



The Fourth Intracellular Src Homology 3 Binding Motif of CD2 is Required for Durable T Cell Activity

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The Fourth Intracellular Src Homology 3 Binding Motif of CD2 is Required for Durable T Cell
Activity

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A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

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Abstract

Recent research from both the bench and clinic have demonstrated the importance of the CD2/CD58 axis in the effectiveness of T cells in diseases where chronic antigen exposure is common. Despite this understanding, the specific mechanisms by which CD2 exerts its effects is uncertain. This study was undertaken to determine the role of specific intracellular polyproline rich motifs of CD2 in T cell signaling and activation after primary challenge as well as activity after multiple rounds of antigen stimulation. Like other costimulatory receptors, CD2 exists in two distinct locations in the Immune Synapse, a structure that is formed in the area of contact between a T cell and an APC. CD2 can be found in the central portion, the cSMAC, where it is believed to be most heavily involved in signal transduction, as well as a peripheral zone referred to as the corolla, where it binds to the cytoskeleton. CD2 contains 4 intracellular polyproline motifs that act as ligands for SH3 domain containing proteins. To study the function of the polyproline motifs, CD2 constructs were designed to disrupt three of these motifs, the first, second and fourth, which have known binding partners involved in signal transduction and interaction with the cytoskeleton. Mutations to motifs 1 and 2 resulted in significant decreases in the ability to bind the Src family kinase Lck, reduced signal transduction downstream of the TCR as well as reducing the activation of the transcription factor NFAT. Mutations to motif 4 had little to no effect in any of these assays. However, the main goal of this study was to determine mechanisms of resistance to exhaustion, so a repeat challenge assay was used to evaluate the effects of the mutants

in a model of chronic antigen exposure. In this assay, motif 4 was critical for maintaining the ability to kill target cells, motifs 1 and 2 were largely dispensable. This suggests that changes to the T cell during a model of chronic antigen exposure increase reliance on motif 4, not motif 1 or 2, which are more heavily involved in signal transduction after initial exposure to antigen. The exact mechanism of this dependency still needs to be determined.

Dedication

This thesis is dedicated to my wife Katherine Corcoran and son Dylan Corcoran. Their constant love and support throughout the past 5 years acted as an emotional anchor in the often-chaotic life of a working parent working towards my degree. Kate, your encouragement and help during the many long days means more to me than you know. Dylan, your willingness and openness to hard work (and fun!) motivated me after many a setback or especially difficult challenge. There is no way I could have come close to finishing without both of you. I hope I can repay their love and support in the same way!

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I would like to thank my advisors Hui Qin Wang and Brian Granda. Their perspectives and guidance helped make this work possible, both challenging and supporting ideas to make them the best possible.

Many of my colleagues at Novartis provided essential guidance during the process of establishing the assays for this work. Melissa Ramones and John Blankenship helped with the generation of the CD2 mutant panel. Mark Ng was invaluable in helping to design CRISPR gRNA and assistance with lentiviral production. Lipi Mukerjee helped with the repeat challenge assay, as well as more general support than she realizes. And I cannot overstate my appreciation to Giri Nathan of the Flow Cytometry core for his advice and teaching of the intricacies of cell sorting and panel creation.

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Chapter I.

Introduction

The immune system has evolved to protect the host from external pathogens and to eliminate damaged or diseased cells and is essential for optimal health. The immune system consists of two main branches, the innate and adaptive immune systems. Cells of the innate immune system such as macrophages and neutrophils are the front line of defense, using non-specific receptors to scan for products from pathogens, such as lipopolysaccharide (LPS) from gram negative bacteria or double stranded RNA (dsRNA) from rotavirus. Binding of these products to their receptors activates the cell, starting a suite of molecular and genetic changes that ultimately lead to the activated cell moving to secondary lymphoid organs such as the lymph nodes or spleen. There, the innate immune cells present protein fragments of the pathogen, called antigen, to cells of the adaptive immune system in a specialized structure called the Major Histocompatibility Complex (MHC) (Jain, 2017).

The adaptive immune system is composed of B cells and $\alpha\beta$ T cells, each of which present specialized receptors on their cell surface that bind non-self antigens. The receptors for both B cells and $\alpha\beta$ T cells are the products of somatic VDJ gene recombination, which generates greater than 10^9 possible protein sequences, giving T and B cells the ability to bind a large repertoire of antigens (Hwang, 2020). Each T cell bears a single version of this specialized receptor, the T cell receptor (TCR), which specifically binds to a non-native polypeptide antigen only when displayed in a MHC from that

individual (Linterman, 2014). The TCR α and β form a complex with the CD3 $\epsilon/\beta/\gamma/\delta/\zeta$ subunits which are required for transducing downstream signaling (Hwang, 2020)

The interactions between T cells and APCs in the secondary lymph organs is a dynamic process with T cells actively scanning APCs for MHC:peptide complex (pMHC) that match its TCR.

Immune Synapse

When a T cell binds to an APC bearing its cognate antigen, several events occur in both the T cell and APC that leads to the formation of a structure called the immune synapse (IS).

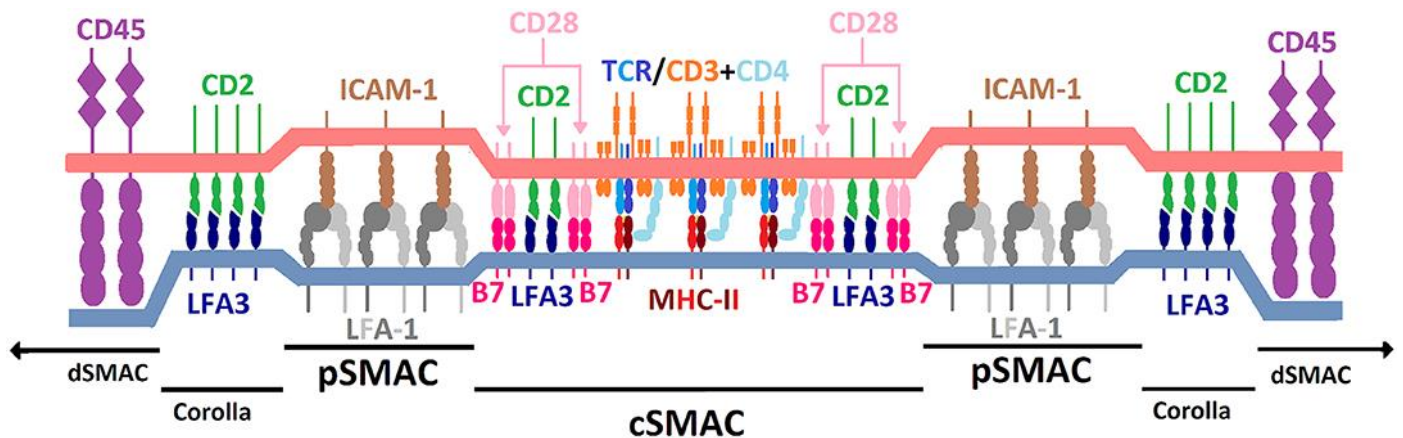


Figure 1. Structure of the mature immune synapse.

Note that CD2 can be found in both the cSMAC as well as the corolla. From “CD2 Immunobiology”, by Binder C, Cvetkovski F, Sellberg F, Berg S, Paternina Visbal H, Sachs DH, Berglund E, Berglund, Front Immunol. 2020 Jun 9

The IS is a complex and well-organized macromolecular complex that controls the interaction between the two cells and activation of the T cell. Also called the supramolecular activation complex, or SMAC, a mature SMAC is composed of a central core containing multiple TCR:pMHC complexes and costimulatory receptors, a surrounding distal zone (dSMAC) primarily composed of adhesion molecules and finally the peripheral zone (pSMAC) composed of dual function adhesion and costimulatory proteins (Figure 1; Binder, 2020; Demetriou, 2020).

