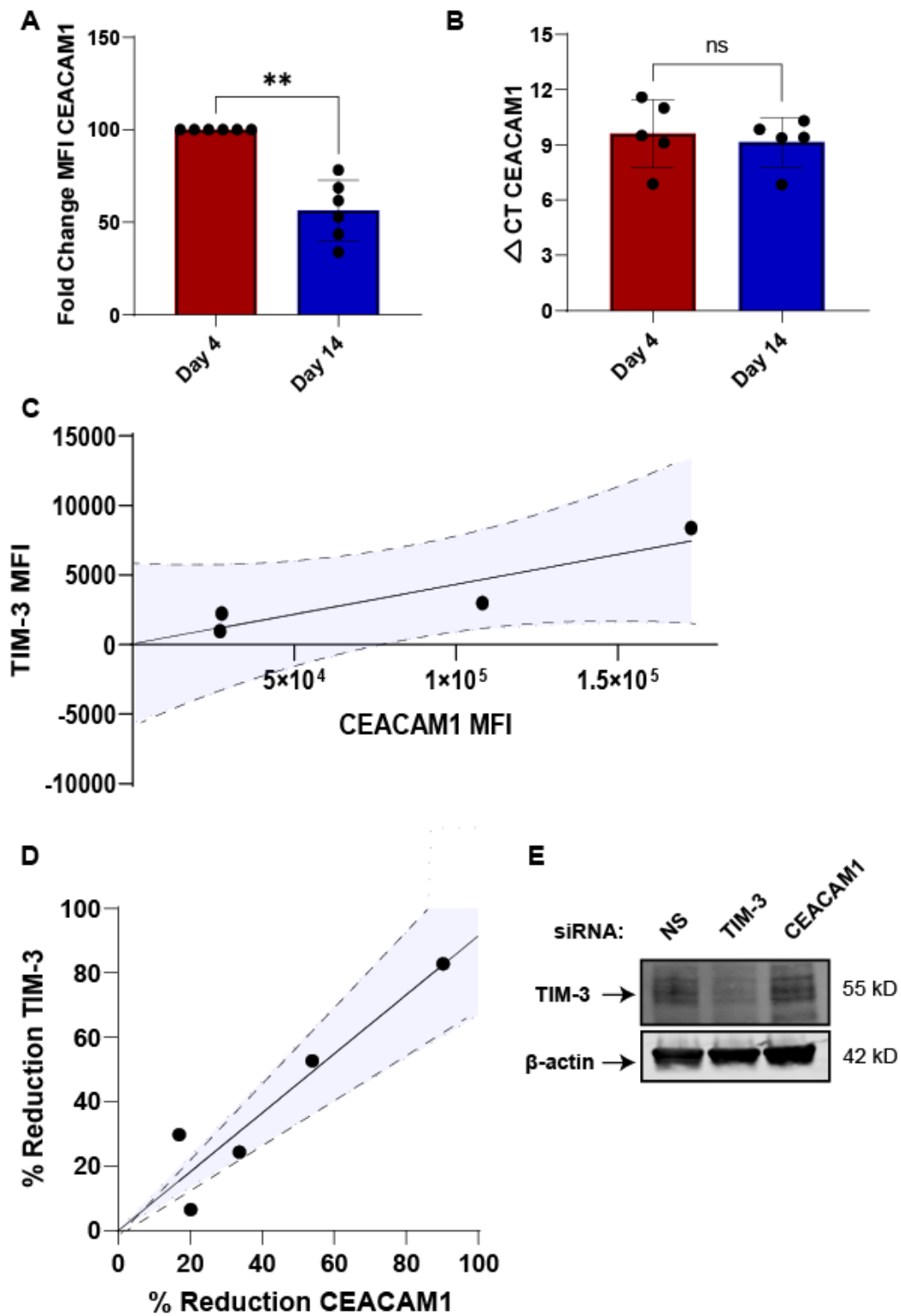
Figure 9: Co-expression of CEACAM1 promotes TIM-3 surface expression on T cells.



(A) Jurkat T cells were transfected with empty vector (EV), TIM-3, CEACAM1, or TIM-3 and CEACAM1 FLAG-tagged expression plasmids and assessed for TIM-3 surface expression. Each point represents separate experiments in which the median fluorescence intensity (MFI) was assessed for each condition. Statistical tests: (A) one-way ANOVA with multiple comparisons; $p < 0.0001$ for EV against TIM-3/CEACAM1. **(B)** Cell lysates were assessed for total CEACAM1 and TIM-3 protein expression by western blot (anti-FLAG); beta-actin served as a loading control. **(C)** Single experiment from (A) plotted as a histogram to highlight the increased % TIM-3⁺ cells with TIM-3 and CEACAM1 co-transfection. **(D)** Dot plot histogram overlay of Jurkats transfected with only TIM-3 (blue) versus both TIM-3 and CEACAM1 (teal). **(E)** Representative flow plots of Jurkats transfected as in (A).

It has been shown that CEACAM1 can effectively “chaperone” TIM-3 to the surface of transfected HEK 293T cells (91); we therefore hypothesized that CEACAM1 also enables TIM-3 surface expression in human T cells. To further investigate the interaction of TIM-3 and CEACAM1, we employed transient ectopic expression in Jurkat T cells, which lack endogenous expression of both proteins. We found that TIM-3 was markedly increased on the cell surface when CEACAM1 was co-expressed, despite similar total protein expression across all conditions (Fig. 9A-B). Not only was the median fluorescence intensity of TIM-3 and CEACAM1 higher with co-expression, but the total proportion of TIM-3⁺ cells was also markedly higher, indicating that CEACAM1 supports TIM-3 surface expression in T cells (Fig. 9C-E). Although transformed Jurkat T cells are unsuitable for RICD analyses, these data confirm that CEACAM1 can escort TIM-3 to the surface of activated T cells, suggesting CEACAM1 may be implicated in changing RICD sensitivity of primary CD8⁺ T cells over time due to its influence over TIM-3 cellular localization.

Figure 10: TIM-3 cellular localization is dependent on CEACAM1 in primary CD8⁺ effector T cells.



(A) CEACAM1 was assessed by flow cytometry (MFI) on days 4 and 14 post-activation; data normalized to day 4. **(B)** CEACAM1 mRNA was collected on days 4 and 14 post-activation and assessed by qPCR, normalized to 18S rRNA, and plotted as DCt values. **(C)** CEACAM1 and TIM-3 MFI were plotted for individual donors on day 4 post-activation. Dotted lines represent 95% confidence intervals. **(D)** Cells from 5 donors transfected with CEACAM1 siRNA on day 0 were assessed for both CEACAM1 and TIM-3 expression on day 4. Dotted lines represent 95% confidence intervals. **(E)** Representative western blot (of 3 donor replicates) probing for TIM-3 following either TIM-3 or CEACAM1 knockdown in newly activated cells (day 4). Statistical tests: (A): Mann-Whitney test, $p=0.0022$; (B): paired t-test, $p=0.6575$; (C) simple linear regression and Pearson correlation, $p=0.0392$; (D) simple linear regression and Pearson correlation, $p=0.0172$.

To verify the ability of CEACAM1 to change TIM-3 localization in primary T cells, we investigated CEACAM1 expression first independently and then in conjunction with TIM-3. We found that while CEACAM1 surface expression mirrored that of TIM-3 over time in culture (Fig. 10A), CEACAM1 total mRNA expression remained steady (Fig. 10B). TIM-3 surface expression was also closely correlated with CEACAM1 surface expression for multiple donors (Fig. 10C). Intriguingly, CEACAM1 knockdown decreased TIM-3 surface expression without affecting total TIM-3 protein levels in early-stage effector T cells (Fig. 10D-E). These results collectively suggest that CEACAM1 drives TIM-3 localization not only in immortalized cell lines, but also in primary cells as well.

RICD.



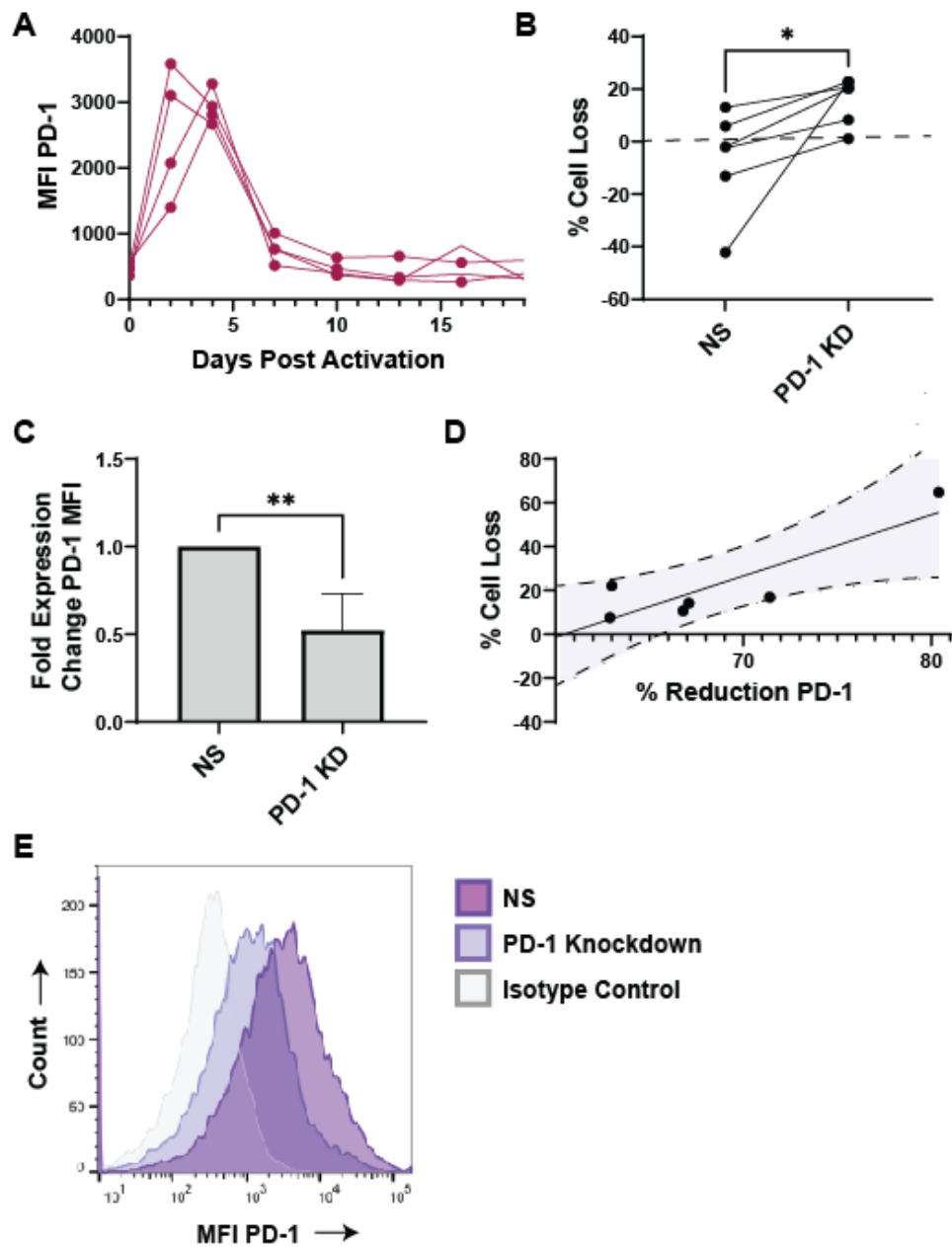
(A) T cells transfected with either CEACAM1-specific siRNA (CEACAM1 KD) or nonspecific siRNA (NS) were assessed for RICD sensitivity on day 4 post-activation as previously mentioned. (B) Cells transfected with siRNA as in (A) on day 10 were assessed for RICD on Day 14 post-activation. (C) CEACAM1 KD efficiency was evaluated using flow cytometry, plotted as fold decrease for individual experiments. (D) Newly activated cells were treated with increasing doses of CEACAM1-Fc chimera for 1 hour prior to restimulation and assessed for apoptosis 24 hours later using propidium iodide staining and flow cytometry. (E) Newly activated cells were treated with CEACAM1-Fc chimera protein, CEACAM3- Fc chimera protein, or solvent control on Day 4 post-transfection and assessed for RICD sensitivity following OKT3 restimulation. (F) Late-stage effector cells (Day 14) were treated as in (E), dose ranging from 10-100 $\mu\text{g/mL}$. Asterisks denote statistical significance using the following tests: (A) paired t-test, $p=0.0007$; (B) paired t-test, $p=0.1843$; (C) Mann-Whitney test, $p=0.0286$; (D) repeated measures one-way ANOVA, control versus 1 $\mu\text{g/mL}$, $p=0.1312$; control versus 10 $\mu\text{g/mL}$, $p=0.0156$, control versus 100 $\mu\text{g/mL}$, $p=0.0127$; (E) ordinary one-way ANOVA with multiple comparisons (corrected), control vs CEACAM1 $p=0.0016$; control vs CEACAM3 $p=0.5216$; (E) ordinary one-way ANOVA with multiple comparisons (corrected), control vs CEACAM1 $p=0.44$; control vs CEACAM3 $p=0.9991$.

To investigate whether CEACAM1 was driving differential RICD sensitivity in conjunction with TIM-3, we first asked whether CEACAM1 influences RICD sensitivity in effector T cells on its own. We found that CEACAM1 siRNA-mediated knockdown increased RICD in newly activated cells, but had no effect on RICD sensitivity in late-stage effector T cells (Fig. 11A-B). This pattern of CEACAM1-dependent protection from premature RICD mirrors the effect of TIM-3 in newly activated cells (Fig. 5B), supporting the hypothesis that they work together to protect new effector T cells from premature RICD during clonal expansion. Administering increasing doses of CEACAM1-Fc during clonal expansion showed modest but effective increases in RICD sensitivity, demonstrating the dependence of RICD sensitivity on CEACAM1 presence at this critical stage (Fig. 11D). Addition of soluble CEACAM1 increased RICD in newly activated cells, while CEACAM3 (an innate immune receptor that does not bind to CEACAM1 (180, 208)) did not (Fig. 11E). Intriguingly, neither CEACAM1 nor CEACAM3 changed RICD sensitivity

late-stage, despite using increasing doses of OKT3 (10-100 $\mu\text{g/mL}$), suggesting that the interaction of TIM-3 and CEACAM1 *in cis* is crucial to providing RICD protection during clonal expansion (Fig. 11F). From these data, we surmise that CEACAM1 promotes TIM-3 surface localization, and that they work as a pair to facilitate protection from premature RICD in early-stage effector CD8⁺ T cells.

It is clear from this work that TIM-3 and CEACAM1 influence RICD sensitivity in effector T lymphocytes. As noted in the introduction, the cellular kinetics of their expression played a role in their activity and RICD influence, which will be discussed in more detail in the Discussion section. We next investigated the protein PD-1 in fine-tuning RICD in effector T lymphocytes, utilizing many of the same methods.

Figure 12: PD-1 is Expressed in Newly Activated Cells and Protects from Premature RICD.