Immediately after a TCR binds to a pMHC with sufficient affinity, a non-covalent bond occurs between the cells, with a slow off-rate, prolonging the contact time between the two cells beyond the normal short duration observed in non-specific interactions. This is the first step in the formation of the IS. Shortly after TCR:pMHC binding, the adhesion molecule LFA-1 on the T cell binds to its counterpart ICAM-1 on the APC, further stabilizing the interaction between the T cell and APC. While ICAM-1 and LFA-1 are binding, the co-receptors CD4 or CD8 on the T cell binds to the MHC on the APC at a secondary site outside of the antigen presentation site of MHC. In resting T cells, CD4 and CD8 non-covalently binds to members of the Src family of kinases, primarily Lck and Fyn (Skanland, 2014; Binder, 2020). The binding of MHC by CD4 or CD8 leads to the reorganization of the T cell surface, separating the inhibitory phosphatase CD45 and Lck and Fyn, allowing Lck and Fyn to remain in an active state (Palacios, 2004). Activated Lck and Fyn are then able to phosphorylate key tyrosine residues in the ITAMs (immunoreceptor tyrosine-based activation motifs) of the CD3 ζ chain of the TCR complex, initiating multiple signaling cascades. The Ras/ERK1/2-AP-1, I κ B, Ca⁺², NFAT, PKC θ , α -I κ B, NF- κ B and TSC1/2-mTOR pathways have been shown to be

activated after TCR-pMHC binding, leading to profound cellular changes ranging from increased cytokine production, upregulation of adhesion and costimulatory receptors to cytoskeletal rearrangements (Hwang, 2020; Binder, 2020; Palacios, 2004; Kaizuka, 2009)

T cell Costimulation

While TCR mediated signaling through the CD3-TCR complex can induce changes to the T cell, a second stimulatory signal is needed to fully activate the T cell. The absence of a second activation signal leads to a hyporesponsive state termed anergy and eventual tolerance to the antigen. These secondary signals can occur from multiple sources, such as cytokines, but the primary mechanism is through costimulatory receptors in the IS (Skanland, 2014; Leitner, 2015). Co-stimulatory receptors fall into three main families, the IgG-like receptors, the TNFR and TIM families (Hwang, 2020). The IgG superfamily consists of 2 subfamilies, the CD28/B7 family and the CD2/CD58 family that are defined by containing extracellular Ig domains, a single transmembrane domain and a cytoplasmic domain containing signaling motifs (Leitner, 2015). Binding of the extracellular Ig-like domain to their respective ligands on the APC restricts the lateral movement of intracellular domain, facilitating binding of signaling and adapter proteins that contain SH3 domains, such as Lck, Fyn and ZAP-70. Unlike the TCR/CD3, CD2 and CD28 interact with actin binding proteins, such as Cdc42, WASp and actinin-1 which links them to the actin cytoskeleton (Zurli, 2020; Banerjee, 2007; Ballek, 2016; Comrie, 2016). This provides additional structure and organization to the nascent IS and provides a physical barrier to reduce the lateral movement of signaling and inhibitory molecules, allowing for prolonged signaling. Additionally, both CD2 and CD28 have been associated with cholesterol and sphingolipid rich cell membrane domains termed “lipid

rafts”. In addition to CD2, CD28 and other costimulatory receptors, these lipid rafts are rich in unbound Lck and Fyn, which facilitates activation of downstream signaling pathways (Binder, 2020; Kaizuka, 2009).

Binding of these costimulatory receptors to their co-receptors increases the physical bonding between the T cell and APC, adds additional stimulatory signals and links the developing IS to the cytoskeleton.

Kinetics of IS formation and maturation

These binding and signaling events cause profound changes to the immune synapse as well as the cell as a whole. At the start of T cell activation, the TCR complexes form small microclusters with little organization of adhesion and regulatory proteins. As activation proceeds, the IS is reorganized, with the TCR complex and costimulatory receptors CD2 and CD28 forming the cSMAC, adhesion molecules such as LFA-1 forming a ring-like structure around it, the dSMAC, which is then surrounded by the CD2 rich pSMAC (Binder, 2020; Demetriou, 2020). The SMAC is anchored to the actin cytoskeleton by binding of adapter proteins to intracellular domains of adhesion molecules and costimulatory receptors such as CD28 and CD2, and the distinct zones formed by the centripetal forces of actin filament formation at the leading edge of the cell pulling the attached proteins towards the center. Anchoring of the IS components to the cytoskeleton serves two purposes, (1) reducing mobility of inhibitory receptors to the cSMAC and (2) acting as scaffolds to optimize the location of intracellular signaling molecules (Binder, 2020; Comrie, 2016). Additionally, CD2 binds to and activates several proteins involved in cell polarity, aligning the MTOC and secretory pathways

towards the IS, enhancing the secretion of toxic granules containing Granzyme B and perforin, while facilitating the endocytosis of TCR complexes away from the cSMAC, modulating the main stimulatory signal (Martin-Cofresces, 2008; Zurli, 2020). Thus, formation of a mature IS is dependent upon multiple binding events between the APC and T cell, and results in a fully active T cell primed to use its full capabilities to fight infection or eliminate damaged cells.

CD28 and CD2 in disease

When the activated T cell enters the circulation to search for cells presenting the cognate antigen, effective costimulation is as necessary for proper CD8⁺ cytotoxic T cell (CTL) function as it was during activation in the secondary lymph organs. Impairment of the CD28/B7 axis, by either downregulation of expression or transcriptional silencing, has been observed in individuals with long term antigen exposure, such as cancer or long-term viral infections (Binder, 2020; Leitner, 2015; McKinney, 2015). Studies of TILs (tumor infiltrating lymphocytes) from cancer patients demonstrated pronounced defects in the ability of CTLs to kill target cells and produce cytokines such as IL-2 and IFN γ (McKinney, 2015). Leitner et al identified CD2/CD58 as the main costimulatory axis in CD28⁻ CTLs and demonstrated that stimulation of CD28⁻ CTLs with CD58 reduced the expression of the senescence marker CD57 and rescued the ability CD28⁻ CTLs to proliferate and produce IFN γ . Additionally, higher than normal CD2 expression has been correlated with autoimmune diseases such as rheumatoid arthritis and multiple sclerosis and in transplant rejection. Based on this data, a CD2 targeting monoclonal antibody, sipilizumab was developed to disrupt the CD2/CD58 axis and is currently undergoing

clinical evaluation (Binder, 2020). Taken together, this demonstrates the significance of properly regulated CD2 activity for health.

Mice lacking either CD2 or CD28 are functionally normal with no adverse phenotype, whereas double knock out mice have profound defects in T cell responses (Binder, 2020). This suggests a redundancy in the pathways activated, which is not surprising since they both contain intracellular motifs that act as binding sites for Src homology domain 3 (SH3) containing signaling proteins, such as Lck and Fyn. Outside of these shared pathways, there are distinct differences and biases between the two. Stimulation of CD28 induces signaling through multiple signaling pathways through its SH2 binding motifs. First, the GRB2/PI3K/PDK1/AKT pathway is activated, leading to upregulation of the transcription factors NFAT and NF- κ B, inducing proliferation and IL-2 secretion. Second, activation of the GRB2/Vav1/JNK pathway has been demonstrated to induce the transcription factor AP-1, which acts synergistically with NFAT to effectively activate T cell (Belizario, 2020). While activation of several pathways are shared, most notably AKT, CD2 primarily induced activation of ERK1/2, p38 MAPK and components of the TCR signaling complex LAT, VAV and ZAP70 (Skanland, 2014) through its SH3 binding motifs. These two studies demonstrate the functional differences between CD2 and CD28 signaling and the benefit of CD2 signaling in CD28⁻ T cells, but the exact mechanisms are not fully understood.

The aim of this study was to elucidate which intracellular SH3 binding domain is driving the rescue of activity of exhausted CTLs by CD2. The intracellular domain of CD2 contains 5 domains with proline rich SH3 binding motifs (PxxP or PxxxP, with x referring to any amino acid), three of which (1, 2, 4) have known interactions (figure 2).

Domains 1 and 2 bind to the SFKs Lck and Fyn as well as CD2BP2. Domain 4 binds to Lck and Fyn, as well as CD2BP1 and 3, CIN85 and CMS (Figure 2; Binder, 2020; Tran, 2005; Dustin, 1998). CD2BP 1 and 3, CIN85 and CMS are scaffolding proteins that directly link CD2 to proteins associated with the actin cytoskeleton and have different and competing effects in different cell lineages, with CMS inducing actin polymerization in T cells (Comrie, 2016; Binder, 2020). Due to the redundancy of Lck and Fyn signaling between Domains 1 and 2 with other IS signaling members, such as CD3 ζ , we hypothesized that Domain 4 is driving the rescue of activity of wild-type CD2 in CD28⁻ CTLs.

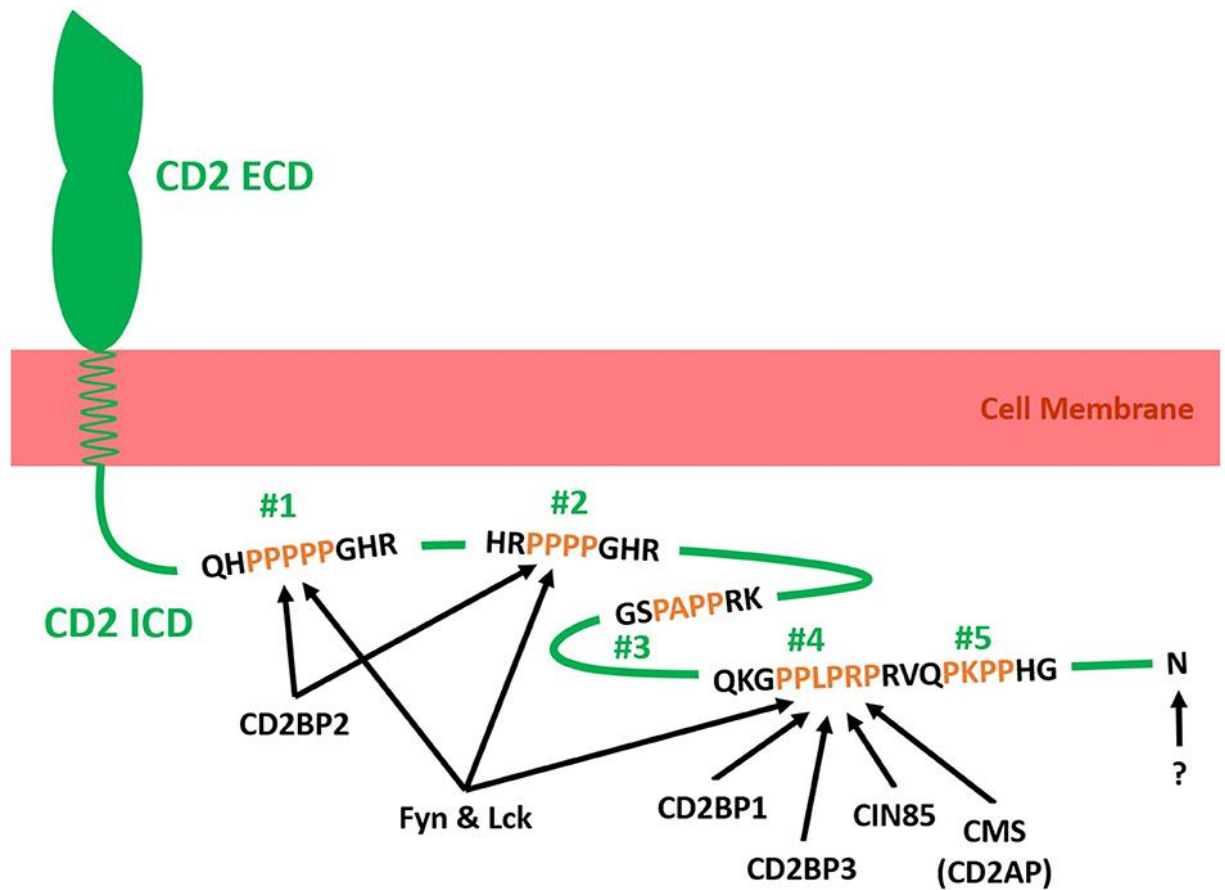


Figure 2. Intracellular motif interactions.

From “CD2 Immunobiology”, by Binder C, Cvetkovski F, Sellberg F, Berg S, Paternina Visbal H, Sachs DH, Berglund E, Berglund, Front Immunol. 2020 Jun 9

Our approach was to generate CD2 constructs with inactivating mutations based on approaches described in Dustin et al and Tran et al to the proline rich SH3 binding motifs, alone or in combination, and transduce them into CD2 knock out Jurkat or primary T cells. We compared the activity of the transduced Jurkat cells by measuring the activation after exposure to a bispecific antibody that binds to CD3ε and the cell surface

protein BCMA expressed on many multiple myeloma cancer cell lines, bypassing natural TCR:pMHC binding to act as a tool to activate T cells. Expression of a luciferase reporter driven by the transcription factor NFAT which is upregulated after T cell activation (Martelli, 2000), T cell derived cytokine production, changes in phosphorylation of a key signaling protein by phosphoflow and changes in cell function as measured by target cell killing were all measured to assess the consequence of the mutations.

While mice are the most common model for immunological studies, there exists several challenges in studying human CD2 biology in mice. First, CD58 is not expressed in mouse, murine CD2 binds exclusively to CD48, and at 100-fold lower affinity. Secondly, expression patterns differ with mice expressing CD2 primarily on B cells, rather than T cells (Binder, 2020). These differences prevent the mouse from being a relevant model system. Therefore, we decided to use in vitro models for our studies. While in vitro models do not fully capture the complexity of immune interactions in situ, they enable us to evaluate the effects of specific interactions in an easily accessible and controllable setting.

Chapter II.

Materials and Methods

The following section describes the materials and experimental methods used in this study. Briefly, lentiviral vectors were created that encoded the CD2 mutants under evaluation as well as eGFP expression and puromycin resistance genes for isolation of transduced cells. These vectors were used to create lentivirus that were used to transduce primary T cells and the Acute lymphoblastic leukemia cell line Jurkat, which was engineered to express luciferase under control of the NFAT promoter. It must be noted that the Jurkat cell line has defects of IFN γ and STAT1 signaling pathways and does not fully recapitulate the signaling of primary T cells but acts as a tool to evaluate specific aspects of CD2 biology (Boselli, 2007). Prior to transduction, the primary T cells and Jurkat cells had endogenous CD2 knocked out using CRIPSR technology. Transduced cells were sorted to capture all CD2 expressing cells, which were then put through a battery of biochemical and cell-based assays to determine the role of the intracellular polyproline motifs in T cell activity in the context of bispecific antibody mediated activation.

Lentivirus Generation

Expression vectors were designed and generated in conjunction with Gencopoeia Inc. (Genecopoeia, Rockville, MD). Customized cDNA encoding the CD2 mutants were introduced into the pReciever-Lv224 vector which has transgene and GFP and puromycin resistance selection marker gene expression driven by the EF1 α promoter, with IRES

sites between CD2 mutant, eGFP and puromycin resistance genes to ensure independent expression of each protein.

The purified plasmids were then used to generate lentivirus. Actively dividing HEK293T cells were plated at 15×10^6 cells per 10 cm tissue culture dish (Corning, Corning, NY). The following day, 2 μ g of lentiviral vector plasmid was mixed with 10 μ g of Packing Plasmid Mix (Cellecta, Mountain View, CA) in 1.02 mL Opti-MEM media (Gibco, Carlsbad, CA) and incubated for 15 minutes at room temperature. In a separate tube, 30 μ L of lipofectamine 2000 (Life Technologies, Carlsbad, CA) was added to 1 mL Opti-MEM medium and incubated for 15 minutes. After incubation of DNA and Lipofectamine, the two reagents were combined and incubated for an additional 15 minutes at room temperature. After incubation, the complexed DNA was added to the HEK293T cells in a dropwise fashion.

After 48 hours, the tissue culture supernatant, containing lentivirus, was collected and filtered through a 0.45 μ m PES filter (Corning, Corning, NY) to remove any cellular debris. Virus was concentrated by using Lenti-X concentrator (Takara Bio, San Jose, CA) at a ratio of 1 part Lenti-X to 3 parts supernatant. After addition of Lenti-X, the mixture was incubated at 4°C for 30 minutes before centrifuging in a Beckman-Coulter (Brea, CA) centrifuge for 45 minutes at 1500 x g. After centrifugation, the supernatant was removed and the viral pellet reconstituted in 50% RPIM1640 (Life Tech, Carlsbad, CA)/50% Fetal Bovine Serum at a volume of 1/10th the initial volume of cell culture supernatant before storage at -80°C.

Cells and Tissue Culture

KMS-11, KMS-11-mCherry, CD2 wild-type and CD2 KO Jurkat-NFAT-luciferase cells were received as a gift from the lab of Dr. Haihui Lu (Novartis Institute for Biomedical Research, Cambridge, MA). Cells were passaged twice weekly in RPMI1640 containing 10% FBS (Life Technologies, Carlsbad, CA), with 0.5 µg/mL puromycin added to the Jurkat-NFAT-Luc cells to ensure expression of the reporter gene.