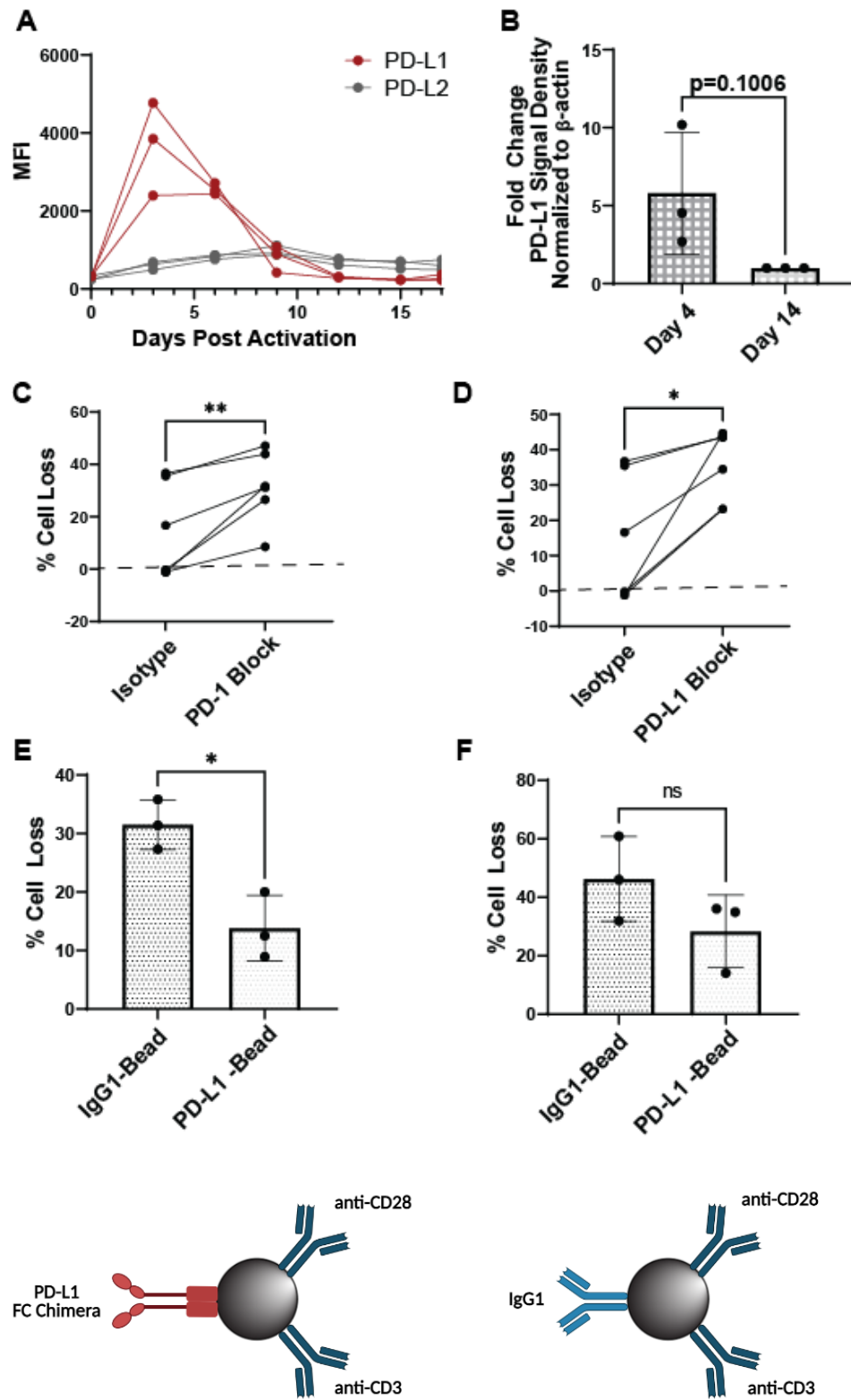


(A) Effector T cells were activated on Day 0 and assessed for PD-1 surface expression for 19 days in culture by flow cytometry. Points represent days on which MFI was taken; each line represents an individual donor. **(B)** Effector T cells were transfected on Day 0 with PD-1-specific siRNA (PD-1 KD) or scramble siRNA control (NS), restimulated on Day 3 post-activation, and assessed for death sensitivity on Day 4 post-activation using propidium iodide staining and flow cytometry. Points connected by lines represent individual donors. **(C)** On Day 3 post-activation, cells were assessed for PD-1 knockdown efficiency through surface staining; graph is representative of 5 donors. **(D)** Individual experiments were tabulated for decrease in PD-1 expression by surface staining (x-axis) with associated % cell loss (y-axis). Error bars indicate 95% confidence intervals. **(E)** Representative histogram of PD-1 knockdown by surface staining. Asterisks denote significance using the following tests: (B) paired t-test, $p=0.0467$; (C) Mann-Whitney test, $p=0.0079$; (D) simple linear regression and Pearson correlation, $p=0.0258$.

It is widely accepted that PD-1 expression is high on terminally differentiated, exhausted CD8⁺ T cells – especially in the setting of cancer immunotherapy or chronic viral infections (2, 53, 241). PD-1 expression has also been described to be intimately tied to the activation status of T cells (207), and more recently has been demonstrated to be immediately upregulated on naïve CD8⁺ effector T cells, following TCR stimulation (4). In order to determine the kinetics of expression of PD-1 on CD8⁺ T cells isolated from primary human donors, we checked PD-1 surface expression by flow cytometry for four separate donors over time post-activation (Fig. 12A). PD-1 is expressed soon after activation and then wanes in expression over time in culture. Because PD-1 is rapidly expressed post-activation, and because during clonal expansion, CD8⁺ T cells are generally much less sensitive to RICD than terminally differentiated cells, we knocked down PD-1 in newly activated T cells and determined their RICD sensitivity using siRNA specific for PD-1 versus a nonspecific siRNA control. PD-1 knockdown (KD) cells showed increased RICD relative to cells from the same donor transfected with negative control siRNA (Fig. 12B). Though knockdown efficiency was greater than 50% for each donor we tested (Fig. 12C, D, E), there was a significant correlation between PD-1 KD efficiency and increased

RICD sensitivity (Fig. 12D). These data collectively suggest that during clonal T cell expansion, PD-1 is highly expressed and acts to protect these expanding cells from premature RICD.

Figure 13: PD-1 Protects Cell Populations by Binding to PD-L1.



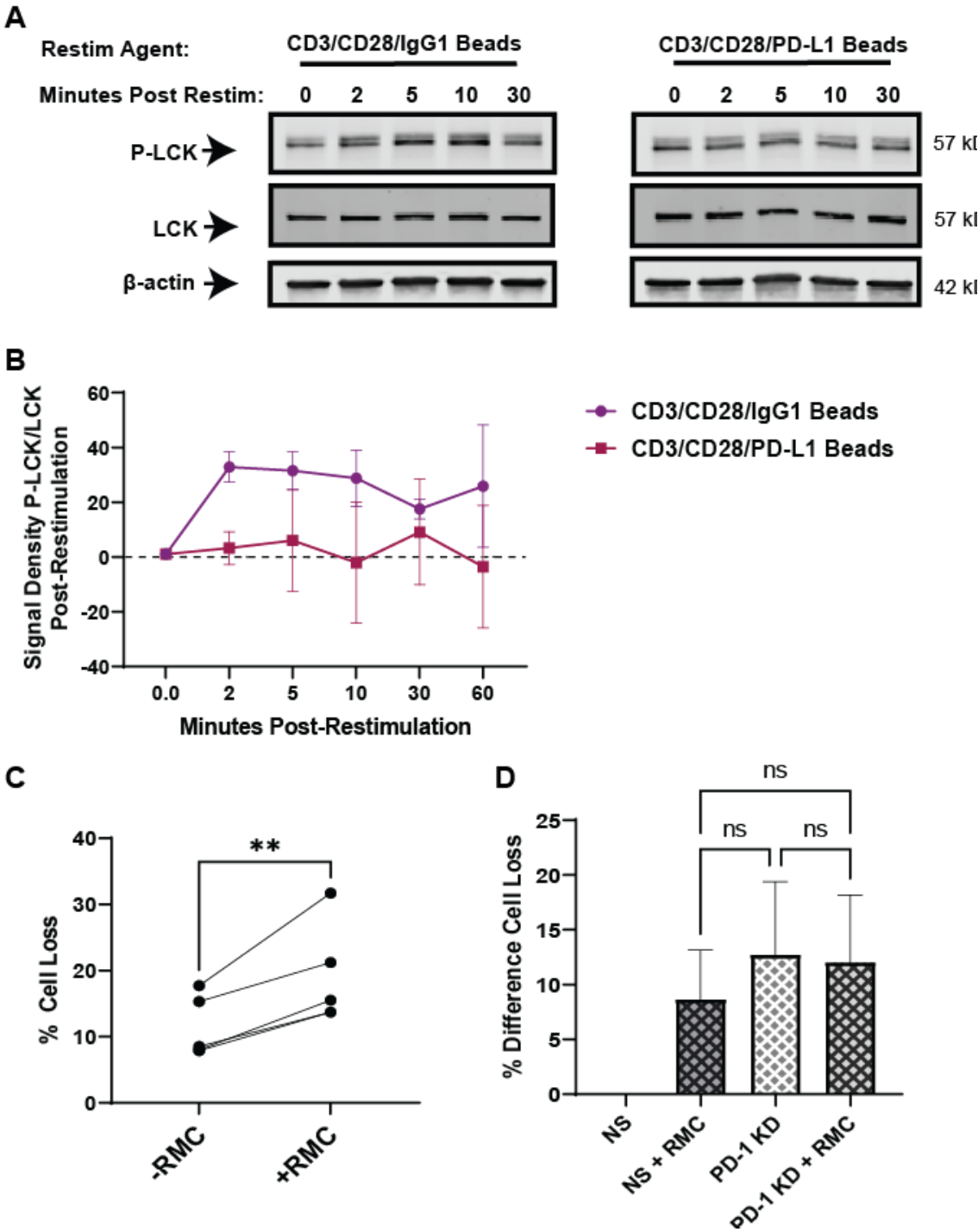
(A) Surface staining of PD-L1 and PD-L2 was conducted starting on Day 0 and up to Day 17 post-activation. Individual dots represent points at which MFI was taken; lines represent individual donors. **(B)** PD-L1 was assessed by immunoblotting on Days 4 and 14 post-activation. **(C-D)** Cells were treated on Day 3 post-activation for 1 hour prior to restimulation with 10 $\mu\text{g/mL}$ PD-1 blocking antibody (C) or PD-L1 blocking antibody (D), or control isotype antibody. Death was assessed using propidium iodide staining and flow cytometry 24 hours post-restimulation. **(E)** Magnetic beads with attached anti-CD3/CD28 and PD-L1-Fc-chimera or IgG1 isotype were incubated with cells at above doses overnight and assessed for death sensitivity with propidium iodide staining and flow cytometry. The following statistical analyses were conducted (with associated p-values): (B) unpaired t-test (C) paired t-test, $p=0.0097$; (D) paired t-test, $p=0.0151$; (E-F) ordinary one-way ANOVA with multiple comparisons (corrected), OKT3 vs. IgG1 beads $p=0.0344$, IgG1 beads vs. PD-L1 beads $p=0.0374$.

(Bottom): Cartoon representation of magnetic beads (as described under Methods) used in this and subsequent assays.

Functional binding of PD-1 to either of its ligands, PD-L1 and PD-L2, has been shown to be paramount in PD-1-driven signaling and increasing effector T cell exhaustion (84, 163, 217, 240). Indeed, PD-L1 blockade is as effective as PD-1 blockade in some contexts as an immunotherapy, demonstrating to importance of blocking the receptor-ligand interaction for immunotherapeutic efficacy (37, 84). We therefore asked whether ligation was an important driver of protection from premature RICD in expanding T cells. We first corroborated previous findings, that the relevant ligand in CD8^+ T cells is PD-L1 (rather than PD-L2, whose expression is restricted to certain dendritic cell types (18, 176)). Primary, effector T cells express PD-L1 over time in culture, and express little to no PD-L2 on their surface (Fig. 13A-B). Blockade of either PD-1 or PD-L1 significantly increased RICD sensitivity for each set of donors CD8^+ T cells (Fig. 13C-D). To provide further evidence that the protection from RICD is mediated by PD-1 and PD-L1 ligation, we employed a system of restimulation in which cells are exposed to magnetic beads which have $\alpha\text{-CD3}$ and $\alpha\text{-CD28}$ antibodies, and either a PD-L1-FC chimera or an isotype control

antibody. PD-1 is localized on the plasma membrane of resting cells and is actively recruited to the immunological synapse during T cell restimulation; therefore we used to this system to provide cells with proximally close activating antibodies and ligands, providing temporally synced signaling of restimulating agents alongside PD-L1 (100, 178). This system has previously been used successfully to initiate activation in cells in the presence of PD-L1 (173), though to our knowledge we are the first to use this system in the context of restimulation. We demonstrated that cells treated with PD-L1 beads were protected from premature cell death to a higher extent than cells restimulated with the isotype control beads, though this effect was lost later stage when cells no longer expression high levels of PD-1 (Fig. 13E-F). These results demonstrate that the binding of PD-1 to its ligand PD-L1 on neighboring CD8⁺ T cells provides crucial protection following activation, and that disrupting this may drive inappropriate premature RICD in expanding populations.

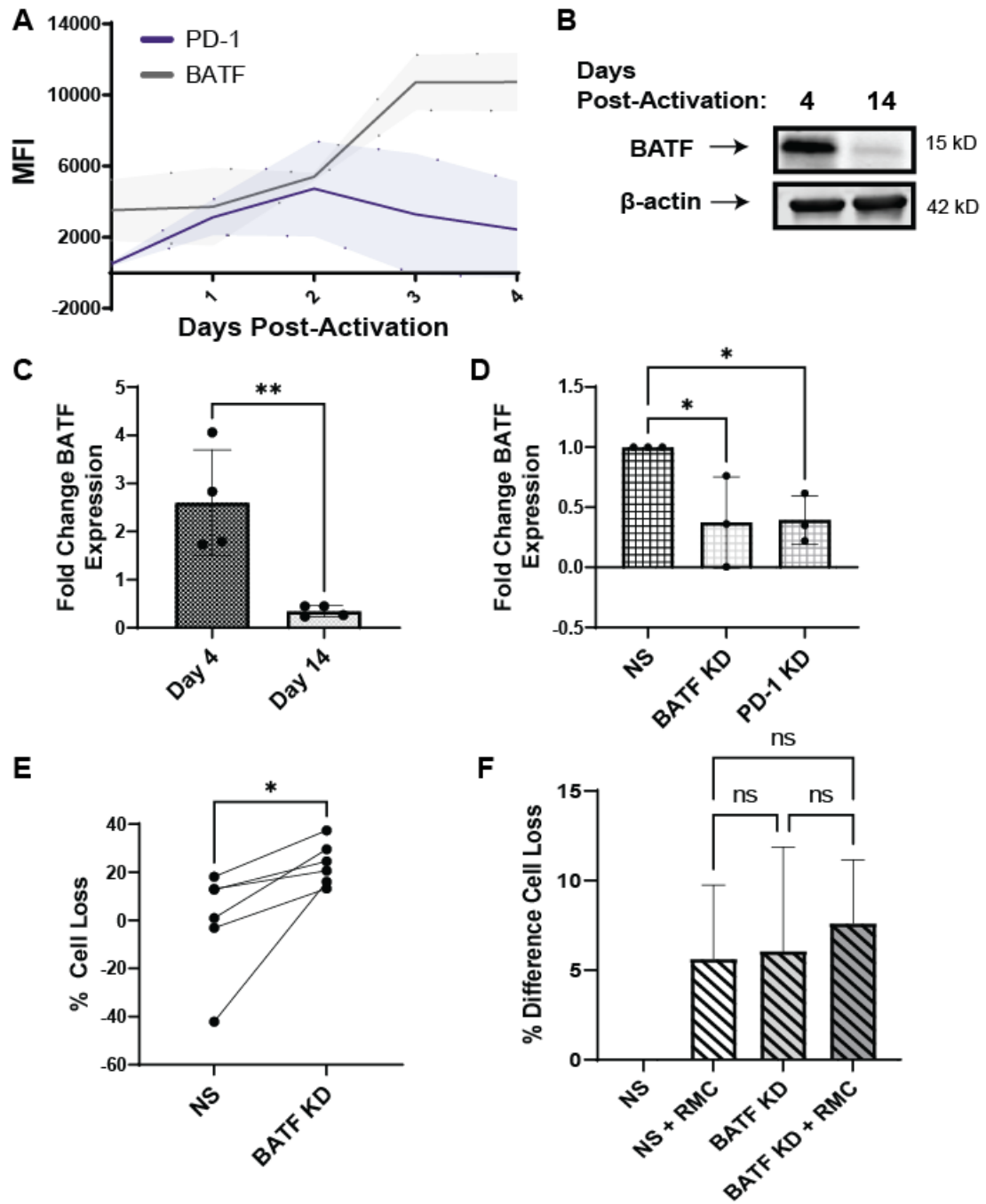
Figure 14: PD-1 Recruits SHP2 to Protect Newly Activated Cells from Premature RICD.



(A) Cells were restimulated with IgG1- or PD-L1-beads as previously described on Day 4 post-activation for the indicated time points. Lysates were collected and probed for phospho-LCK (P-LCK) or total LCK (LCK). (B) Three individual donors were assessed for overall changes to P-LCK following restimulation. To calculate the change, P-LCK and LCK signal densities were collected; for each individual time point, the ratio of P-LCK to total LCK was taken and plotted per time point. (C) Cells were treated with the SHP2 inhibitor RMC-4550 for 1 hour prior to restimulation and assessed 24 hours later for cell death using propidium iodide staining and flow cytometry. (D) Cells were transfected with PD-1 siRNA (PD-1 KD) or scramble siRNA control (NS) on Day 0, assessed for knockdown efficiency on Day 3, and treated with RMC-4550 1 hour prior to restimulation. Death sensitivity was assessed 24 hours post-restimulation using propidium iodide staining and flow cytometry. The following statistical tests were used: (B) (C) paired t-test, $p=0.0091$; (D) repeated measures one-way ANOVA with multiple comparisons (corrected).