Primary human PBMCs from anonymized donors were purchased from HemaCare (South San Francisco, CA). CD3⁺ T cells were isolated from bulk PBMCs using the pan T cell negative isolation kit (Life Technologies, Carlsbad, CA). 100 µL of a biotinylated antibody cocktail was added to 1×10^8 PBMCs in a 5 mL facs tube and incubated at RT for 15 minutes. The cells were washed once with 5 mL PBS containing 1% FBS (facs buffer) by centrifuging at 1200 rpm for 5 minutes. After centrifugation, the supernatant was aspirated off and the cell pellet resuspended in 100 µL of facs buffer before 100 µL of magnetic streptavidin beads were added. The bead:cell mixture was incubated for 15 minutes, then the volume was adjusted to 2.5 mL with additional facs buffer. The facs tube was transferred to a magnetic separator (Thermo Fisher, Carlsbad, CA) and incubated for 5 minutes to allow the beads and non-T cells to bind to the walls of the tube. The non-adherent cells containing the untouched T cells were transferred to a tissue culture flask and volume adjusted to 1×10^6 cells per mL in T cell media (RPMI1640 containing 10% FBS, 1% NEAA, 1% sodium Pyruvate; Life Technologies, Carlsbad, CA).

CD2 KO in Primary T cells

Endogenous CD2 was knocked out using spCAS9 and a dual gRNA approach using the NEON transfection system (Thermo Fisher, Carlsbad, CA). Guide RNA 1 (AAGCTGGCTACAAATTTACA) and 2 (CTGACCTGTGAGGTAATGAA), along with tracrRNA, were purchased from IDT (Coralville, IA). Upon receipt, the RNA was reconstituted in nuclease-free duplex buffer at a concentration of 100 μ M.

One day prior to electroporation of primary T cells with CD2 gRNA: tracrRNA:spCas9 RNP complexes (1:1:1 molar ratios), freshly isolated primary T cells were cultured with CD3/CD28 dynabeads (Thermo Fisher, Carlsbad, CA) at a 1:1 bead:cell ratio in TCM supplemented with 5 μ g/mL each of IL-7 and IL-15 (PeproTech, Cranbury, NJ).

gRNA:tracrRNA complexes were formed by combining 5 μ L of gRNA 1 and 2 with 10 μ L tracrRNA and heating to 95°C for 2 minutes before allowing to cool to room temperature over 5 minutes. 7.7 μ L of a 62 μ M spCas9 stock (IDT) solution and 22.7 μ L of Opti-MEM were added to the cooled complexes and incubated at room temperature for 15 minutes to form RNP complexes.

While the RNP complexes were forming, the T cells were debeaded by centrifuging the cell suspension for 5 minutes at 1500 rpm, at room temperature, followed by resuspending in 4 mL fresh TCM in a 5 mL facs tube. The tube was then placed in a magnetic separator for 5 minutes before the free T cells were decanted off. The cells were adjusted to 60×10^6 cells per mL in buffer T from the NEON kit and an equal number of fresh CD3/CD28 beads added. 100 μ L of cell suspension was added to and equal volume of RNP complex, and the cell:RNP suspension was added to the

NEON device. Electroporation was conducted for 3 x10 ms pulses at 1600 mV. The electroporated cells were then transferred to a 6-well plate (Corning, Corning, NY) containing 3 mL TCM. This process was repeated until all T cells were electroporated. T cells were cultured at 37°C for 4 days before sorting of CD2 knockouts.

On Day 4 post electroporation, the T cells were centrifuged at 1500 rpm for 5 minutes and debeaded as per the method described above. Cells were stained with live/Dead stain (Near-IR Fixable Viability Dye, Thermo Fisher, Carlsbad, CA) and anti-human CD2 antibody conjugated to V450 (Clone RPA.T10, Thermo Fisher, Carlsbad, CA) before sorting for live, CD2 negative cells on a BD FACSAria cell sorter (Becton-Dickinson, Franklin Lakes, NJ). A portion of live, CD2 positive cells were collected as well, to serve as CD2 positive controls. Sorted cells were cultured in TCM supplemented with 5 ng/mL IL-7 and 15 until lentiviral transduction.

Lentiviral transduction and Isolation of transduced cells

Both CD2KO Jurkat-NFAT-Luc and CD2 KO primary T cells were transduced to express the mutant CD2 constructs using a modified spinoculation approach. 24-well non-tissue culture treated plates (Corning, Corning NY) were coated with 150 µL of a 1 µg/mL solution of Retro-Nectin (Takara) and incubated at 4°C overnight. The following day, the retronectin was removed and the plates blocked by adding 500 µL of 2% BSA in PBS (Gibco, Brea, CA) and incubating at room temperature for 1 hour. After blocking, the BSA solution was removed and 400 µL of lentivirus stock was added, 4 wells per vector. The plates were then centrifuged at 2,000 x g for 2.5 hours at room temperature. After centrifugation, all but 100 µL of the virus stock was removed, and 1 million CD2 KO primary T cells or Jurkat CD2KO cells were added. They were then centrifuged for

30 minutes at 800 x g at 32°C to improve infection. After centrifugation, the plates were incubated at 37°C for 4 days.

4 days after spinoculation, all cells transduced with the same construct cells were pooled. T cells were then sorted for double positive eGFP and CD2 as described previously. The sorted eGFP⁺ CD2⁺ T cells were cultured in TCM + IL-7 and IL-15 for an additional 8 days, expanding with fresh TCM + IL-7 and IL-15 as needed to keep the cells between 0.5 and 1 x 10⁶ cells/mL. The CD2KO Jurkat cells were cultured in RPMI 1640 supplemented with 10% FBS and 8 µg/mL puromycin to kill non-transduced cells. Eight days later, they were sorted for live, eGFP⁺ CD2⁺ cells and passaged with twice weekly subculturing.

Lck binding assay

To determine the effects of the CD2 mutations on Lck binding we developed an ELISA based assay. Cell lysates from sorted WT, CD2 KO or transduced Jurkat-nFAT-Luc cells were made by adding 300 µL of Triton-X lysis buffer containing Protease and phosphatase inhibitors (Thermo Fisher, Carlsbad, CA) to 6 million cells. The cells were incubated at 4°C on a rotator before sonication with a probe sonicator for 3 cycles of 0.5 sec at 20 kHz, on ice. The samples were then centrifuged at 10,000 x g for 20 minutes to pellet debris. The supernatant was transferred into multiple aliquots before storage at -20°C. Protein concentration was determined by BCA assay after thawing on ice for 1 hr.

High protein binding Nunc Immulon 96-well plates (Nunc) were coated with 100 ng of either recombinant human Lck (Thermo) or anti-CD2 antibody (Clone RPA.2.10) in 100 µL PBS at room temperature for 2 hours. After incubation, the coating solution was decanted, and the plates washed 3 times with 300 µL PBS. After washing, the plates

were blocked with 300 µL of 5% BSA in PBS overnight at 4°C. The next day the BSA was aspirated, and the plates washed three times with a wash buffer consisting of PBS + 0.5% tween 20.

Samples were diluted to 1000 µg/mL and 100 µL was added to both Lck and anti-CD2 antibody coated wells. The samples were incubated for 4 hours at room temperature for 4 hours before washing 3 times with wash buffer. 100 µL of a 2 µg/mL solution of biotinylated polyclonal anti-CD2 antibody (Abcam) was then added, and incubated for 2 hours at room temperature. After another wash step, a 1:1000 dilution of streptavidin-Horseradish Peroxidase (thermo) was added and incubated for 15 minutes. After washing, 100 µL of TMBOne reagent was added and the reaction allowed to progress for 10 minutes before quenching with 100 µL 1N HCl. The plates were then read on a Spectramax (Molecular Devices,) plate reader, reporting OD₄₅₀.

Binding of Lck was calculated as a ratio of CD2 bound to Lck to total CD2 by the following formula:

$$\text{Ratio}_{\text{Lck}} = \frac{\text{Sample Lck OD}_{450} - \text{CD2KO Lck OD}_{450}}{\text{Sample CD2 OD}_{450} - \text{CD2KO CD2 OD}_{450}}$$

The CD2KO Lck and CD2 OD₄₅₀ values were used to correct for any nonspecific binding for these antibodies to yield the true binding values for each variable.

Phosphoflow

Phosphorylation of the cell signaling/adaptor protein SLP-76 was measured using a protocol derived from Skanland et als. Briefly, WT, CD2KO or CD2 mutant transduced Jurkat-nFAT-luc cells were plated in a 96-well tissue culture plate (Corning, Corning, NY) at 50,000 cells per well in RPMI1640 media and incubated for 2 hours at 37°C.

After incubation, biotinylated anti-CD3 (Biolegend, San Diego, CA) and anti-CD2 (Thermo Fisher, Carlsbad, CA) antibodies were added to give a final concentration of 1 and 5 $\mu\text{g/mL}$, respectively. Two minutes later, avidin was added to a final concentration of 50 $\mu\text{g/mL}$ and allowed to bind for 1 minute before adding 200 μL of prewarmed Phosflow Fix I solution (BD Biosciences, Franklin Lakes, NJ) to stop the reaction. After fixing for 15 minutes, the cells were washed twice with Phosflow perm wash buffer I (BD Biosciences, Franklin Lakes, NJ). Cells were then stained with APC-conjugated anti-human SPL-76 (Y128)(Thermo Fisher, Carlsbad, CA) for 30 minutes at 4°C, protected from light, before washing 3 times with Phosflow Perm Wash Buffer I. After the final wash, the cells were resuspended in 200 μL of facs buffer and stored at 4°C until analysis on a BD Fortessa LSRII flow cytometer. Results were analyzed using flowjo software (BD, Franklin Lakes, NJ), gating for the lymphocyte fraction and single cells before determining the geometric mean of the APC signal.

Jurkat-NFAT-luciferase Assay

KMS-11 cells, which express the CD2 ligand CD58, and Jurkat cells expressing CD2 WT or CD2 KO or CD2 mutants were mixed at a 1:3 ratio consisting of 10,000 KMS-11 cells to 30,000 Jurkat cells in a 96-well plate (Corning). A dose range of the bispecific antibody (BSP) (a generous gift from Novartis Biologics Center) which has one arm that binds to a tumor associated antigen (TAA) on KMS-11 target cells and CD3 ϵ on Jurkat cells, starting at 100 nM and diluted 1:2 to give concentrations ranging from 100 nM to 0.00325 nM, was added to the cells. The plates were incubated for 24 hours before adding 150 μL of BrightGlo reagent (Promega) and reading on the Envision luminometer (Perkin Elmer, Waltham, MA).

Repeat Challenge

KMS-11-mCherry, which expresses CD58, the primary ligand for CD2, and WT, CD2KO or CD2 mutant transduced T cells were mixed at 10,000 cells each in a 96-well black sided, clear bottom plate (Corning), in triplicate, avoiding the exterior wells. A bispecific antibody (courtesy of Novartis Biologics Center), which binds to a tumor associated antigen expressed on the target cells with one arm and T cells at the CD3 ϵ subunit of the TCR complex, was added to give a final concentration of 1 nM. The plate was then placed into an IncuCyte S3 (Sartorius, Goettingen, GER), which recorded whole well images every 4 hours. Three days later, the T cells were challenged with fresh target cells and BSP. The plate was centrifuged at 1500 rpm for 5 minutes and 100 μ L of supernatant removed. Fresh KMS-11-mCherry cells (10,000 cells in 50 μ L) and BSP (50 μ L of a 3 nM solution) were added and the plate was mixed before returning to the IncuCyte. The T cells were rechallenged three more times for a total of five challenges. At the last time point, 100 μ L of cell suspension was collected for cytokine analysis.

Cytokine ELISA

Cell culture supernatants from the final challenge were analyzed for IFN γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF α levels using the human Proinflammatory I panel from MSD (Meso Scale Discovery, Rockville, MD). The lyophilized standard vial was reconstituted with 250 μ L of Buffer 45 and rested for 15 minutes to allow complete dissolution of all analytes. The standards were then serially diluted one to three 6 times to generate a full standard curve for sample quantifications. 100 μ L of standard was added to a well of a pre-washed MSD plate and a one to five dilution of each sample added to its corresponding well. The samples and standards were

then incubated for 2 hours, shaking on an orbital shaker at 60 rpm. After incubation the plates were washed thrice with 300 μ L PBS containing 0.2% tween 20. Then, 100 μ L of a 1:500 dilution of capture antibody was added and incubated for 2 hours as above. The plates were washed after incubation and 200 μ L of detection reagent added, and the plates were then read on the MSD reader. Concentrations were determined using MSD Workbench software.

Graphing and Statistical Analysis

All graphs were generated in Prism (Graphpad, San Diego, CA). Student's t-test and One-way ANOVA with multiple comparisons between each group were also calculated in Prism.

Chapter III.

Results

In order to determine the role of specific CD2-effector protein interactions on T cell activity we used a panel of CD2 constructs with specific mutations to key amino acids in proline-rich SH3 domain binding motifs (PxxP or PxxxP) in the intracellular domain of CD2. These constructs were used to generate a panel of lentiviral vectors that encoded the CD2 variants and then transduced both the acute lymphoblastic leukemia cell line Jurkat or primary T cells, each with endogenous CD2 knocked out, with the vectors and subjected them to a battery of biochemical and cellular assays to assess the roles of the SH3 domain binding motifs in T cell activity. Key processes in T cell activity were measured by multiple assays describe in the Materials and Methods section, including ligand binding, phosphorylation of signaling proteins, activation induced changes in gene transcription and control of target cell growth in model of chronic antigen challenge. This series of experiments allowed us to determine the contribution of each CD2-effector protein interactions on different aspects of T cell activity.

CD2 mutant Panel

In order to evaluate CD2-effector protein interactions mediated by SH3 domain binding, we created a panel of CD2 constructs where alanine was substituted for proline at specific positions in motifs 1 and 2 or 4, or all three, to disrupt binding to SH3 domain containing proteins based on the work described in Binder and Freund (Binder,

2020; Freund, 2002). Table 1 lists the sequences of the intracellular domain of CD2 for each of the 4 constructs created.

Table 1: Amino acid substitutions in CD2 intracellular proline-rich motifs

	Sequence
Consensus sequence	...QHPPPPPGHRSQAPSHRPPPPGHRVQHQPQKRPPAPSGTQVHQQKGPPPLRPRVQPKPPHGA AENSLSPSSN
CD2-1 WT	...QHPPPPPGHRSQAPSHRPPPPGHRVQHQPQKRPPAPSGTQVHQQKGPPPLRPRVQPKPPHGA AENSLSPSSN
CD2-2 (m 1, 2, 4)	...QH APPAAG HRSQAPSHR APPAAG HRVQHQPQKRPPAPSGTQVHQQKG APLAAP RVQPKPPHGA AENSLSPSSN
CD2-3 (m 1, 2)	...QH APPAAG HRSQAPSHR APPAAG HRVQHQPQKRPPAPSGTQVHQQKGPPPLRPRVQPKPPHGA AENSLSPSSN
CD2-4 (m 4)	...QHPPPPPGHRSQAPSHRPPPPGHRVQHQPQKRPPAPSGTQVHQQKG APLAAP RVQPKPPHGA AENSLSPSSN
	1 2 4

The first mutant contained the consensus sequence for human CD2 and represents the wild-type CD2 and will be referred to as WT. The second mutant contained alanine substitutions in motifs 1, 2 and 4 and represents the negative control, where all three domains under evaluation are mutated, and is referred to as m1_2_4. The third mutant has alanine substitutions to motifs 1 and 2 only, with motif 4 containing the wild type sequence, and will be referred to as m1_2. The final mutant has alanine substitutions only to motif 4, motifs 1 and 2 with wild type sequence and will be referred to as m4.

Lentiviral vectors containing these sequences, with independent eGFP expression to facilitate transduced cell isolation, were used to transduce the CD4 ALL cell line Jurkat or primary human T cells, both with endogenous CD2 knocked out. The resulting cell lines were used in a panel of functional assays to evaluate the roles of specific interactions between CD2 and intracellular domains of effector molecule in T cell activity.

Expression of CD2 mutants in Jurkat and primary T cells

After lentiviral infection of CD2KO Jurkat cells and primary T cells, the transduced cells were isolated by sorting for dual CD2 and eGFP⁺ cells. Transduced Jurkat cells were enriched prior to cell sorting by addition of 8 ug/mL puromycin to the cell culture media to eliminate non-transduced cells due to a puromycin resistance gene in the vector construct. Primary T cells transduced with the vectors did not tolerate 2 ug/ml of puromycin, the lethal dose identified in preliminary trials, even with integration of the transgene.

After sorting, the expression levels of CD2 were determined by flow cytometric analysis and compared to either parental Jurkat or primary T cells. Table 1 lists the mean fluorescence intensity (MFI) for each transduced cell line, Figure 3 shows the histogram for each sample. Wild-type Jurkat Cells had a MFI of 3589, compared to 193 for the CD2 knock-out Jurkat cells. This value for the CD2 knock-out is identical to the isotype control, indicating a complete knock-out of endogenous CD2. The transduced Jurkat CD2KO lines had variable CD2 expressions, with MFI values ranging from a low of 1127 for m1, 2, 4 to a high of 2370 for WT. This demonstrates that the CD2 mutants are expressed in Jurkat CD2KO cells, although at a lower level than endogenous CD2. This may be due to the strength of the promoter used or the mutations may not be as well tolerated as the endogenous CD2 protein during translation and processing.