Downstream of PD-1 and PD-L1 ligation, SHP2 is recruited to the ITSM of the tail of PD-1, leading to a cascade of proximal signaling events and culminating in a damped overall TCR signal strength (164, 170, 221). We found that ligating PD-1 during TCR/CD28 stimulation modestly decreased LCK phosphorylation in our system, confirming what others have previously documented (Fig. 14A-B) (12, 83). Because PD-L1 ligation is important for driving this difference in RICD sensitivity, we next asked whether recruitment of SHP2 activity likewise drives protection from premature RICD during expansion. Treatment with the SHP2 inhibitor RMC-4550 led to increased RICD sensitivity for each donor we tested (Fig. 14C). Moreover, treating cells transfected with PD-1 siRNA with RMC-4550 did not increase RICD further beyond single treatment alone, implying that PD-1 and SHP2 are part of the same pathway of protection from premature RICD (Fig. 14D). Though our system is limited in that we cannot say with certainty that the *recruitment* of SHP2 to the ITSM of PD-1 is responsible for driving protection from RICD, this work suggests the importance of SHP2 activity in preventing RICD.

Figure 15: BATF is Downstream of PD-1 and Protects Cells from Premature RICD.

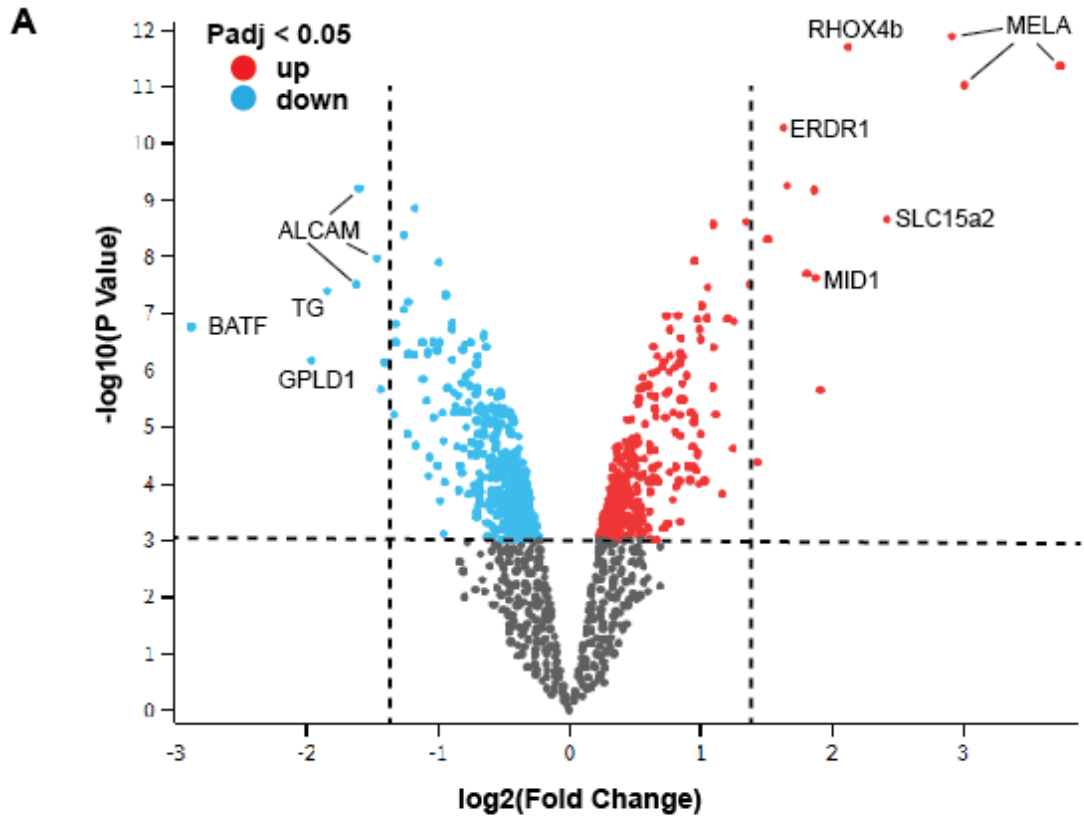


(A) Purified CD8⁺ T cells were activated on Day 0 and assessed for PD-1 (surface staining) and BATF (intracellular staining) each day post-activation. 4 donors were assessed total. Lines represent the average MFI of the 4 donors, shading represents the 95% confidence interval. **(B)** BATF was assessed by immunoblotting on Days 4 and 14 post-activation; representative blot of 1 donor. **(C)** 4 donors total were assessed for BATF densitometry compared to β -actin on Days 4 and 14. **(D)** Cells from 3 donors were transfected with either BATF siRNA (BATF KD), PD-1 siRNA (PD-1 KD) or scramble siRNA control (NS) and assessed for changes in BATF mRNA by qPCR. All CT values were normalized to housekeeping gene 18S. **(E)** Cells were transfected with BATF-specific siRNA (BATF KD) or scramble siRNA control (NS) on Day 0 and restimulated for 24 hours prior to death assessment on Day 4 post-activation. Cell loss was assessed by propidium iodide and flow cytometry. **(F)** Transfected cells were treated with SHP2 inhibitor RMC-4550 for 1 hour prior to restimulation on Day 3 post-activation and assessed for death 24 hours post-restimulation by propidium iodide staining and flow cytometry. The following statistical tests were used: (C) unpaired t-test, $p=0.0064$; (D) ordinary one-way ANOVA with multiple comparisons (corrected), NS vs. BATF KD $p=0.0372$, NS vs. PD-1 KD $p=0.0422$; (E) paired t-test, $p=0.0254$; (F) repeated measures one-way ANOVA with multiple comparisons (corrected).

The protein BATF, a “master-regulator” transcription factor, has been demonstrated to be increased in expression following PD-1 ligation with its ligands, and potentially plays a role in driving exhaustion of CD8⁺ T cells (132, 188). We next queried whether BATF was responsible for driving differential RICD sensitivity downstream of the PD-1/SHP2 signaling axis. Upon investigation, we confirmed that BATF expression is increased immediately following PD-1 surface expression, and that it is most highly expressed when CD8⁺ T cells are undergoing rapid expansion in culture (day 4) versus later stages of terminal effector differentiation (day 14) (Fig. 15A-C). We also confirmed that PD-1 KD cells express less BATF than their negative control counterparts (Fig. 15D). Knockdown of BATF revealed that BATF does indeed protect expanding cells from premature RICD (Fig. 15E) and that it is likely dependent upon the PD-1/SHP2 signaling axis, as treatment with RMC-4550 does not increase RICD sensitivity greater than the single treatment cohorts (Fig. 15F13F). These results collectively suggest that PD-1 works

through recruitment of SHP2 and downstream increases in BATF expression to mitigate RICD sensitivity in newly activated effector T lymphocytes.

Figure 16: Murine Microarray Analysis Reveals Transcriptional Changes which may Protect Cells from Premature RICD Downstream of PD-1 and BATF.



B

Gene Name	Fold Change	Gene Name	Fold Change
BATF	-2.877	MELA	3.739
GPLD1	-1.962	SLC15a2	2.419
TG	-1.843	RHOX4b	2.121
ALCAM	-1.625	MID1	1.878
ITGAE	-1.436	ERDR1	1.633
RAB4a	-1.402	SCD2	1.511
ELL2	-1.322	IFITM3	1.43
PDGFRB	-1.322	RPL391	1.373

(A) Murine microarray analysis was conducted using published data (117), as described in the Materials and Methods. Dotted lines represent arbitrary cut-off points used to determine most highly significant genes. (B) Genes which were most differentially expressed in BATF^{-/-} cells are represented. Negative values indicate a decrease in expression in BATF^{-/-} compared to wild type.

As a master-regulator transcription factor, BATF plays a crucial role in providing the necessary signals for cell growth, differentiation, and survival (117, 242). Analysis of published murine microarray data from Kurachi et al. (117) demonstrated that BATF does indeed restructure the transcriptional landscape of expanding T cells. (In their analysis, CD8⁺ T cells from BATF^{-/-} were compared to BATF^{+/-} mice following LCMV infection (Armstrong strain) in an effort to understand how BATF affected the activation and survival of this immune compartment.) We investigated this dataset in order to find genes which might feasibly play a role in mediating RICD sensitivity in T cells. In order to narrow down genes of importance, we focused on the top 16 genes which showed the highest statistical likelihood of being differentially expressed in BATF^{-/-} versus wild type and the highest overall amount of differential expression (both positively and negatively) (Figure 14A). Of these genes (listed in Figure 14B), Activated Leukocyte Cell Adhesion Molecule - CD166 (ALCAM) has been demonstrated to play a significant role in mediating the survival of breast cancer cells by increasing the concentration of the pro-survival protein BCL-2 as well as basal autophagy levels (8, 50), both of which have previously been demonstrated to be key contributors to cell survival and RICD protection, respectively (86, 127, 248). In addition to ALCAM, other candidate genes involved in survival and signaling were differentially expressed, though not in the top 16 differentially expressed genes. Though these results are preliminary, we hypothesize that the cumulative activity of ALCAM and others (explained further in the Discussion section) may be playing a significant role in providing protection from premature RICD.

Chapter 4: Discussion

TIM-3

In this study, we reveal a novel role for TIM-3 as a key regulator of restimulation-induced cell death (RICD) in CD8⁺ effector T cells. Surprisingly, our findings indicate that TIM-3 affects RICD differently at distinct timepoints following T cell activation. In newly activated CD8⁺ effector T cells, TIM-3 partners with CEACAM1 to shield them from premature RICD, helping to ensure proper clonal expansion. For late-stage effector T cells, however, TIM-3 potentiates RICD by enhancing TCR signal strength. We posit that RICD resistance may be driven in part by CEACAM1 serving to chaperone TIM-3 to the plasma membrane early after initial TCR stimulation; an effect lost in late-stage effectors wherein TIM-3 remains largely intracellular.

Ultimately, our results demonstrate that TIM-3 helps to tune RICD sensitivity of effector CD8⁺ T cells. To this point, TIM-3 has most thoroughly been characterized as an exhaustion marker on tumor infiltrating T lymphocytes. Indeed, severely exhausted cells are marked by elevated TIM-3 expression (19, 126, 174, 254). However, collective studies of TIM-3 have highlighted considerable functional variability in different contexts. Kane et al. first described the ability of TIM-3 to enhance TCR signaling in Jurkat T cells (125). These findings appear contradictory to the body of literature suggesting that TIM-3 inhibits T cell responses (104, 147, 201, 262), coincident with the hypothesis that TIM-3 contributes to exhaustion. However, recent work from Dr. Rafi Ahmed and others has demonstrated that TIM-3⁺ CD8⁺ cells in chronic viral infections are severely exhausted and beyond the rescue of anti-PD-1/PD-L1 checkpoint blockade, considering TIM-3 blockade alone is insufficient to rescue these cells from senescence and apoptosis commitment (103).

We posit that these late-stage “terminally exhausted” cells, in which TIM-3 may be primarily intracellularly localized, could be accelerated towards their death through TIM-3-enhanced RICD, though this requires further investigation. Our data therefore helps to clarify some of these conflicting points in the literature, and highlights a novel role for TIM-3 as a key regulator of normal CD8⁺ T cell homeostasis.

Our work further emphasizes the nuances of TIM-3 function and the role that interaction with specific ligands plays in defining its functions. We show that TIM-3 can augment TCR signaling following restimulation in late-stage effector T cells, leading to increases in downstream pro-apoptotic protein expression; this is congruent with the aforementioned study by Kane et al. (125). It is important to note that in that study, TIM-3 ligands were not necessary for driving differences in signaling output, a finding which is corroborated by our data (125). Downstream of these events, TIM-3 has been documented to promote PI-3K and AKT signaling (63, 218). We found that while treating newly activated cells with PI-3K and AKT inhibitors had no appreciable effect on RICD, late-stage effectors showed decreased RICD following inhibitor treatment (Supp. Fig. 2A-B). Further work will be needed to determine whether TIM-3 is facilitating increased proximal PI-3K and AKT signaling as part of its role in potentiating RICD.

In newly activated T cells, the mechanism by which TIM-3 and CEACAM1 protect cells from premature RICD also remains unresolved. CEACAM1 harbors a cytoplasmic immunotyrosine inhibitory motif (ITIM) for phosphatase recruitment, working to dampen the strength of the TCR signal (39). On the other hand, TIM-3 ligand engagement is known to displace Bat3, leaving LCK more vulnerable to dephosphorylation and diminished proximal TCR signaling (190). We found that silencing TIM-3 during initial T cell

activation did not have a measurable impact on proximal TCR signaling (data not shown), although more detailed studies are warranted. One could hypothesize that TIM-3 is needed on the surface merely as a ligand for CEACAM1, which is dampening proximal signaling via its ITIM alone. However, addition of recombinant CEACAM1 enhanced RICD, ostensibly by outcompeting TIM-3-CEACAM1 interactions *in cis*. These data indicate that CEACAM1 engagement via homotypic interaction does not confer RICD protection, suggesting that surface TIM-3 and CEACAM1 must cooperate to enforce RICD resistance. Indeed, CEACAM1 knockdown changed TIM-3 surface localization but not total expression in early-stage effectors (Fig. 10), making it plausible that increased TIM-3 intracellular localization in newly activated cells forced them to behave more like late-stage effectors with elevated RICD sensitivity. Additional experimentation will be required to ascertain the proportional inputs of TIM-3 and CEACAM1, both individually and together, in fine-tuning RICD sensitivity over time. Despite these unknowns, our data indicate that TIM-3 function in human CD8⁺ T cells is intimately tied to its temporal expression, cellular localization, and ligand availability.

CEACAM1 is a member of the carcinoembryonic-antigen-related cell adhesion molecule family, known to be involved in influencing a wide number of normal cellular processes through both hetero- and homophilic interactions (81). The isoform predominantly expressed in T cells – CEACAM1-4L – has a long cytoplasmic tail containing ITIM motifs which dampen proximal T cell receptor signaling by targeting ZAP-70 (39). Because of its status as a coinhibitory protein, CEACAM1 has been tested as a potential therapeutic target alongside other checkpoint blockade regimens, with some success (49, 62, 129, 165). However, its function with regard to programmed cell death is

unclear. One study found that CEACAM1 expression in Jurkat T cells prevented FAS-mediated apoptosis by directly redistributing beta-catenin in the actin cytoskeleton (131), whereas another study by Nittka et al. described that CEACAM1 can promote apoptosis in Jurkat cells through direct activation of caspases (159). Much like TIM-3, the cellular context within which CEACAM1 is studied is likely important for understanding its full functional capacities within the immune system. Our study demonstrates a novel function for CEACAM1 in protecting expanding cells from premature RICD, working in concert with TIM-3. This finding also sheds new light on patients with specific germline TIM-3 mutations, a significant proportion of whom suffer from subcutaneous panniculitis-like T cell lymphoma (SPTCL) (72, 182, 231). In these patients, TIM-3 cannot localize to the plasma membrane, which the authors noted led to “persistent immune activation and increased production of inflammatory cytokines... promoting SPTCL” (72). While inflammatory cytokines may be induced by multiple TIM-3⁺ immune cell types, CD8⁺ T cells are the primary driver of pathogenesis in SPTCL. Indeed, TIM-3 mutations noted in SPTCL are predicted to disrupt CEACAM1 association (Supp. Fig. 3). These results are consistent with our findings in late-stage effectors, in which TIM-3⁺ lymphocytes have increased proximal T cell signaling output. We predict that although more effector CD8⁺ T cells may undergo RICD at early stages, a pool of over-activated clones lacking TIM-3/CEACAM1-dependent signal modulation can drive damaging immunopathology in the form of SPTCL. In all, these results suggest that TIM-3 and CEACAM1 must work cooperatively to maintain immune system homeostasis via RICD regulation.

Finally, this study has revealed that co-inhibitory proteins like TIM-3 can fine-tune RICD sensitivity in effector CD8⁺ T cells. Early studies offered conflicting results on how

CD28 co-stimulation and CTLA-4 expression may influence activation-induced cell death/RICD, particularly in CD4⁺ T cells (23, 168, 210, 246). RICD has long been recognized as a key homeostatic process for constraining effector T cell expansion (227). Several factors govern RICD sensitivity in late-stage effector T cells, but little is known about mechanisms of RICD resistance in early expanding T cells. A recent study from our group showed that early expanding conventional human T cells are less susceptible to RICD in part due to the influence of the transcription factor FOXP3 and autophagy (248). It is also plausible that newly activated T cells avoid RICD via downregulation of TCR/CD3 expression (71). Indeed, we have found that effector CD8⁺ T cells do express less CD3 at early versus late timepoints after activation (Supp. Fig. 2C). However, early effector T cells also express higher levels of CD25 after stimulation (Supp. Fig. 2D), which should enhance IL-2 responsiveness and cell proliferation (128). Intriguingly, CEACAM1 has also been documented to decrease IL-2 production in expanding T lymphocytes, which may act to keep clonal expansion and RICD sensitivity in check despite high CD25 expression (34). Because TIM-3 and CEACAM1 work together to protect newly activated cells from RICD, our results underscore a mechanism by which expanding CD8⁺ T cells modulate responses (including apoptosis) by fine-tuning TCR signal strength via co-inhibitory proteins. Indeed, our results corroborate the “Tide Model” of T cell immunity, in which dynamic, tightly regulated expression of costimulatory and coinhibitory proteins dictate T cell fate and ensure optimal control of population expansion and contraction (36, 267). Our results suggest this process also governs RICD sensitivity in CD8⁺ T cells, whereby signal calibration via coinhibitory and costimulatory proteins helps to determine their temporal susceptibility to cell death in response to TCR re-engagement. Future studies

should expound upon this hypothesis to better understand how other co-inhibitory proteins can shape the overall T cell response by promoting RICD resistance, particularly during clonal expansion.