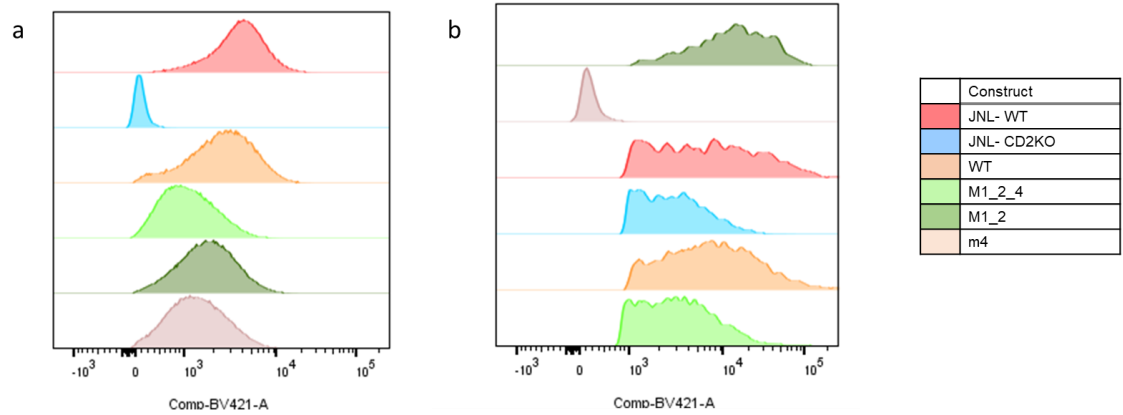


Figure 3. Expression of CD2 mutant constructs in Jurkat (a) and primary T cells (b). CD2 KO cells were transduced with lentiviral vectors encoding CD2 mutants, sorted for eGFP expression and then analyzed for CD2 expression by staining with an anti-CD-V450 antibody. All constructs were expressed, but at lower levels than endogenous CD2 (top histogram in each graph).

Table 2 MFI values of Transduced Jurkat and Primary T cells

Construct	CD2MFI	
	Jurkat	Primary T cells
<i>Jurkat Parental</i>	3985	11943
<i>Jurkat CD2KO</i>	193	269
WT	2370	6428
M1_2_4	1122	2797
M1_4	1721	7096
M4	1366	3196

Mean Fluorescence Intensity Values for Transduced Jurkat and Primary T Cells. Parental, CD2 KO and CD2 mutant transduced CD2KO Jurkat and primary T cells were analyzed for CD2 expression by flow cytometry.

Table 2 and Figure 3 show the CD2 mutant expression data from transduced CD2 KO primary T cells. CD2 WT primary T cells have a MFI of 11943, and CRISPR mediated CD2 KO primary T cells had an MFI of 293, indicating a complete elimination

of all CD2 expression. CD2KO primary T cells transduced with the CD2 mutants had expression levels lower than the endogenous, with values ranging from a low of 2797 for m1, 2, 4 to 7096 for m1, 2. These values are dramatically lower than the endogenous expression observed in the WT primary T cells, with all constructs being expressed at 27-59% of endogenous levels, if not lower. The lower expression levels in the primary T cells may be due to the same factors as the Jurkat cells or a byproduct of lower transduction efficiency.

This data demonstrates that the CD2 mutants are expressed on the cell surface in both CD2KO Jurkat and primary T cells, although at lower levels than endogenous CD2 in parental Jurkat cells or primary T cells.

Lck binding

Next, we evaluated the ability of the CD2 mutants to bind Lck, a Src-family kinase member that contains a SH3 domain and is responsible for TCR proximal signal transduction in T cells. We developed an ELISA based assay to measure binding of the mutant CD2 from the transduced Jurkat cells to immobilized recombinant human Lck. Due to the varying expression levels of the CD2 mutants possibly affecting the measurement of Lck binding by the mutants, we incorporated a CD2 binding arm to normalize the binding of Lck to the expression of CD2.

Endogenous CD2 from parental Jurkat cells was able to bind immobilize Lck at an OD Lck/OD CD2 ratio of 0.54 (Figure 4). CD2KO Jurkat cells transduced with WT CD2 had a similar Lck/CD2 ratio as the endogenous CD2, with a value of 0.62, 116% of the endogenous ratio. This demonstrates that the WT CD2 is able to bind Lck at least as

well as endogenous CD2. Mutations to motifs 1, 2 and 4 resulted in a relative decrease of 81% compared to endogenous CD2, demonstrating a clear decrease in the ability to bind Lck when the polyproline motifs in CD2 are disrupted with alanine substitutions. Mutations to motifs 1 and 2 similarly decreased Lck binding, but at a lower level, with decrease of 64.4% The increase in Lck binding compared to the triple mutant is most likely due to wild-type motif 4. Mutations to motif 4 had no measurable effect on Lck binding with a ratio of 0.60, 115% of endogenous CD2, nearly identical to the WT construct (Figure 4). This suggests that CD2 binding to Lck is driven by motifs 1 and 2, with motif 4 having a minor contribution to the binding, primarily when there are mutations to motifs 1 and 2.

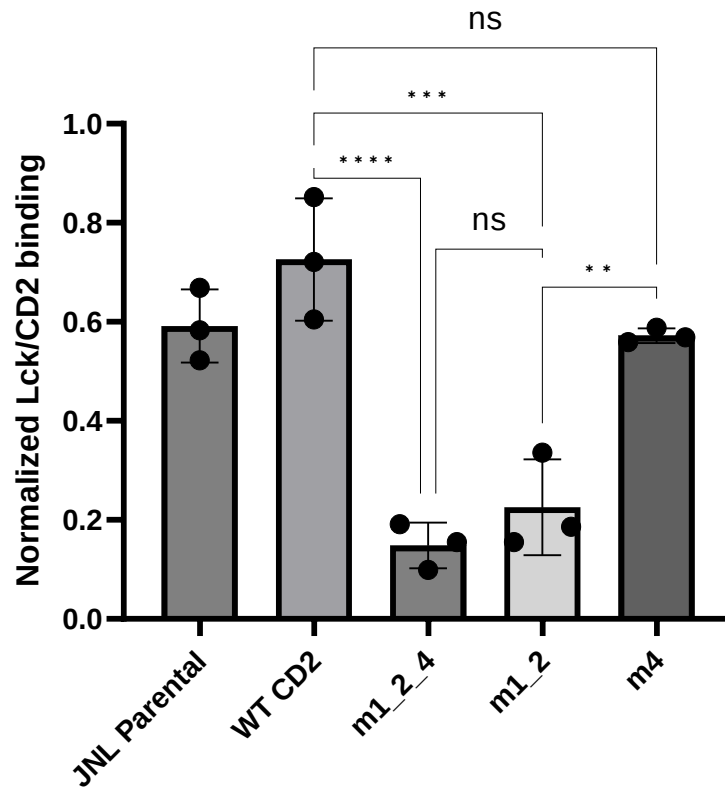


Figure 4. Effects of proline substitutions on Lck binding.

*Cell lysates from the CD2 mutants transduced CD2KO JNL cells were tested for their ability to bind recombinant human Lck in a modified ELISA. Mutations to motifs 1 and 2 led to a significant reduction in the ability to bind Lck compared to endogenous or WT CD2. Mutations to motif 4 had no significant effect on Lck binding. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$*

SPL-76 phosphorylation

Next, we evaluated the impact of the CD2 mutants on phosphorylation of the T cell receptor associated adaptor-signal transduction protein SLP-76. SLP-76 is

downstream of Lck and is essential for T cell functioning (Burns, 2011) Using the methods described in Skanland et al (Skanland, 2014), biotinylated anti-CD3 and CD2 antibodies were added to Jurkat parental, CD2KO and WT, m1_2_4, m1_2 and m4 cells. Avidin was added to induce CD3ε and CD2 crosslinking, mimicking natural T cell activation via TCR:pMHC interactions with APCs, before fixing the cells to stop the reaction and staining for phosphorylated SLP-76.