In conclusion, this study describes a novel function for the co-inhibitory proteins TIM-3 and CEACAM1 in mitigating RICD in expanding effector CD8⁺ T lymphocytes during clonal expansion (Fig. 17). In contrast, TIM-3 alone potentiates RICD in late-stage effectors when decoupled from CEACAM-1. This study implies that the utilization of checkpoint blockade antibodies may have broader effects on the immune response than previously appreciated, including dysregulation of cell death sensitivity. In fact, a recent report showed loss of CEACAM1 leaves mice more susceptible to enteropathogenic bacterial infection, specifically through a dysregulated, hyperactive CD8⁺ T cell response in the colon (269). These concepts may warrant further consideration for those studying adverse outcomes and variable efficacy associated with checkpoint blockade regimens (1, 85, 143, 179, 185), with the goal of specifically reinvigorating exhausted cells without inducing overt immunopathology or compromising nascent T cell responses.

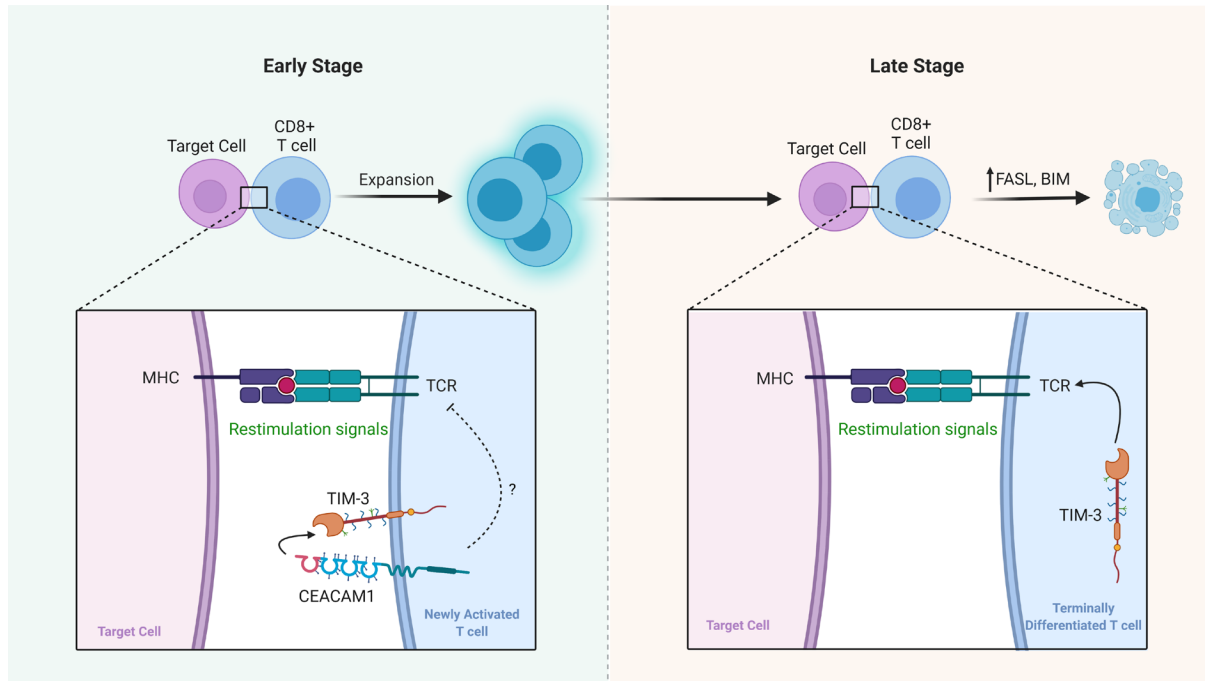


Figure 17: Graphical Summary of Aim 1. Soon after activation, TIM-3 and CEACAM1 co-localize to the surface of T cells, promoting protection in the face of heavy restimulation and expansion. Over time, terminally differentiated cells become more susceptible to RICD partially due to the influence of TIM-3, which remains predominantly intracellular, increases proximal TCR signaling, and leads to increased FASL and BIM expression.

PD-1

In this study, we aimed to understand the role of the co-inhibitory protein PD-1 in mediating sensitivity to restimulation-induced cell death (RICD) in effector T lymphocytes. We found that PD-1 plays a crucial role in providing protection from RICD in newly activated T cells, primarily through decreasing the overall signal strength of the T cell receptor (TCR) and through the increased expression of the transcription factor BATF. BATF ostensibly governs transcriptional changes which we hypothesize mediate differential RICD through the enhanced expression of modulatory proteins such as ALCAM, CTLA-4, TIM-3 and others, though these targets must be validated in future investigations. The findings herein illuminate a hitherto unknown role for PD-1 in effector T cells, and suggest that PD-1 can work in multiple contexts to maintain immune system balance and optimal function while maintaining similar mechanisms of signaling.

PD-1 (programmed cell death – 1), as its name suggests, was initially thought to drive death mechanisms in T cells (64, 67, 99, 113, 162). The original studies suggesting the ability of PD-1 to increase cell death were performed through subtractive hybridization of RNA on cell populations in the murine thymus (99). PD-1 expression was discovered to be upregulated in murine T cells following *in vivo* infusion with anti-CD3 antibodies, a phenomenon which correlated with thymocyte death in their model. These results left the authors to conclude that PD-1 must be implicated in driving increased cell death, although it was only based on a correlation. In contrast, our study suggests that stimulation through the T cell receptor increases PD-1 expression, and that the presence of PD-1 actually protects these expanding cells from premature RICD. We propose that these early experiments documented an important physiological phenomenon related to PD-1, which

is corroborated by our data: that T cells, in the face of restimulation, upregulate PD-1 as a protective mechanism against death, leading to protection from RICD in cells which express PD-1 over those that do not. This model would predict that cells which express high levels of PD-1 would be protected from RICD and would persist in the population, explaining why the original papers documented such high PD-1 expression in the cells that survived in the face of restimulation. We introduced this hypothesis previously, extrapolating that the metabolic reprogramming driven by PD-1 may be playing a crucial role in providing protection from RICD in PD-1^{hi} lymphocytes (249). Importantly, this model does not contradict the more well-known phenomenon of exhaustion, to which PD-1 is a significant contributor (53, 69, 88, 223). Tumor infiltrating lymphocytes (TILs) which are functionally exhausted and express high levels of PD-1(3) may have been the original population which were prevented from dying prematurely to RICD, and thus, remain alive in the face of constant restimulation, yet lacking functional capabilities due to the inhibitory nature of PD-1 signaling. Further work will need to verify that this is indeed the case *in vivo*. Such work could include the reintroduction of varying levels of PD-1 in specific clonal lineages of murine T cells (from a *Pdcd1*^{-/-} background) in the context of various doses of anti-CD3-mediated injections. Specific clonal lineages could be tracked over time to determine their apoptotic commitment as well as exhaustion propensity to determine the exact contribution of PD-1 to both RICD and exhaustion simultaneously. Overall, our work clarifies some of the earlier work done regarding PD-1 and expands upon the functional capabilities of this protein in providing immune homeostasis.

The transcription factor BATF, while more recently discovered, is quickly being recognized as a major driver of T cell population expansion and dynamics (98, 117, 151,

247). Though originally discovered to control Th17 cell differentiation (211), its role has expanded to include functions across a variety of T cell populations, including CD8⁺ T cells (118, 148, 261). The functional scope of BATF include regulating processes like metabolism, cellular differentiation, and viral immunity (117, 242). In a recent publication by Kurachi et al., the authors demonstrate that BATF is critical not only in allowing cells to differentiate properly, but to survive as well (117). Indeed, the CD8⁺ T cells from BATF^{-/-} mice did not survive past 3 days *in vivo* when the mice were challenged with LCMV, demonstrating the necessity of functioning BATF to promote cell survival in the face of viral infection. These results suggested to us that the cells lacking BATF could potentially be dying through RICD – as viral infections initiate a robust CD8⁺ T cell response through an abundance of antigen, which persists until viral clearance - though this was not demonstrated directly in their paper. Indeed, we discovered that knocking down BATF in expanding T cells increases their sensitivity to RICD. Investigation of the role of transcription factors in RICD has so far only extended to FOXP3 (248); we now report an additional transcription factor that is also mediating RICD sensitivity. As a master-regulator transcription factor, BATF is also significant in that it must work with other transcription factors such as IRF4 and JUN to mediate differential transcription of genes (43, 77, 130, 243). This suggests that other transcription factors may partner with BATF as crucial regulators of RICD, though this hypothesis remains to be further explored.

We and others have shown that death sensitivity in T cells is dependent upon *de novo* transcription (181, 248), indicating that active transcription of pro- or anti-apoptotic proteins plays a central role in fine-tuning RICD sensitivity. We hypothesized that BATF was either promoting transcription of genes which protect expanding effector T cells from

premature RICD, or repressing pro-apoptotic genes that potentiate RICD. Investigation of the top 16 differentially expressed genes from the dataset supplied by Kurachi et al. (117) suggest a novel hypothesis: that Activated Leukocyte Cell Adhesion Molecule - CD166 (ALCAM) may protect expanding cells from premature RICD (as this protein was significantly downregulated upon knockout of BATF). ALCAM is a member of the immunoglobulin superfamily of receptors, and has been documented to play important roles in T cell activation, development, and migration both through homophilic interactions and heterophilic interactions with the protein CD6 (24, 172, 253). ALCAM has most thoroughly been investigated in the setting of cancer progression; it has been labeled as a valuable prognostic marker in various tumor types (50). Interestingly, ALCAM has been investigated alongside PD-L1 expression in tumors; the authors discovered that tumors positive for ALCAM or PD-L1 independently predicted lowered survival rates among study participants with malignant plural mesothelioma (96), demonstrating the vast constellation of signaling parameters that could be related under similar circumstances, such as the tumor microenvironment. Importantly, ALCAM was discovered to play a major role in providing survival for breast cancer cells through an increase in BCL-2 expression (8). Traditionally, RICD has been contrasted from other mechanisms of propiociidal cell death, such as Cytokine-Withdrawal Induced Death (CWID), by the type of apoptosis mechanism employed: RICD is traditionally thought to be carried out through the extrinsic mechanism of apoptosis (death-receptor mediated), whereas CWID is typically carried out through the intrinsic mechanism of apoptosis (mitochondria-mediated) (227). Although BCL-2 is a pro-survival protein related to mitochondrial commitment to apoptosis, we now appreciate that the mechanisms employed by RICD and CWID - which were hitherto

thought to be completely separate – actually employ much overlap in order to carry out functional apoptosis (204, 226, 227). Indeed, is it now recognized that BIM, a BCL-2 family member and a key mediator of the intrinsic mechanism of apoptosis, works alongside the death receptor FAS to promote RICD sensitivity in CD8⁺ T cells (227). This finding offers the hypothesis that other BCL-2 family members may also play pivotal roles in mediating RICD. It is therefore possible that increased levels of BCL-2 following ALCAM expression may be promoting survival in the face of heavy restimulation leading to decreased RICD sensitivity, though this hypothesis warrants further investigation. In addition, ALCAM has also been demonstrated to increase basal levels of autophagy in breast cancer cells (8). Autophagy plays an important role in tumor cell survival by suppressing p53 (257); we previously also showed that autophagy plays a protective role in expanding T cells from premature RICD through a similar mechanism (248). We propose that ALCAM might protect expanding T cells from premature RICD through increased BCL-2 expression – counterbalancing the expression of BIM and other pro-apoptotic proteins increased with TCR signaling – while simultaneously increasing autophagy, promoting the suppression of p53 and overall survival of cells in the face of TCR restimulation.

It is worth noting, however, that other genes of potential significance were identified in our analysis of the dataset as supplied by Kurachi et al., including ITGAE, IL-7R, and TNFRSF4, CTLA family members -2 and -4, and TIM-3. Alternative hypotheses to ALCAM being the sole contributing factor to RICD sensitivity (mediated by BATF) are that another single gene is driving protection, or that a network of mechanisms is employed during expansion to allow cells to grow despite heavy antigenic burden. This hypothesis is

explored further in the Overall Significance section of this Dissertation. What remains clear is that further investigation will need to be done in order to determine whether these hypotheses are correct, which are expounded upon in the Future Directions section of this dissertation.

In this study, we determined that PD-1 – previously believed to promote programmed cell death in T cells – protects cells during clonal expansion (Fig. 18). This occurs through recruitment of the phosphatase SHP2 and the downstream increase the transcription factor BATF, which facilitates extensive transcriptional changes to allow cells to remain resistant to RICD during this critical period. This discovery is perhaps most significant because of its implications regarding checkpoint blockade immunotherapies. Within the last few years, researchers have discovered that blocking PD-1 signaling can promote tumor regression by reversing TIL exhaustion and promoting cytotoxic effector function, yielding greater cancer remission in certain contexts (240, 240, 268, 270). The administration of this monoclonal therapy is as a bolus into the bloodstream of individuals undergoing checkpoint blockade, suggesting that cells expressing PD-1 other than TILs could be affected by this therapeutic intervention. This study demonstrates that PD-1 is important for driving T cell expansion following activation, suggesting that some proportion of expanding T cells may be at risk of premature RICD following therapeutic blockade. As discussed in the next chapter, future studies are needed to understand how to better target exhausted TILs for blockade therapy so that newly activated T cell populations are protected during the critical expansion phase, as well as extrapolate on the full role of co-signaling proteins in driving critical immune homeostasis mechanisms.

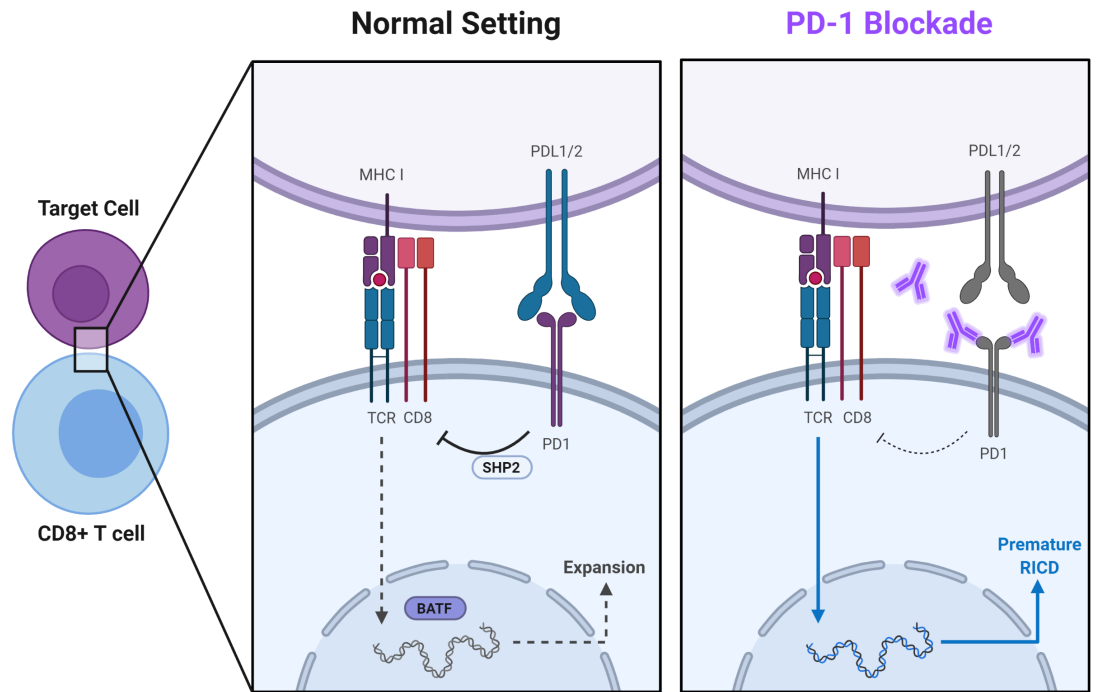


Figure 18: Graphical Summary of Aim 2. Without PD-1 manipulation, restimulation recruits PD-1 signaling. PD-1 dampens proximal signaling through recruitment of SHP2, while also promoting BATF transcriptional activity. The cumulative effects of these events is dampened RICD sensitivity soon after activation. These effects are mitigated during the process of checkpoint blockade, which abrogates PD-1 signaling.