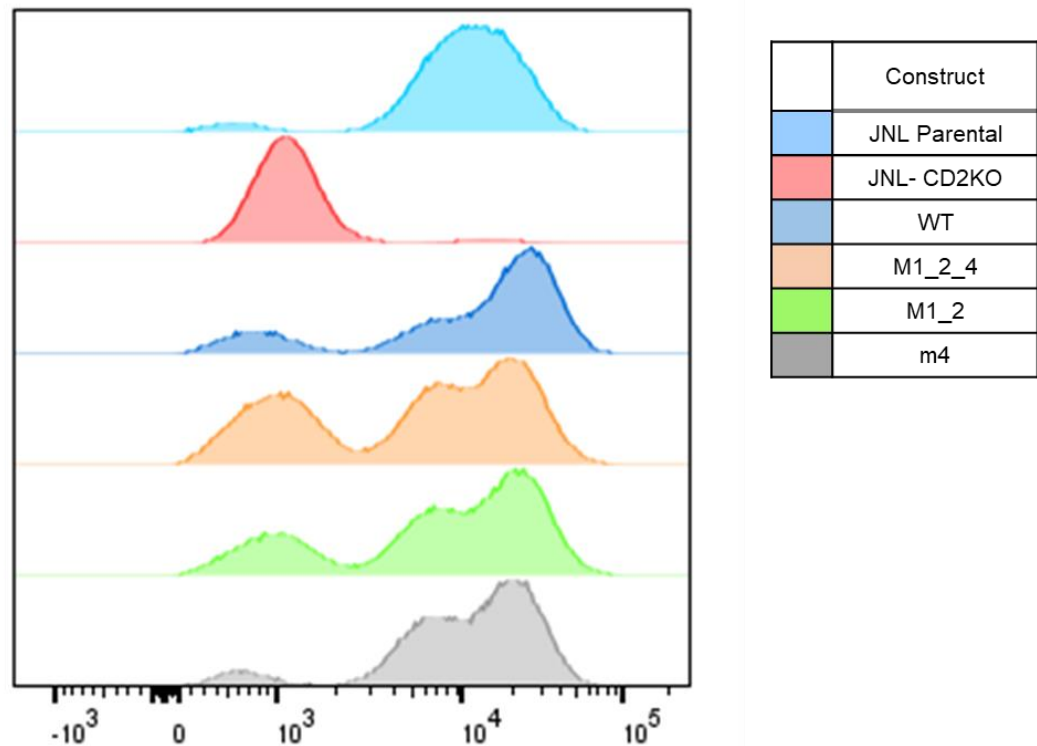


Figure 5. Effects of CD2 mutations on SLP-76 phosphorylation.

The peak on the right side of the histogram represents cells with high levels of phosphorylated SLP76, compared to the peak on the left which are cells with low or no phosphorylated SLP-76. We can see impairment of SPL-76 phosphorylation was observed in JNL CD2KO cells expressing CD2 with mutations to motifs 1 and 2, but not 4, by the distribution of cells in the phosphorylated/non-phosphorylated peaks.

Table 3 Effects of CD2 Mutants on SLP-76 (Tyr141) Phosphorylation

Construct	pSLP-76 MFI	Change From JNL-CD2KO	Percent of JNL Parental
JNL Parental	9560	8322	NA
JNL CD2KO	1238	NA	12.3
WT	9772	8534	102
M1_2_4	4844	3606	43.3
M1_2	6692	5454	58.5
M4	9573	8335	100

Parental Jurkat cells were uniformly activated after CD3:CD2 ligation, with a MFI of 9560 (Figure 5, Table 3), where the peak on the right hand side of the histogram in Figure 5, representing phosphorylated SLP-76, is much larger than the peak on the left, representing unphosphorylated SLP-76. CD2KO Jurkat cells had minimal SPL-76 phosphorylation, with an MFI of 1238, demonstrating the need for CD2 to induce this signaling event, as CD3 stimulation by itself is not able to induce SPL-76 phosphorylation (Figure 5). CD2KO Jurkat cells transduced with WT CD2 had an MFI of 9772, similar to the parental Jurkat cells with endogenous CD2. This suggests that the WT construct is able to induce downstream signaling to the same extent as endogenous CD2, even with lower expression levels. We must consider that the effects of the CD2 mutations on SLP-76 phosphorylation may be greater than the data suggests, since the expression level of the CD2 mutants is lower than endogenous CD2, and the assay conditions were established with WT primary T cells. The m1_2_4 construct, with mutations to motifs 1, 2 and 4, leading to a relative reduction of pSLP-76 levels by 57%, with an MFI of 4844 (figure 5, table 3). The m1_2 construct also had pSLP-76 levels decreased by 41% compared to endogenous CD2. Mutations to motif 4 had no

measurable effects on pSPL-76 levels compared to endogenous CD2, with a MFI of 9573 compared to 9560 (Figure 5, Table 3). These data confirm the role of CD2 intracellular SH3 binding motifs 1 and 2 in Lck binding and downstream signaling, with motif 4 having no effects on SLP-76 phosphorylation.

NFAT Activation

Expression of the Nuclear Factor of Activated T cells (NFAT) family of transcription factors are upregulated after T cell activation and induce the expression of multiple cytokines and effector molecules (Macian, 2005) needed for full immune activation. Using Jurkat cells that have stable expression of luciferase reporter gene under the control of a NFAT responsive promoter, JNL cells, we then evaluated the effects of our SH3 domain binding motif mutants on late T cell activation. After incubation of the JNL cells with a bispecific antibody (BSP) which binds CD3 ϵ with one arm and the B Cell Maturation Antigen (BCMA) on the other a luciferin containing reagent was added and luminescent signal measured. Binding of the T cell to the BSP and target cell mimics natural TCR:pMHC interactions, giving the primary signal for T cell activation, and secondary signals are provided by the costimulatory ligands on the target cell. In humans, one such costimulatory ligands is CD58, the natural ligand for CD2.

JNL cells expressing WT CD2 showed dose dependent NFAT activation, with maximal signal starting at 11 ng/mL of BSP. Knockout of CD2 in JNL cells resulted in a 15% decrease in signal (Figure 6a) compared to endogenous CD2. JNL CD2KO cells transduced with the WT CD2 construct had a similar dose response as parental JNL cells, but at a much higher signal (Figure 6b). This may be an artifact of cell line generation, since both the NFAT reporter construct and CD2 mutant constructs are under puromycin

selection, and very high levels of puro were needed for CD2 purification and may have led to selection of subclones of JNL cells with inherently higher expression of the reporter construct. JNL cells with m1_2_4 had significantly reduced signal at 11 ng/mL compared to the WT construct (25%, $p=0.008$). Mutations to motifs 1 and 2 only led to a non-significant 15% reduction in signal compared to WT. Mutations to motif 4 alone had no effect on reporter expression (Figure 6b). These data show that disrupting intracellular SH3 domain binding motifs 1, 2 and 4 leads to a significant decrease in T cell activation. This decrease appears to be primarily driven by the first two motifs, since rescue with a functional motif 4 partially rescued the decrease, whereas functional motifs 1 and 2 paired with mutated motif 4 had levels of reporter expression nearly identical to the WT. This suggests that inhibiting CD2 from associating with SH3 domain containing proteins such as Lck can reduce the levels of effector molecules such as cytokines produced (Macian, 2005, Martelli, 2000). Orthogonal assays such as direct measurement of IL-2 in the cell culture supernatants or changes in the expression of the transcription factor AP-1 protein after stimulation would further confirm these findings.

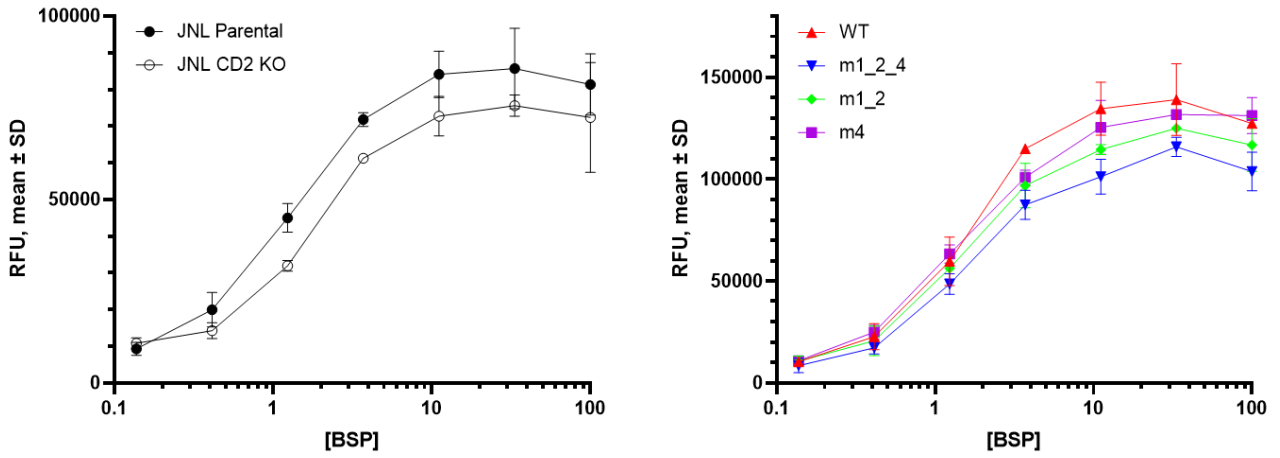


Figure 6. Effects of CD2 mutants on NFAT activation.