Overall Significance

The Influence of Co-Signaling Proteins on Immune System Dynamics: An Updated Tide Model of Immunity

For my thesis project, I set out to determine whether the proteins TIM-3 and PD-1 fine-tuned the process of restimulation-induced cell death (RICD) in effector T lymphocytes through their influence over T cell receptor (TCR) signal strength. My overarching hypothesis was that co-inhibitory proteins would protect cells from RICD by dampening the TCR signal, preventing the cells from reaching the signal amplitude necessary for RICD commitment. Both of my specific aims detailed an experimental approach to address the goal of determining whether this overarching hypothesis was consistent with the biological function of each protein in the context of RICD. Surprisingly, I discovered that TIM-3, though traditionally considered a co-inhibitory protein, functions differently depending upon the time tested post-activation. In newly activated cells (day 4 *in vitro*), TIM-3 localizes to the plasma membrane and protects expanding cells from RICD in conjunction with CEACAM1. At later stages of effector T cell differentiation (day 14 *in vitro*), TIM-3 remains intracellular and promotes RICD independently of CEACAM1 interaction. PD-1, once thought to be a mediator of cell death, clearly protects early expanding T cells from premature RICD through its influence over the overall TCR signal strength as well as induction of BATF expression. Therefore, we can conclude that – like the majority of immune system dynamics – multiple contextual factors influence the ability of these proteins to fine-tune RICD sensitivity in T lymphocytes, including the time at which they are expressed, ligand availability, as well as their unique mechanisms of influencing TCR signal strength.

T cell receptor recognition of the pMHC complex was originally believed to be the sole determinant of a T cell's activation status. The influence of co-signaling proteins on the overall activation status of T cells gained prominence in the field of immunology with the necessity to build a model which allowed the immune compartment to discriminate between self and non-self (35). The theory of tolerance induction was first applied to B cells (150) and then to T cells (120, 213), in which it was recognized that sufficient co-stimulation – in the form of co-stimulatory signaling and subsequent IL-2 consumption – must occur during antigen presentation as a necessary “second signal” preventing T cells from undergoing functional anergy. However, as Zhu et al. point out in a seminal review article (267), co-inhibitory proteins are often expressed at similar time points as co-stimulatory proteins, and their presence often dictates the functional outcomes of signaling – with associated larger implications in T cell tolerance, anergy, plasticity, and effector function.

In order to bring together the fundamental paradigms of initiating signals of immune responses, as well as incorporate current knowledge of co-signaling proteins and their influence over T cell activation, Zhu et al. described an updated model, which they termed the “Tide Model of Immunity” (267). In this model, they propose that the primary signal that T cells receive is composed of the first two necessary signals mentioned above: TCR-pMHC binding and recognition, and co-signals in the form of CD28-B7 binding and IL-2 consumption. The second signal actually is comprised of the presence (or absence) of other co-signaling proteins – both co-stimulatory and co-inhibitory – which often balance each other out and compete for ligands on the adjacent cell. Their model builds on the paradigm that while the primary signals that T cells receive are the gatekeepers of an initial

response, the secondary signals from co-stimulatory and co-inhibitory proteins actually determine the fate of the T cell in question through their influence over the signal strength downstream of the T cell receptor. These co-signaling dynamics, including positive and negative feedback mechanisms provided by co-stimulatory and co-inhibitory proteins, collectively determine the outcome of an immune response. Their dynamic interplay and expression patterns are similar to the rise and fall of the tide, giving this model its unique and descriptive name (267).

This model is supported by the observation that T cells, and other members of the adaptive arm, express a myriad of unique co-signaling proteins at various stages following cellular activation. Many members of the co-signaling family have redundant functions but are expressed at different times; for example, while CTLA-4 and PD-1 both act to dampen overall TCR signal strength, they are both expressed at different points and play different roles in various homeostatic processes. CTLA-4 is expressed in naïve T cells, but remains intracellular and only traffics to the surface following the initial events of T cell activation to compete with the ligand of CD28 and recruit phosphatases to the immunological synapse (209); PD-1 is expressed only after T cell activation and is most highly expressed on cells with chronic antigen exposure, implicating PD-1 in T cell exhaustion (17). Beyond the canonical proteins CTLA-4 and PD-1, many co-inhibitory proteins are unique in their structure, mechanism of action, overall effect on the function of the cell population in question, and time post-activation at which they are expressed. These insights suggest that, much like the phosphorylation cascades following TCR activation, the scope of co-inhibitory expression is a finely regulated process in order to provide optimal signaling and outcomes for the cell population in question.

The Tide Model of Immunity is important because it outlines the significance of co-signaling proteins as the key determinants of the outcomes of an immune response, across both the adaptive and innate arms (267). Of late, the collective actions of these proteins have been focused on the mechanisms of T cell activation and exhaustion; however, the regulatory network provided by co-signaling proteins extends to the innate system as well. Though the innate system does not require complex antigen processing prior to action, many coreceptors are still present on the surface of dendritic cells and other innate immune cells in order to curb excessive inflammation (267). For example, Ig Superfamily member CD200R provides coinhibitory signals to tissue macrophages (87), the lack of which drives autoimmune encephalomyelitis (EAE). Interestingly, a similar phenotype is observed in mice treated with TIM-3 blockade (147).

We now posit that this paradigm extends to T cell homeostasis, including propiocietal cell death. Indeed, the co-signaling proteins TIM-3 and PD-1 both fine-tune the process of RICD. Intriguingly, these proteins fine-tune RICD sensitivity at unique points in time following T cell activation, suggesting that their expression – and that of other co-inhibitory proteins – may be temporally regulated as part of a larger network of T cell regulation, akin to the “ebb and flow” of a tide. Hence the Tide Model of Immunity can be extended to help explain the key homeostatic process of RICD; more specifically, that T cells temporally regulate their RICD sensitivity through a multitude of mechanisms – including co-signaling protein expression – in order to optimally expand and contract most beneficially for the host.

Newly activated CD8⁺ T cells must be protected for specific reasons: first, they must be able to expand appropriately to enable responses to the infecting pathogen, and to

build a strong enough effector cytotoxic T lymphocyte (CTL) pool (in terms of numbers) to effectively control the infection; second, a proportion of the clonal population must be spared from death to promote the formation of long-term pathogen-specific memory (107). Late-stage effector cells must contract in order to prevent immunopathology of the host, as demonstrated in individuals who lack effective SAP (225). At the crux of our model is the idea that strong measures to prevent RICD are “turned on” in the several days following TCR engagement, including FOXP3 expression (with associated changes in protective autophagy), and rapid attenuation of TCR signal strength after initial antigen encounter – achieved primarily through the internalization of TCR with subsequent signal downmodulation (Supp. Fig. 2C), as well as co-inhibitory protein expression and signaling. We showed that FOXP3 is transiently expressed in expanding conventional T cells to provide protection from premature RICD immediately following activation (248). We have now demonstrated with this work that multiple measures which modulate the signal strength of the TCR are also employed early during clonal expansion, corroborating the plausibility of our model. In terminally differentiated cells, our model suggests that mechanisms which sensitize T cells to RICD must be deployed in order to constrain effector T cell responses. We have shown previously that increased glycolysis in terminally-differentiated “late- stage” CD8⁺ effector T cells enhances RICD sensitivity through a mechanism linked to increased FASL expression (122). With this work, we have demonstrated that key proteins (e.g. PD-1, BATF) which reduce RICD sensitivity are no longer present in older effector T cells, and that co-signaling proteins like TIM-3 can promote increased TCR signal strength, thus facilitating RICD and curtailing effector T cell expansion while antigen is still present, prior to clearance and contraction via CWID.

Early-Stage Cells: Balancing TCR Signal Strength to Prevent RICD and Facilitate Survival

Immediately following activation (pMHC-TCR binding, co-receptor recognition, and appropriate co-signaling activity), T cells begin the process of clonal expansion. This process is dependent on a number of factors to deploy swiftly and correctly, including TCR signal strength amplitude, leading to translocation of key transcription factors into the nucleus of cells to begin the process of transcriptional reprogramming and differentiation. However, the necessity for counterbalancing an overactive immune response is evident in the rapid, subsequent upregulation of co-inhibitory proteins, which act fundamentally to dampen T cell signal responses and prevent autoimmunity. PD-1^{-/-} mice suffer from autoimmunity (30, 158), and individuals with germline mutations in TIM-3 suffer from subcutaneous panniculitis-like T cell lymphoma – at its core, a disease facilitated by overactive CD8⁺ T cells attacking normal tissue (72, 182). The unique mechanisms by which TIM-3 and PD-1 act to fine-tune RICD sensitivity suggest that the events following activation are heavily regulated – primarily, at the level of TCR signal strength – in order to allow cells to expand properly while simultaneously preventing premature RICD.

Though we hypothesized that TIM-3 would prevent RICD in effector T lymphocytes directly through mitigating TCR signal strength amplitude, we found that this was only reflected at distinct times following T cell activation. The true effects of TIM-3 are dependent upon a number of factors, two of which are the expression and availability of one of its ligands, CEACAM1, and the localization of TIM-3; we found that these effects are also inextricably linked together in T cells. Following activation, TIM-3 is shuttled to

or retained on the plasma membrane through the actions of CEACAM1. We posit that this likely promotes TIM-3/CEACAM1 association *in cis* and a modulatory signal that remains to be defined, ultimately contributing to the protection of expanding CD8⁺ T cells from premature RICD. While TIM-3 and CEACAM-1 may also interact *in trans* across adjacent T cells, previous work demonstrates that an *in cis* CEACAM-1/TIM-3 heterodimer facilitates TIM-3 surface expression (91), consistent with our data in Jurkat T cells. We hypothesize that the true effects of dampened TCR signal strength are likely facilitated by CEACAM1 utilizing TIM-3 (and other CEACAM1 proteins *in cis* or *trans*) as its binding partner, as we found that modulating TIM-3 expression did not dramatically change the phosphorylation status of key proximal signaling proteins in newly activated cells (data not shown). In contrast to TIM-3, CEACAM1-4L has been documented to be a co-inhibitory protein across a wide variety of cell types and environments, and carries two canonical ITIM signaling motifs in its tail, allowing it the ability to dephosphorylate ZAP-70 (31, 39). Like TIM-3 and PD-1, loss of CEACAM1 has been associated with autoimmunity (154), and recent reports have also shown the promise of pharmacological blockade of CEACAM1 in promoting anti-tumor responses, aligning CEACAM1 with PD-1 and TIM-3 as mediators of dampened immune responses at distinct points following activation (141, 165). Our model would suggest that proteins which modulate TCR signaling would be expressed primarily in newly activated cells, and would fundamentally act to dampen TCR signal strength in order to protect at least a proportion of cells from premature RICD. Though TIM-3 and CEACAM1 are a unique case in their expression pattern and mechanism of preventing RICD, they do follow the tenants laid out by our model, and thus support our hypothesis.

In direct contrast to TIM-3 and CEACAM1, PD-1 follows the canonical principles laid out by our model. PD-1 is expressed during T cell expansion and wanes over time without restimulation, binds to its ligand PD-L1 on neighboring CD8⁺ T cells *in vitro*, and mediates effects through dampened TCR signal strength (through SHP2) to decrease RICD sensitivity. RICD is potentiated by a robust TCR signal, as previously demonstrated (219); however, this early study concluded that the influence of the TCR signal strength on RICD was a direct result of a quantitative increase in CD3 ITAM phosphorylation, rather than differential phosphorylation of specific ITAMs. This finding was revised when we and others demonstrated that RICD is intrinsically associated with signaling by SAP and NTB-A, proteins which act to directly boost TCR signal strength regardless of CD3 expression (110, 181, 225). While we have also demonstrated that CD3 expression is increased in terminally differentiated cells *in vitro* (Supp. Fig. 2C), our work suggests that direct dephosphorylation of proximal signaling proteins also plays an important role in determining RICD sensitivity, supporting published data from our group delineating how SAP and NTB-A act to enhance TCR signal strength (110, 225). PD-1 recruits SHP2 to its tail, the outcome of which is decreased phosphorylation and activity of ZAP-70 (221, 263). We demonstrated that pharmacological inhibition of SHP2 increased RICD in newly activated cells, leading us to conclude that both the expression patterns of CD3 over time as well as fine-tuning RICD signal strength play cooperative roles in dictating RICD sensitivity over time. This finding further supports our model of the influence of co-signaling proteins over T cell population dynamics and RICD sensitivity, as their main form of influence is typically through modulation of TCR signal strength (152).

Interestingly, PD-1 signaling also facilitates increased expression of the

transcription factor BATF, which we have demonstrated also functions to dampen RICD sensitivity during expansion. Historically, BATF has been associated with T cell differentiation and lineage commitment of TH17 cells, though its role in a number of other important aspects of T cell biology has become increasingly apparent over the years (98, 118, 211). In this study, we identified another role for BATF, adding to the ever-growing body of literature regarding its function: that of providing protection from premature RICD in effector T lymphocytes. We confirmed in this study that PD-1 signaling promotes BATF expression as previously shown (188); however, further work is required to ascertain whether BATF is directly downstream of PD-1-dependent SHP2 recruitment and activity. Overall, this finding is highly significant for two reasons: first, it sheds further light on mechanisms facilitating apoptosis protection that are potentially downstream of TCR signaling; second, it adds BATF to the list of transcription factors which have been shown to influence RICD sensitivity.

Meta-analysis of previously published murine microarray data (117) revealed genes that BATF regulates. Beyond ALCAM, additional differentially expressed target genes remain to be explored to understand how BATF influences apoptosis sensitivity. For example, closer inspection of the genes targeted by BATF in the murine microarray include ITGAE, IL-7R, and TNFRSF4, CTLA family members -2 and -4, and TIM-3. ALCAM and ITGAE (CD103) are both cell-adhesion proteins, and function to promote interaction with adjacent T cells and tissue retention (24, 68). It is worth noting that CD103 is descriptive marker of specific subsets of cells which have been documented to survive for long periods of time, including tissue resident-memory T cells, as well as tumor infiltrating regulatory T cells (10, 45). We and others have shown that regulatory T cells are highly

resistant to RICD largely through suppression of SAP expression, however, other mechanisms may be in place – such as upregulation of CD103 – which may further protect this cellular compartment from RICD sensitivity (112). Further work exploring how CD103 might support T cell survival will be needed, as the above conjectures are largely associative in nature. The IL-7/IL7R signaling axis has been demonstrated across numerous studies to be critical for maintenance of BCL-2 and MCL-1 expression in T cells (6, 29, 140). Interestingly, this signaling axis also significantly contributes to the commitment between memory precursor versus short-lived effector cell subsets (MPEC, SLEC, respectively (106)). Memory subsets have traditionally been thought to be less sensitive to apoptosis (107), providing a potential link between BATF and survival in conventional effector T cells. TNFRSF4, also known as OX40L, is part of a critical co-signaling pathway during cellular expansion that promotes the expression of Survivin following T cell activation (230), and has even been documented to drive anti-tumor responses (102). Finally, we and others have shown that CTLA-4 and TIM-3 drive changes in apoptotic sensitivity (48). Based on these insights, we hypothesize that BATF may be driving larger transcriptional changes through a variety of ways – relating to cell-adhesion, signaling, and apoptosis – in order to protect expanding cells from premature RICD, and that the culminating effect of this transcriptional remodeling allows cells to expand even in the face of strong restimulation. Indeed, the abrupt decline of the CD8⁺ effector T cell response in *Batf*^{-/-} mice challenged with LCMV (117) underscores this point; our findings clarify this phenotype further in implicating premature RICD as a likely cause for this phenomenon.

BATF has been described as a master-regulator transcription factor, in that it binds

cooperatively with other transcription factors to initiate differential gene expression. Members of the IRF and JUN transcription factor families have been implicated in working with BATF to drive changes in T cells, including tolerance, plasticity and cell lineage commitment, and class-switching in B cells (98, 130, 151, 211). Unlike other members of the AP-1 transcription factor family, BATF does not have a transcriptional activation domain, thus requiring the partnership of BATF with other transcription factors to function (151). This suggests that partnering transcription factors participate in BATF-dependent regulation of RICD in T cells, warranting further investigation. Though FOXP3 was the first transcription factor discovered to play a role in mediating RICD sensitivity in T cells, it is clear that BATF and others play a role in protecting expanding cell populations as well, demonstrating the dynamic complexity of RICD sensitivity in T cells. Further work extending from these studies may delineate exactly how transcription factors like FOXP3, BATF, and others interact and cooperate to induce and repress genes that shape apoptosis susceptibility.

Late-Stage Cells: Contracting the T Cell Response through Co-Signaling Protein Expression

In this discussion, an updated Tide Model of Immunity has been applied to understand the temporal regulation of propriocidal T cell death mechanisms that maintain immune homeostasis. Following pathogen clearance, two cooperative pathways and mechanisms contribute to the contraction of the T cell pool to prevent autoimmunity and immunopathology of the host. Canonically, a proportion of activated T cells undergo RICD at the peak of the response to prevent excessive accumulation of effector T cells, while

CWID is responsible for culling the majority of effector T cells during contraction (Fig. 3). Initial data suggested that RICD was facilitated primarily through extrinsic death receptors FAS and FASL, and that CWID was mediated by pro-apoptotic proteins such as BIM that destabilize mitochondrial membrane potential, thus leading to the release of cytochrome C, apoptosome formation, and intrinsic apoptosis (227). We now understand that RICD and CWID employ overlapping mechanisms to drive apoptosis, differing slightly in T cell lineages: CD4 T cells execute RICD primarily through FASL upregulation; CD8 T cells utilize both death receptors and intrinsic mechanisms of apoptosis (e.g. BIM) to induce RICD and set the upper limit of expansion. A critical and largely underappreciated component which separates RICD from CWID is time: as mentioned, RICD occurs first to set the limit of expansion while antigen is present, whereas CWID follows to delete the majority of remaining T cells once antigen is cleared and cytokine production wanes. Largely, the mechanisms that facilitate RICD and CWID are intrinsically dependent upon environmental conditions, particularly antigen abundance and the presence of IL-2, however, other factors also play a major role. We now appreciate that glycolysis is intrinsically tied to RICD sensitivity of late-stage effector T cells in culture through FASL; the availability of glucose and other molecular fuels dictates the primary form of metabolism utilized by the cell population, and influence their RICD sensitivity *in vivo*. In contrast, the availability of IL-2 plays a major role in determining whether cells succumb to CWID (93, 196). Though the presence of T-regulatory cells plays a major role in determining the overall amount of IL-2 in the environment (74), relative CD25 expression theoretically should have a key role in sensitizing T cells to CWID. Intriguingly, we found in this study that CD25 expression is much lower in older, late-stage effector T cells (Supp.

Fig. 2D); this highlights the idea that mechanisms may exist which facilitate RICD and CWID that are yet to be discovered. In this study, we have determined that co-signaling proteins TIM-3 and PD-1 play unique roles in driving differential RICD sensitivity in early versus late-stage effectors. We now appreciate that changes in the temporal expression or cellular localization of these molecules and others is critical in determining whether or not a T cell undergoes apoptosis.

Since its initial discovery in the 1990s, the literature regarding the functional capability of TIM-3 has been riddled with discrepancies and inconsistencies. Perhaps the most striking of these is the disagreement about whether TIM-3 is actually co-stimulatory or co-inhibitory in nature. Although proteins are often ascribed with a single function, many others carry out multiple divergent functions (i.e. pleiotropy); indeed, in the immune system, the canonical death-inducing proteins FAS and FASL (234, 235) function differently depending upon the time at which they are expressed and their environment, including non-apoptotic signaling. We have shown in this study that TIM-3 may also exhibit temporal pleiotropy: in expanding cells, TIM-3 protects burgeoning populations to prevent premature RICD; in terminally-differentiated effectors, TIM-3 promotes RICD to facilitate full contraction of the T cell pool. This work therefore helps to clarify conflicting points in the literature by demonstrating that the time of expression as well as the localization of TIM-3 are key factors in determining its function.

RICD is initiated through restimulation through the TCR; as aforementioned, we and others have shown that the strength of signal received through the TCR is a key determinant of RICD sensitivity (219, 248). It therefore follows that proteins which increase TCR signal strength should also increase T cell sensitivity to RICD. Indeed, this

was demonstrated through investigation of the cooperative function of SAP and N-TBA in the context of X-linked lymphoproliferative disease (110, 225). In this study, we have shown that TIM-3 boosts the signal strength of the TCR in late-stage effectors, with concomitant increases in FASL expression and cleavage, as well as increased levels of BIM (Fig. 6). This function of TIM-3 was tied to its expression in late-stage effectors, and occurred without ligand-binding (Figs. 7-8). In the recently published review article by Das et al., the authors proposed that the function of TIM-3 was intimately tied to its environment and ligand-availability. They suggested that in the absence of a ligand, TIM-3 increases T cell receptor signaling by stabilizing proximal TCR signaling components within the immunological synapse; in the presence of a ligand, residues within the tail of TIM-3 become phosphorylated, destabilizing proximal signaling components and leading to an overall dampened signal response (51). Overall, our work supports this theory in the case of late-stage effectors with the caveat that in our model, TIM-3 was not localized to the surface to increase proximal signaling, a finding which warrants further investigation (experimental ideas and hypotheses are included under Limitations and Future Directions). Critically, our work adds that the location of TIM-3 as well as the binding of specific ligands (CEACAM1) play a role in driving these effects on TCR signaling, which had not been included in their model. Finally, our work also demonstrates the importance of the timing at which TIM-3 is expressed as being a key driver of the signaling outcomes in T cells, and highlights the idea that co-signaling proteins may work to increase TCR signaling in late-stage cells for the express purpose of contracting the T cell pool as part of a larger homeostatic process. This work is significant in its assertion that co-signaling proteins may have different functions depending upon the environment in which they are expressed,

prompting further investigation.

We discovered in this study that PD-1 is an important protecting factor against premature RICD in newly activated cells. We also confirmed that PD-1 is primarily expressed soon after T cell activation in *in vitro* culture, with little to no expression in terminally differentiated effectors in the absence of chronic TCR stimulation. Based on this expression pattern, in the absence of PD-1, our data suggest that effector T cells reaching terminal differentiation are no longer afforded the protection from RICD that PD-1 offered during the clonal expansion phase. This temporal association clarifies mechanisms which protect and sensitize T cells to RICD at distinct points following activation, primarily in relation to TCR signal strength, metabolism, and BATF expression.

PD-1 has long been established to dampen TCR signal strength by recruiting SHP2 to the immune synapse to dephosphorylate multiple proximal signaling proteins (217). In this study, we have shown that PD-1 works in association with SHP2 to decrease TCR signal strength modestly, which we associated with protection from premature RICD. In late-stage effectors, it can be inferred that SHP2 is not functioning to the same extent due to the absence of PD-1; the TCR signal therefore reaches the threshold necessary for apoptosis commitment. This finding underscores the impact of overall TCR signal strength as a governor of RICD sensitivity at all stages post-activation. Based on this finding, we hypothesize that while the signal threshold for apoptosis commitment does not change significantly post-activation, the regulation of both TCR and co-signaling protein expression ultimately determines TCR signal strength, and, by proxy, RICD sensitivity. Indeed, other co-inhibitory proteins are expressed in newly activated cells more so than late-stage, corroborating this hypothesis (Supp. Fig. 2E).

PD-1 is also known to regulate metabolic changes in activated T cells. PD-1 signaling has been associated with increased levels of fatty acid oxidation and decreased levels of glycolysis when PD-1 signaling is present during activation (173). Though we did not investigate this in our study, we hypothesize that PD-1 may protect a proportion of cells from premature RICD through increased fatty acid oxidation, even while cells are switching to glycolysis to meet the ATP requirements of rapid division and proliferation following activation (136); in this same vein, the absence of PD-1 in late-stage cells leads to increased glycolysis, driving increased RICD sensitivity. This hypothesis was first generated by Voss et al. (249), and our work in this study at least partially supports it in our investigation of the timing of PD-1 expression following T cell activation. Further work will be needed to tie together the interwoven mechanisms of PD-1 signaling, metabolism, and RICD sensitivity at various time points of a T cell response.

BATF has largely been explored for its role as a master regulator transcription factor, working in tandem with other members of the AP-1 family to promote broad transcriptional changes following activation. Importantly, BATF is highly expressed following PD-1 ligation with PD-L1 and is undetectable in late-stage effectors (Fig. 15). This observation aligns with its description as an important transcription factor in driving early differentiation mechanisms in T cells. We have demonstrated in this work that BATF protects cells from premature RICD, an effect which may be facilitated through the collaborative action of multiple proteins working to tip the scale in favor of survival even in the face of strong restimulation. Without the presence of BATF, effector T cells may be unable to overcome strong pro-death signals, such as increased expression of pro-apoptotic mitochondrial proteins often associated with RICD in CD8⁺ T cells, including BIM (204,

226); this is strongly corroborated by the findings from Kurachi et al., in which the vast majority of BATF^{-/-} T cells succumbed to apoptosis within two to three days following viral infection (117). Collectively, our work linking transient expression of BATF and FOXP3 in newly activated cell populations provides the foundation for the concept that a complex network of transcription factors prevents premature RICD through differential transcription of pro-survival proteins, metabolic signatures, and autophagy during proliferation, an effect which is lost or attenuated at later stages of terminal differentiation when their expression wanes. This idea supports our updated Tide Model of Immunity, and implies that a potential significant driver of temporal regulation of RICD sensitivity may be differential expression of pro- and anti- apoptotic proteins – an effect facilitated in part by co-signaling proteins like PD-1.

Implications of the Tide Model: T Cell Exhaustion and Checkpoint Blockade

In the setting of a natural infection, T cell populations become activated, grow, and expand to protect the host. In situations where the pathogen is cleared, the cell numbers return to normal through the utilization of propioidicidal apoptotic processes that T cells engage, including RICD and CWID. However, chronic infections as well as tumor growth and metastasis dramatically change the immunological landscape in multiple ways (255, 265). Constant restimulation of T cells through long-term infections and growing tumor microenvironments causes cells to become “exhausted”, a term which has developed to identify subsets of T cells which persist in the face of constant antigenic restimulation but are functionally anergic (254). Exhausted cells are defined by a distinct transcriptional program which includes diminished expression of cytokines and pro-apoptotic proteins

which allow the full orchestration of an immune response to occur, as well as promote tumor lysis. Over time, an exhaustion signature has been discovered using murine models of chronic LCMV infection which allows labeling of these populations both *in vivo* and *in vitro*. Exhausted cells are characterized by their expression of multiple co-signaling proteins, including PD-1 and TIM-3; the downregulation of genes involved in processes downstream of TCR signaling and which are important for promoting key cytokine production; and the dampened expression of genes involved in migration, adhesion, and chemotaxis (255). Further investigation has revealed that this group of exhausted cells actually is composed of multiple unique gene expression signatures defining degrees of exhaustion and reversibility of this state, highlighting the plasticity of the immune system even under extreme circumstances (146, 252, 260).

Over the years, cumulative data from many groups has shown that these long-term infections and illnesses have important effects on the overall health of the immune system, and have provoked a new field of focus in immunotherapy which seeks to manipulate the exhausted state to clear pathogens and promote cancer remission. Checkpoint blockade is an immunotherapeutic cancer treatment which is characterized by the utilization of monoclonal antibodies which block co-signaling protein activation, thus bringing about a reversal of the exhausted state of tumor infiltrating lymphocytes (TILs) and promoting cancer remission. The primary focus of checkpoint blockade regimens have focused on targeting PD-1 and CTLA-4, both of which have demonstrated success in the clinic (184, 193). At the molecular level, these monoclonal antibodies target and disrupt the binding of co-signaling proteins to their binding partners, thus “releasing the brake” on TCR signal inhibition and promoting the reversal of the exhausted state. Investigational focus into

manipulating the immune system to benefit patients has blossomed in the past 20 years, but does not come without costs to those undergoing treatment. While checkpoint blockade is generally considered more mild than chemotherapy, an entirely new subset of side effects has been defined by utilizing this immunotherapeutic, termed “immune-related adverse events” (irAEs) (258). Typically, these irAEs are characterized by overactivation of the immune system with associated autoimmune-like toxicities. These side effects offer a unique insight into the molecular mechanisms that checkpoint blockade therapy activates.

An important aspect of this therapy is its means for delivery, which is typically as a bolus of monoclonal antibody into the bloodstream, without actually targeting exhausted TILs. Because we and others have now shown that the expression of co-signaling proteins TIM-3 and PD-1 are not exclusive to the exhausted phenotype, but rather, are important drivers of cell proliferation and homeostasis, utilization of this therapeutic has significant implications relating not just to exhausted cells, but to cells across a wide variety of states post-activation. We have shown in this study that both TIM-3 and PD-1 are important drivers of RICD protection in newly activated cell populations; utilizing TIM-3 and PD-1 blockade regimens (which are currently being tested as a dual therapy (201)) to target and reverse exhaustion may also inappropriately target expanding T cell populations regardless of antigen specificity. We hypothesize T cells undergo 1 of 3 fates during checkpoint blockade therapy (with potentially some overlap of function) (Fig. 17):

Fate 1. Proportions of newly activated populations – which may have gone on to become TILs – undergo inappropriate RICD during polyclonal expansion under this therapeutic regimen.

Fate 2. T cells which survived premature RICD become hyperactivated cells which engage in immunopathologic destruction to the host.

Fate 3. Some engage in tumor cell lysis, promoting cancer remission; these also work alongside cells which were “exhausted” – i.e., the original targets of checkpoint blockade therapy.

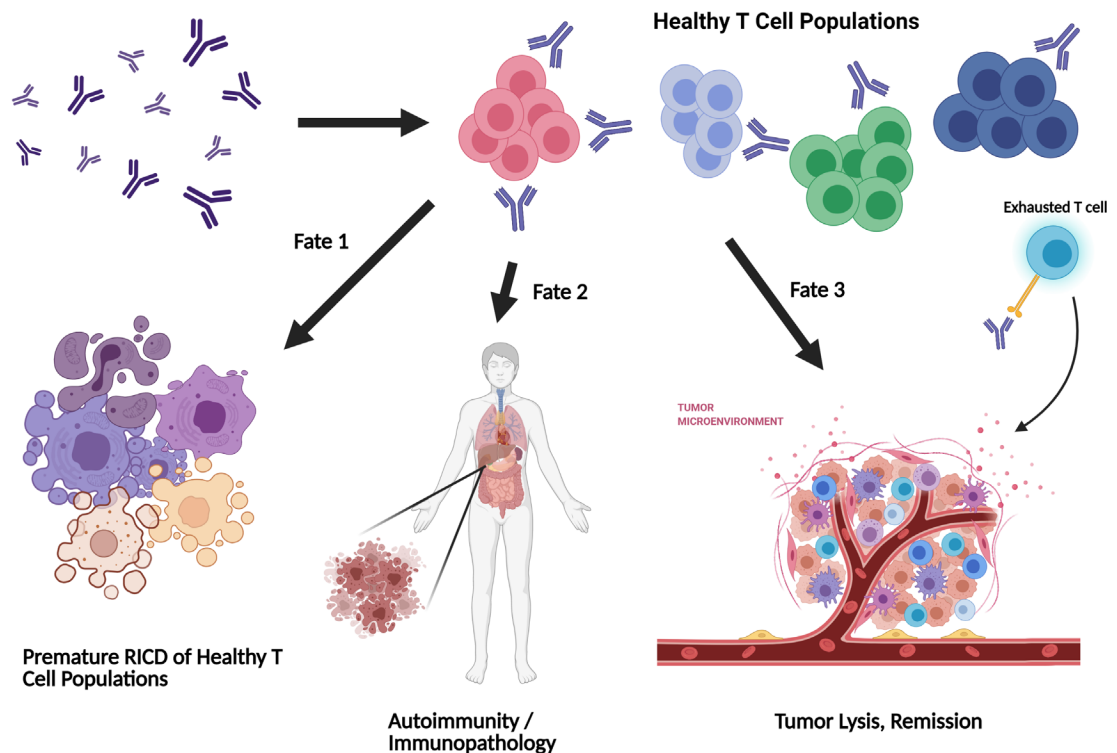


Figure 17: Checkpoint blockade affects T cell populations indiscriminately and likely increases RICD sensitivity of newly activated T cell populations.

In regards to the first fate, premature RICD could result in functional clonal deletion of T cells with high avidity for pMHC, with a consequent reduction in overall breadth of the T cell repertoire. The adaptive immune system is built on the premise of having hundreds of millions of unique T cell receptors, increasing the chance that random T cell receptors will exactly match a specific MHC-peptide combination. If the eventual memory TCR repertoire is depleted in any way, this could compromise recall responses to certain antigens. Though we did not directly test the effects of PD-1 or TIM-3 blockade on CD4⁺ T cells, one might hypothesize that this compartment would also be affected since they also express these proteins, and thus the full breadth of an immune response – from orchestration to enacting cell lysis – might be compromised through checkpoint blockade. Indeed, this idea is corroborated by the recent discovery that while checkpoint blockade provided an overall benefit for mice with high tumor burdens through the reinvigoration of highly exhausted cells, mice with lower tumors burdens underwent clonal deletion of their CD8⁺ T cell compartment following combinatorial regimens of checkpoint blockade, facilitating tumor escape and growth (167). The authors speculated that this process involved activation-induced cell death, a process that is generally synonymous with RICD but more appropriately used for naïve T cells upon initial activation (227). Though the authors never directly tested that RICD was playing a role in their model, their findings suggest that RICD could be a driving factor behind the clonal deletion they saw following checkpoint blockade. Further work will be needed to determine if this was indeed the case, and whether large-scale clonal deletion could have lasting impacts on the immune landscape even after checkpoint blockade therapy.

In regards to the second fate, we hypothesize that cells which survive premature

RICD may ultimately be hyperactivated and engage in host immunopathology, with the resulting effects similar to autoimmunity. This is not only corroborated by the reporting of irAEs in patients undergoing checkpoint blockade therapy, but also by patients suffering from TIM-3-associated SPTCL (72). People with germline TIM-3 mutations have hyperactive CD8⁺ T cells which attack their own tissue, primarily localized to adipose tissue under the dermis. As previously described, many of these mutations render TIM-3 unable to localize to the plasma membrane, which we believe facilitates increased TCR signaling and RICD in healthy late-stage effector T cells. However, we posit the loss of surface TIM-3 expression during clonal expansion, likely due to abrogated CEACAM1 interaction, could drive CD8⁺ T cells to become hyperactivated and indiscriminately attack healthy tissue, resulting in SPTCL or similar phenotypes (e.g. hemophagocytic lymphohistiocytosis (124)). Though an increased susceptibility to infections was not reported by the authors, it would be interesting to determine the clonal diversity of SPTCL clones relative to the bulk CD8 T cell pool in these patients. We hypothesize that they may have a restricted clonal repertoire, with select clones expanding in the population and driving host immunopathology in specific tissues (e.g. subcutaneous fat), though this remains to be further tested.

The third fate, involving release from exhaustion to drive target cell lysis in the tumor microenvironment or eradicate a chronic infection, is the aim of checkpoint blockade. Even this pathway, however, warrants serious consideration for revision of checkpoint blockade. Emerging evidence suggests that only specific populations of exhausted T cells are reinvigorated by PD-1 checkpoint blockade, such as PD-1⁺ TIM-3⁻ cells marked by TCF1 expression that retain stem cell-like properties (94). These

populations, despite having similar phenotypes, display distinct epigenetic and transcriptional profiles (41, 144). Indeed, even among these “progenitor” or “stem-like” exhausted CD8⁺ T cells, varying degrees of PD-1 and CXCR5 expression dictate differential sensitivity to checkpoint blockade (144). The expression of a multitude of co-inhibitory proteins – including TIM-3 – promotes “severe” exhaustion, and is characteristic of terminally-differentiated T cells destined to die, perhaps by RICD – although the relative ratio of surface versus intracellular TIM-3 expression remains to be elucidated (144). By contrast, cells which have not reached an exhaustion threshold may succumb to apoptosis, as shown by Pai et al. (167), leading to increased tumor evasion and metastasis. In addition, a study published by Kurtulus et al. in 2019 demonstrated that even in the absence of PD-1 expression, TILs of patients who have undergone anti-PD-1/TIM-3 therapy have dramatically different transcriptional profiles post-therapy, demonstrating that checkpoint blockade affects “bystander” cell populations (119). Indeed, not only are the transcriptional profiles affected, but the overall clonality of TILs is affected: Yost et al. demonstrated in 2019 that TILs entering the tumor post checkpoint blockade therapy are clonally distinct from those that occupy the tumor microenvironment prior to therapy (264). These results collectively suggest that checkpoint blockade therapy dramatically changes T cells and the tumor microenvironment, and support the necessitation of modifying our current approach to checkpoint blockade, given that only a small proportion of all circulating T cells are susceptible to reinvigoration (144). Although these therapies have demonstrated remarkable success in driving remission of specific tumors in a subset of patients, consequential side effects of checkpoint blockade (e.g. autoimmunity, potential clonal deletion, compromised nascent T cell responses), arguably necessitate a serious revision of

our approach to checkpoint blockade therapy.

Looking Ahead: Revising Our Approach to Checkpoint Blockade

Based on our understanding of how PD-1 and TIM-3 influence the key homeostatic process of RICD, we hypothesize that taking a more targeted approach to checkpoint blockade would greatly benefit patients; at best, helping prevent clonal deletion of nascent T cell populations, and at the very least reducing some of the unwanted side effects of autoimmunity. In order to more specifically target exhausted cells, patients could undergo apheresis, exhausted cells could be selected from their white blood cell population, and the rest of the cells returned. (It is worth noting that CD103⁺ TILs – which are a positive prognostic indicator in patients with esophageal squamous cell carcinoma (42) – have been found in increased numbers outside the tumor microenvironment, indicating that distinct TIL clones can be found in recirculation. Additionally, Rahir et al. assessed that increased tumor vasculature was associated with better clinical outcomes, specifically due to the increased infiltration of CD8⁺ T cells (189). With this information in mind, methods which target TILs remaining in the tumor microenvironment should be explored.) These exhausted cells could be separated specifically through their expression of PD-1 and TIM-3 (or other co-signaling proteins) – thus securing the population needed while simultaneously ensuring that these cells will more susceptible to checkpoint blockade therapy. Modified versions of this type of procedure have been done, with notable success (33, 212). Checkpoint blockade could be performed *in vitro* and these reinvigorated cells returned to the patients, preventing healthy cells from experiencing any form of blockade at any point. This modified version of checkpoint blockade therapy would have the benefits

of specifically targeting exhausted cells, bypassing healthy populations, and ensuring that premature RICD is avoided. In addition, monoclonal antibody therapies are currently delivered systemically via direct intravenous injection into patients; this therapeutic approach could also be modified through direct injection into solid tumors, if the situation warranted.

Limitations & Future Directions

In this work, we have provided new insights into the mechanisms underlying RICD sensitivity in effector T lymphocytes, including the influence of time. Importantly, our data have also helped explain some of the conflicting literature regarding the full function of TIM-3. This study, however, has limitations which should be taken into consideration when interpreting our results.

Limitation 1: Drawing Conclusions from a Polyclonal Population of Bulk CD8⁺ T Cells.

In order to procure sufficient cell numbers from human donor blood, our experiments relied on the use of bulk CD8⁺ T cell populations, which are composed of naïve, effector, and memory cell subsets. While we do not believe this a major limitation of the study – as the trends in the data were generally the same for the majority of the experiments we ran – it is clearly a consideration in interpreting the results.

Indeed, we did notice that some cell populations derived from different donors were more sensitive to RICD than others at baseline (i.e., without manipulation of co-signaling proteins) – this could be due to the donor cell population diversity. Naïve and central memory T cell subsets are relatively resistant to RICD in comparison to effector T cells, and CD8⁺ T cell subsets express varying levels of co-inhibitory proteins, as aforementioned (11, 67). *Therefore, a clear next experimental step would be to purify these various populations from individual donors and determine if effectors derived from these subsets are differentially sensitive to RICD following co-signaling protein manipulation or blockade.*

Limitation 2: Lack of Insight in an *In Vivo* Setting

Another obvious limitation to this study is our exclusive use of *in vitro* experiments. While we were able to effectively culture and manipulate primary human T cells from many donors for numerous experiments, we never translated our findings to an *in vivo* system. Indeed, the entirety of this work was performed in optimal *in vitro* conditions, using only purified CD8⁺ T cells. The clear limitation here is that all cellular dynamics – whether in normal tissues and/or a tumor microenvironment – are lost. As an example, in our system, we provide a single dose of restimulating antibody to a diverse polyclonal population of CD8⁺ T cells pre-treated for one hour with blocking monoclonal antibodies. In the human body, these blocking monoclonal antibodies potentially target many cells that express the targets of these antibodies, such as tumor epithelial cells that express PD-L1 (38)). One question therefore pertains to the bioavailability of these monoclonal antibodies in the human body, and the influence that differential binding of these antibodies has on RICD. We have demonstrated that the PD-1/PD-L1 signaling axis is important while isolated T cells are being restimulated. But what happens when ligands for other co-signaling proteins are present on different surrounding cells, and in various amounts? Is there a threshold for blocking PD-1 and TIM-3 interactions in lymphoid tissues or tumors that impact RICD sensitivity? What other tumor-derived signals or metabolites might work to dampen or exacerbate RICD in the presence of checkpoint blockade? Many of these questions can only be answered by extending our experimental approaches to *in vivo* murine models. Furthermore, although our work demonstrates that while TIM-3 and PD-1 clearly influence RICD sensitivity, the magnitude of the difference in RICD sensitivity is

relatively small (~10-20% change with manipulation of these proteins). Does this small amount of increased sensitivity have an actual impact on the overall ability of the immune system to kill tumor cells? While initial studies of human T cells must arguably be conducted in controlled *in vitro* culture conditions, a clear next step would be to understand how our findings translate to the *in vivo* setting. This could be achieved by utilizing sophisticated murine models, including relevant infections (e.g. LCMV) and TCR transgenic mice where antigen specificity is pre-defined. Alternatively, there may be opportunities for exciting *ex vivo* experiments utilizing tumor biopsy tissues or 3-dimensional organoids derived from real patients with or without checkpoint blockade therapy, in which TIL survival and function can be interrogated after polyclonal restimulation.

Future Directions: Outstanding Questions

1. How does intracellular TIM-3 promote strong TCR signaling in late-stage effectors?

We hypothesize that intracellular TIM-3, likely decorating the membrane of the Golgi or endoplasmic reticulum, is recruited to the immunological synapse following restimulation in late-stage effector T cells, but never traverses the plasma membrane (to be available for surface staining). To test this hypothesis, we propose performing confocal microscopy, co-staining for TIM-3 and CD3/LCK/ZAP-70 (or other members recruited to the synapse), in the presence and absence of TCR restimulation. To determine if TIM-3 associates with or directly promotes enhanced recruitment and phosphorylation of proximal signaling proteins, we propose performing co-immunoprecipitations (co-IPs) of

TIM-3 from early versus late-stage effector T cells, perhaps further refined by cellular fractionation or acid stripping of membrane proteins to isolate surface versus intracellular pools of TIM-3. In addition to probing for CEACAM1 and proximal signaling molecules like BAT3 and LCK, we would also take an unbiased approach such as mass-spectrometry to identify the proteins that are being pulled down with TIM-3. To determine which interacting proteins may be particularly critical for influencing the TCR signal, we could specifically silence or pharmacologically inhibit individual targets in late-stage effectors in the setting of TIM-3 manipulation (knockdown or blockade).

2. Is CEACAM1 acting as a chaperone to bring TIM-3 to the surface?

To test this question experimentally, we propose first determining the localization of TIM-3 and CEACAM1 in newly activated cells through confocal microscopy. We hypothesize that they will co-localize in newly activated cells pre- and post- restimulation. Apparent co-localization via microscopy is only a suggestive correlation, however, and therefore we propose including the following experiments to define this interaction more mechanistically and with more certainty.

To fully define their interactions, TIM-3 and CEACAM1 binding must be disrupted before the effect on RICD can be assessed in early-stage effector T cells. Indeed, we have begun to probe this question using the biochemical modeling software ChimeraX (Supp. Fig. 3). We found that SPTCL patient-derived I97M and Y82C mutations in TIM-3 (72) affect the putative binding site for CEACAM1, and prevent the surface localization of TIM-3 upon co-expression of CEACAM1 in Jurkat T cells (Supp. Fig. 3). We propose extending these studies to primary T cells by creating lentiviral constructs which harbor

wild type or mutant forms of TIM-3 fused to GFP, and testing their cellular localization in a CEACAM-dependent manner. Alternatively, unlabeled TIM-3 could be ectopically expressed after removal of endogenous TIM-3 via CRISPR/Cas9 RNP-based editing or silencing via an siRNA targeting the 3' untranslated region. If our preliminary results with these mutant constructs were verified, we would then determine the RICD sensitivity of newly activated primary cells following expression of mutant and WT TIM-3 constructs. We hypothesize that cells expressing the mutant forms of TIM-3 would have increased RICD sensitivity in comparison to cells expressing the wild type construct of TIM-3, due to a loss of TIM-3/CEACAM1 heterodimer expression *in cis* on the plasma membrane.

3. How does TIM-3/CEACAM1 signaling protect newly activated cells from premature RICD?

Though we assessed (albeit preliminarily) the pattern of signaling in newly activated cells following TIM-3 siRNA-mediated silencing (data not shown), further exploration should include probing the effects of CEACAM1 on signaling in newly activated cells. CEACAM1-4L (the predominant isoform in T cells (81)) harbors two ITIM motifs in its tail which have been implicated in SHP1 recruitment and dephosphorylation of ZAP-70 (39). We therefore hypothesize that binding of TIM-3 and CEACAM1 in newly activated cells protects them from premature RICD via recruitment of SHP1 to the tail of CEACAM1 and subsequent dephosphorylation of proximal signaling proteins. To test this experimentally, we can inhibit SHP1 through pharmacological intervention and assess RICD in the presence or absence of CEACAM-1 silencing or blockade.

4. What genes are important and involved in BATF-mediated protection from RICD in newly activated cells?

To answer this question in a non-biased way, we propose deleting BATF in newly-activated T cells and performing bulk RNA-sequencing, with or without TCR restimulation as separate comparison groups. Our laboratory recently optimized gRNA-loaded CRISPR/Cas9-based deletion of target genes, including BATF, in primary T cells using ribonucleoprotein nucleofection. Because of the influence of BATF on a number of key cellular processes (117), we propose looking both at individual genes which are differentially up- or down- regulated following BATF deletion, as well as larger signaling pathways revealed by gene-set enrichment analyses and/or Ingenuity Pathway Analysis (97). Individual genes of interest (for example, if they are directly related to apoptosis, such as BCL-2 family members) could be experimentally verified through manipulation (siRNA knockdown) and testing RICD sensitivity. To connect these targets with BATF, dual manipulation experiments could be performed in the setting of RICD assessment (i.e., if the protein was upregulated in the presence of BATF, “rescuing” the cells from apoptosis through the overexpression of the target gene could be tested in BATF knockout cells).

Because we and others have shown that there is increased BATF expression following PD-1 signaling (Fig. 15D), another insightful experiment would be in the comparison of the transcriptional profiles of PD-1 versus BATF knockdown cells. The impetus for this analysis would be in finding overlapping genes or pathways which might influence RICD sensitivity. The vast majority of studies involving transcriptional analysis of T cells following PD-1 manipulation have been performed in mice; however, some have been done in humans, and could serve as the platform for the initial analyses (188). A more

thorough and controlled experiment would be a repetition of these analyses (PD-1 silencing or blockade in CD8⁺ T cells) alongside BATF deletion.

Chapter 5: Conclusion

We have now shown that co-inhibitory proteins TIM-3 (with CEACAM1) and PD-1 work directly following activation – albeit in distinct ways – to reduce RICD sensitivity and provide a measure of protection to expanding T cells. In addition, late-stage effector T cells must be constrained in order to prevent autoimmunity and associated immunopathology of the host; we have demonstrated that PD-1 is no longer present during this time point to inhibit T cell responses, and TIM-3 works intracellularly to promote T cell signaling, the cumulative effect of which is the promotion of RICD sensitivity in late-stage cells. We believe that these findings may be part of a larger orchestration of co-signaling protein expression dynamics, which act to collectively regulate RICD sensitivity at various stages of the T cell response post-activation.

Interfering with the natural homeostatic processes of the immune system has consequences, which may take the form of host tissue destruction and associated autoimmunity. We hypothesize that immune imbalances experienced by patients on checkpoint blockade therapy may involve the inadvertent targeting of nascent cell populations, promoting increased RICD of expanding effector T cell populations and/or selecting for hyper-stimulated clones that cause collateral damage to the host. This hypothesis is supported not only by the irAEs that patients have reported (185), but also by the recently reported evidence that the proportion of T cells which are functionally exhausted are a distinct lineage separate from effector and memory T cells, rather than a collective attribute of cell populations when exposed to chronic infection (61, 214). This premature RICD of healthy populations has important implications for overall immune system dynamics and the health of the immune system post-checkpoint blockade.

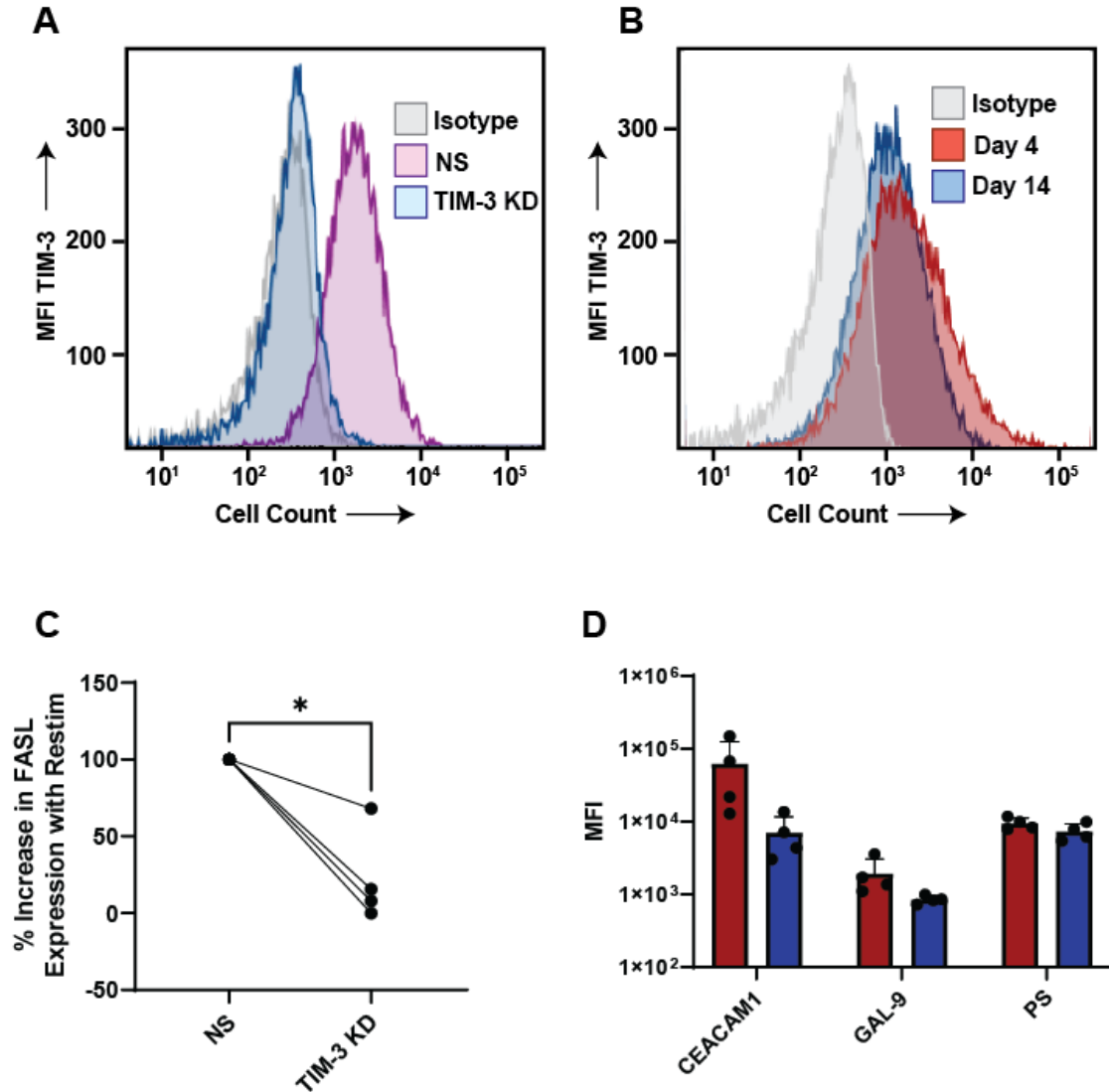
The Tide Model of Immunity – which we updated to include the critical homeostatic process of RICD – warrants further investigation, particularly in light of the recent utilization of checkpoint blockade therapy to promote cancer remission. Most particularly, our model was developed through our interpretation of immune dynamics in culture, which has significant limitations when compared to the totality of interactions *in vivo*, and most especially the differences that the setting of chronic infections introduces. We believe that this updated version of the Tide Model of Immunity – while limited – does provide the foundation for understanding how co-signaling proteins affect multiple facets of the immune-regulatory network, and highlights the unique and diverse functions of co-signaling proteins. Future work will be needed to fill in more details about the tide model that are currently not well explained, including the full effects of TIM-3 binding to different ligands in different cellular compartments following activation, the functional scope of PD-1 activity beyond the tumor microenvironment, and the contribution of other co-signaling proteins to RICD sensitivity following T cell activation.

Chapter 6: Appendices

Supplemental Table 1: Primer and Oligo Sequences Used in this Study

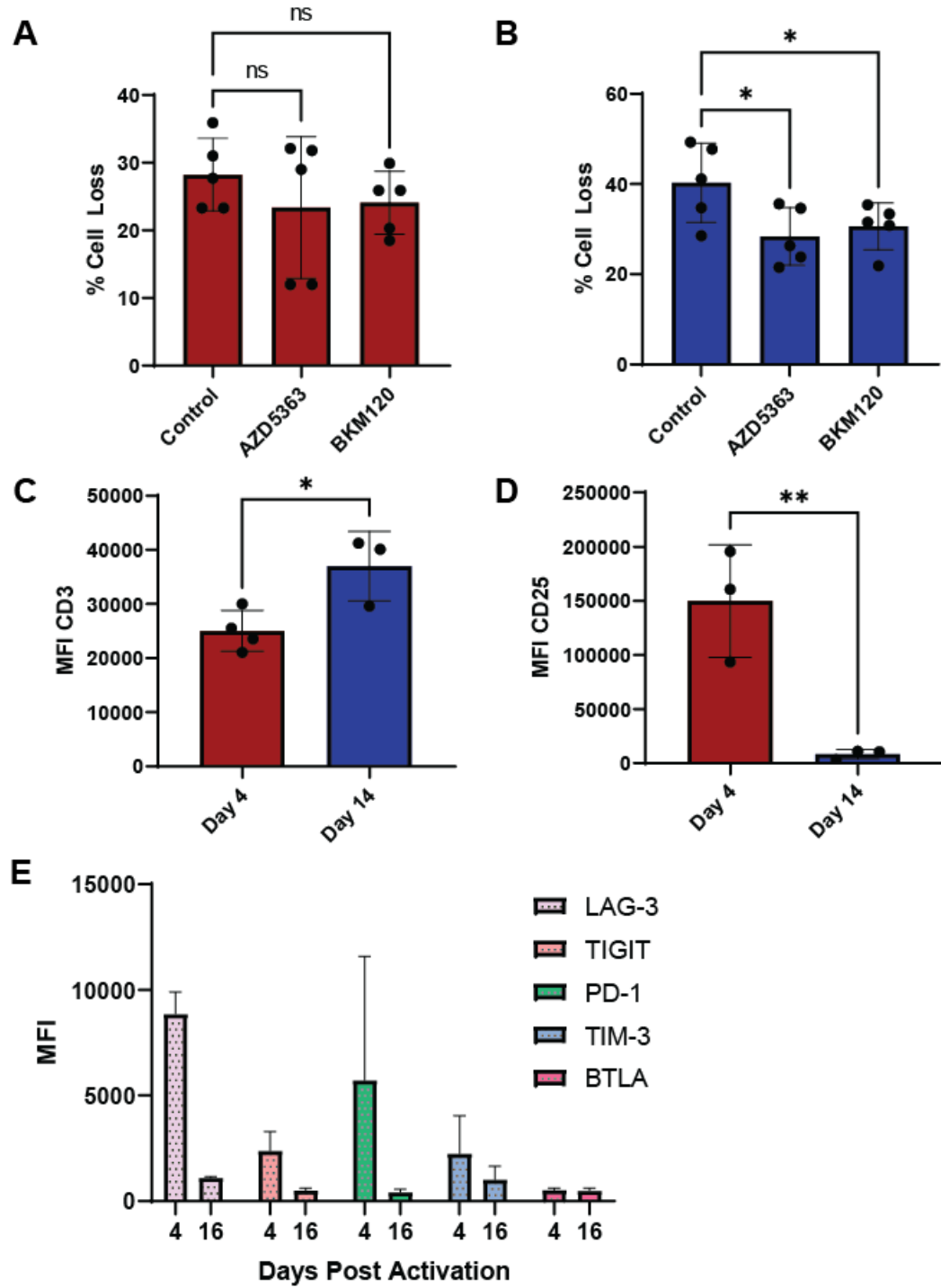
Supplementary Table 1: Primer and Oligo Sequences Used in this Study				
Target	Use	Primer	Sequence (5'-3')	Reference
TIM-3	qPCR	Forward	GGAGCCTGTCCTGTGTTTGA	NCBI Primer BLAST
TIM-3	qPCR	Reverse	AGGGACACATCTCCTTTGCG	NCBI Primer BLAST
18s	qPCR	Forward	ACCCGTTGAACCCCATTCGTGA	NCBI Primer BLAST
18s	qPCR	Reverse	GCCTCACTAAACCATCCAATCGG	NCBI Primer BLAST
CEACAM1-4L	PCR	Forward	GCAACAGGACCACAGTCAAGACGA	Singer et al., 2002
CEACAM1-4L	PCR	Reverse	GTGGTTGGAGACTGAGGGTTTG	Singer et al., 2002
CEACAM1-4L	PCR	Forward	CACTTCACAGAGTGCCTGTA	NCBI Primer BLAST
CEACAM1-4L	siRNA knockdown	Sense	ACUGAAUCCAUGCCAUUCAUGUTG	IDT siRNA Design
CEACAM1-4L	siRNA knockdown	Antisense	CAACAUUGAAUGGCAUGGAUUCAGUAG	IDT siRNA Design
PD-1	qPCR	Forward	AAGGCGCAGATCAAAGAGAGCC	NCBI Primer BLAST
PD-1	qPCR	Reverse	CAACCACCAGGGTTTGGAAGT	NCBI Primer BLAST
BATF	qPCR	Forward	GATGTGAGAAGAGTTCAGAGGAG	NCBI Primer BLAST
BATF	qPCR	Reverse	GTTTCTCCAGGTCTTCGCTCTC	NCBI Primer BLAST

Supplemental Figure 1



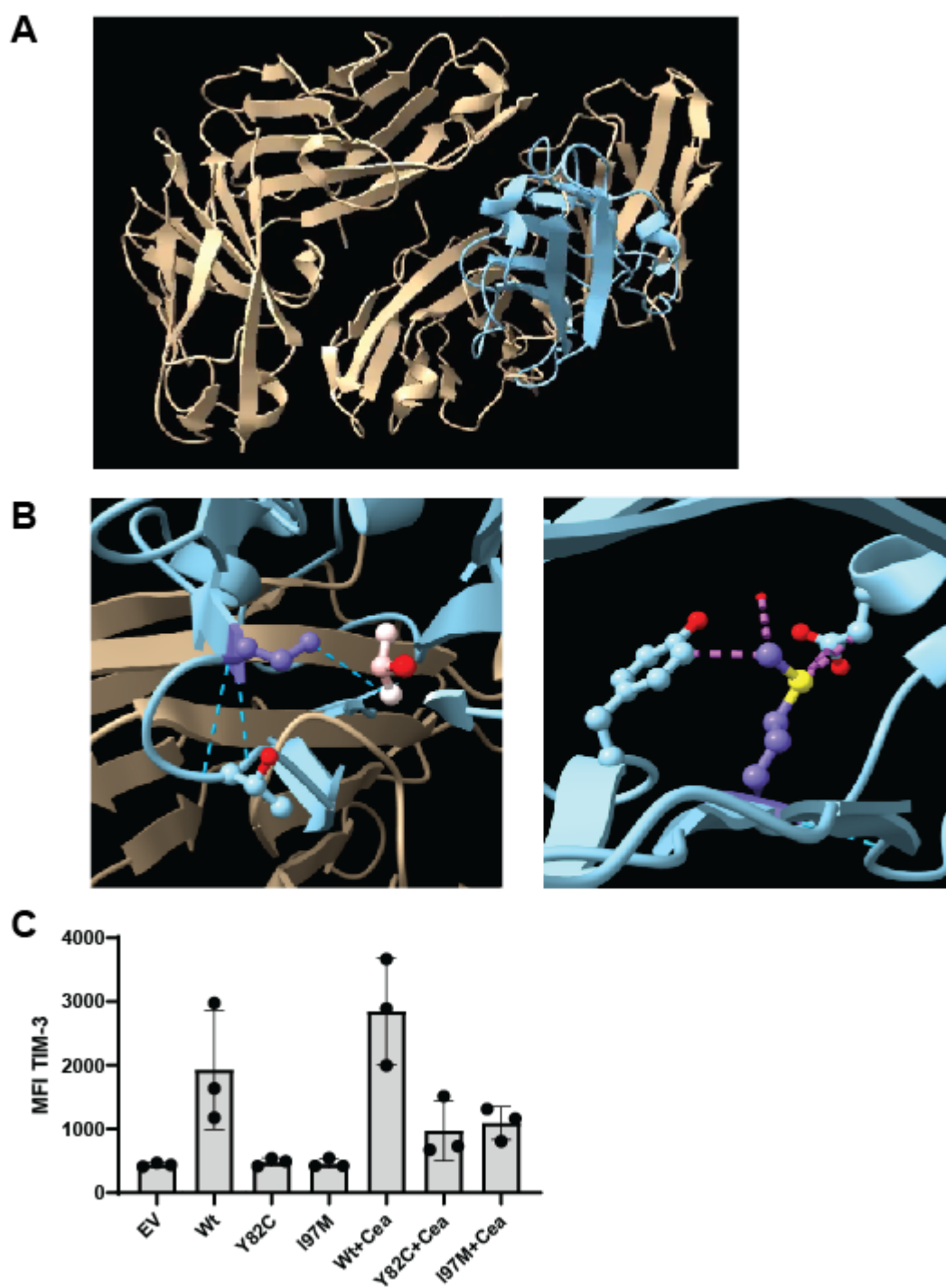
(A) CD8⁺ T cells were assessed for TIM-3 knockdown efficiency following siRNA transfection. Representative histogram displays single donor TIM-3 MFI late-stage. (B) CD8⁺ T cells were assessed for TIM-3 expression on Day 4 and Day 14 post-activation by flow cytometry. Representative histogram overlay of a single donor with both time points. (C) Late-stage effector T cells were transfected with TIM-3-specific siRNA (TIM-3 KD) or nonspecific siRNA control (NS) and restimulated for 4 hours with 100 ng/mL OKT3. Cells were assessed for FASL expression by flow cytometry, and % increase in FASL MFI from baseline was calculated per donor (represented by connecting lines). (D) CD8⁺ T cells were assessed on Day 4 (red) and Day 14 (blue) for TIM-3 ligand expression by flow cytometry; the MFI was quantified and plotted. A total of 4 donors were tested for each ligand.

Supplemental Figure 2



(A) Newly activated (day 4) or **(B)** late-stage (day 14) effector CD8⁺ cells were treated with 1 μ M AKT inhibitor AZD5363, 1 μ M PI-3K inhibitor BKM120, or DMSO solvent control for 1 hour prior to restimulation with 100 ng/mL OKT3, followed by RICD assessment 24 hours later using propidium iodide staining and flow cytometry. **(C)** Effector T cells were assessed for CD3 surface expression by flow cytometry on days 4 and 14 post-activation. Points represent individual donors. **(D)** Effector T cells were assessed for CD25 surface expression by flow cytometry on days 4 and 14 post-activation. Points represent individual donors. **(E)** Effector T cells from 3 donors were assessed early (day 4) versus late (day 16) for expression of co-signaling proteins LAG-3, TIGIT, PD-1, TIM-3, and BTLA. Statistical tests: One-way repeated measures ANOVA with multiple comparisons (corrected): (A) control versus AZD5363, $p=0.2482$; control versus BKM120, $p=0.3649$. (B) Control versus AZD5363, $p=0.0387$; control versus BKM120, $p=0.0394$; asterisks denote statistical significance using unpaired t-tests: (C) $p=0.0262$. (D) $p=0.0093$.

Supplemental Figure 3



Molecular modeling was performed using the protein modeling software program ChimeraX. TIM-3 and CEACAM1 interactions were modeled using this software program; above, TIM-3 in aqua and CEACAM1 in beige **(A)**. **(B)** Left, simulating a mutation in TIM-3, I97M, led to creation of new intramolecular H-bonds within TIM-3 itself. Right, simulating the mutation Y82C in TIM-3 led to new intramolecular clashes with neighboring residues within TIM-3. Collectively, these mutations led to misfolding of TIM-3. **(C)** Mutants constructs were created which were ectopically expressed in Jurkat T cells; see below for detailed key for proteins expressed above. Expression of mutant TIM-3 constructs both demonstrated lack of TIM-3 surface expression both with and without co-expression of CEACAM1. EV: empty vector; Wt: wild-type TIM-3; Y82C: mutant TIM-3 construct; I97M: mutant TIM-3 construct.

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