(a) CD2 KO results in significant decreases in NFAT reporter expression after stimulation with BSP and target cells compared to WT. In JNL cells transduced with the CD2 mutants (b), we observed significant effects with mutations to motifs 1 and 2, but not motif 4, when compared to cells transduced with the wild-type CD2.

Table 4 Effects of Mutants on NFAT activation

Construct	AUC	Percent of WT	P-Value vs WT
JNL Parental	8205690	NA	NA
JNL CD2KO	7211293	87.9	0.01
WT	13093827	NA	NA
M1_2_4	10628329	81.1	0.0168
M1_2	11724307	89.5	0.045
m4	12695325	96.9	0.602

*Repeat Measures ANOVA used for analysis

Repeat Cell Killing Assay

Repeated exposure to antigen is known from experimental evidence, as well as from the clinic, to lead to T cell exhaustion. One of the hallmarks of T cell exhaustion is the downregulation of the costimulatory receptor CD28. In this setting, multiple groups have demonstrated the increased role of CD2 in maintaining T cell activity (Majzner, 2020, Yan, 2022). In order to evaluate the role of CD2 SH3 Domain binding motifs in T cell exhaustion, we utilized a repeat challenge assay. Every 3 to 4 days, new antibody and target cells, which express CD58, the natural ligand for CD2, were added to CD2 WT, KO or CD2KO with CD2 mutant primary T cells to mimic repeated antigen exposure and T cell activation.

After the first two challenges, all CD2 mutants, CD2 KO and CD2 WT primary T cells had similar levels of killing, eliminating nearly all target cells (Figure 7). After the third challenge, we observed reduced target cell killing in the CD2 KO primary T cells. Target cell killing in the CD2 WT primary T cells and CD2 mutant transduced cells remained unchanged. After the fourth challenge, the CD2 KO primary T cells lost the ability to kill all target cells, as the TII remained unchanged throughout. The m1_2_4 transduced cells also exhibited impaired T cell mediated cytotoxicity, with static levels of target cells observed. WT and m1_2 and m4 T cells maintained similar capacity for

controlling target cell growth as the Primary T cell (Figure 7).

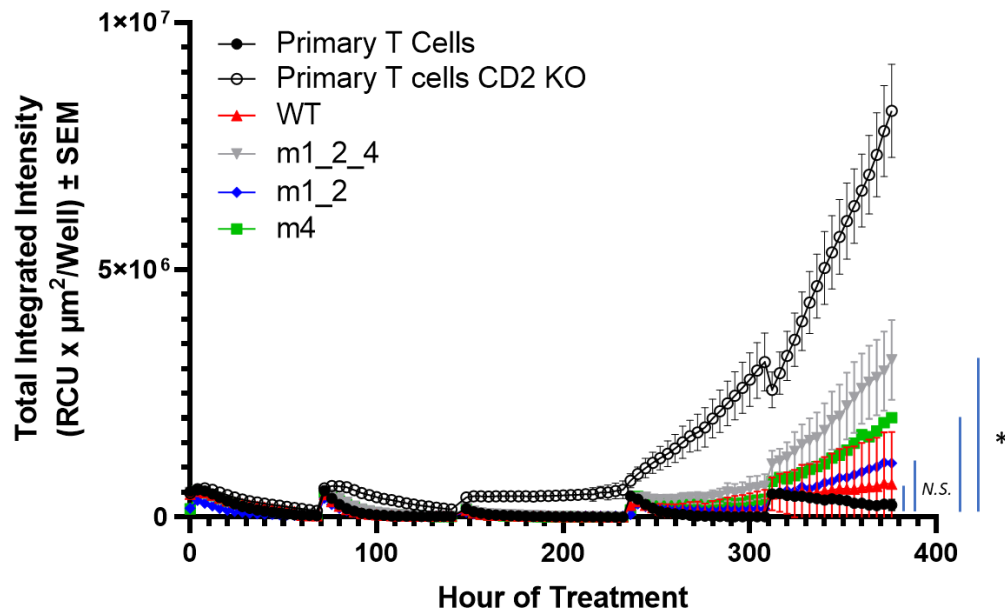


Figure 7. Motif 4 is critical to chronic target cell killing.

*In the repeat challenge assay, loss of CD2 resulted in total loss of the ability to eliminate target cells after 3 rounds of challenge. Rescue with all CD2 mutants significantly restored killing ability compared to the knockout, but only the cells with a wild-type motif 4 had levels of growth control similar to the primary T cells. * $p < 0.001$*

After the 5th challenge, we observed dramatic differences between the different CD2 constructs. The triple mutant lost all ability to reduce target cell growth, with terminal Total Integrated Intensity (TII) values of 3.2×10^6 RFU $\times \mu\text{m}^2/\text{well}$, 12.7-fold higher than the value for the WT CD2 primary T cells (2.5×10^5 TII). The WT transduced T cells maintained their killing ability, with terminal TII value of 6.7×10^5 . The m1, 2 transduced T cells had minimally reduced target cell killing, with terminal TII values of 1.1×10^6 , 64% higher than the WT transduced T cells. Interestingly, while the

motif 4 mutant, with its WT m1 and 2, had minimal impacts in SLP-76 phosphorylation and NFAT activation, mutations to this motif significantly reduced the ability of T cells to kill target cells in this assay (Figure 7). We observed terminal TII values of 2.0×10^6 , a three-fold increase in signal compared to the CD2 WT transduced T cells. This suggests that unlike signaling and T cell activation after a single exposure, motif 4 is essential for durable control of target cell growth after multiple rounds of challenge.

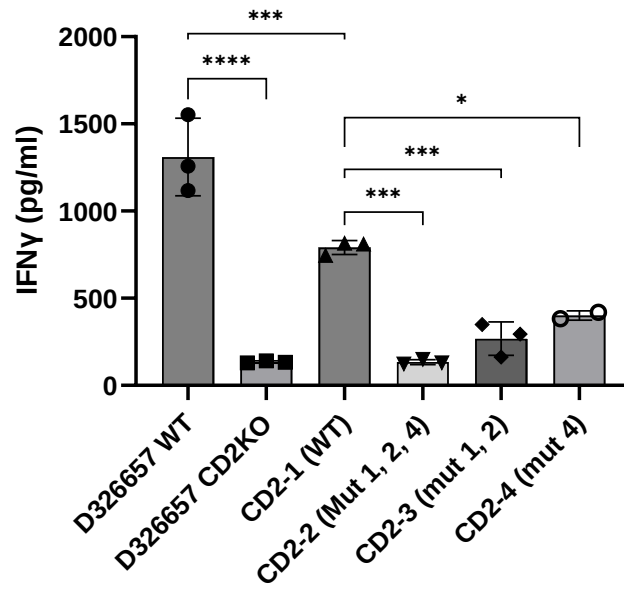


Figure 8. Effects of mutations on IFN γ production after final challenge.

*Transduction of CD2 KO primary T cells restored IFN γ secretion in cells with intact motifs 1, 2 and 4. Combinations of wild type motifs 1 and 2 or 4 alone was not able to rescue cytokine secretion to the same level. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, Student's t -test.*

After the final challenge, cell culture supernatants were collected and analyzed for cytokine secretion. Supernatants from CD2 WT primary T cells had 1310 pg/mL of IFN γ and the CD2 KO had a mean of 134 pg/mL, nearly a 10-fold decrease (Figure 8). The transduced T cells had IFN γ levels that associated with their killing activity, with the WT construct giving the highest levels of IFN γ , 791 pg/mL, followed by 268 and 400 pg/mL for the m1, 2 and m4 constructs. The triple mutant had IFN γ levels nearly identical to the CD2 KO primary T cells, 133 pg/mL, further demonstrating the requirement for at least 1 one of the CD2 intracellular SH3 domain binding motifs for IFN γ secretion. We observed no significant levels of IL-2, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13 or TNF α in the supernatants. We must note that these effects were observed even with expression levels of the mutants lower than endogenous CD2, potentially minimizing the impact of the mutations in this assay.

Chapter IV.

Discussion

T cell activation is a highly dynamic and well-organized series of events and depends on the coordinated rearrangement of molecules present on the cell surface prior to TCR ligation to peptide loaded MHC complexes on the APC or target cells. If the strength of the TCR:pMHC interaction is sufficiently strong, binding of costimulatory receptors and adhesion molecules on T cells can bind to their cognate ligands on the APC, providing the necessary second activation signal as well as stabilizing the nascent Immune Synapse (IS). CD28 is known to be a primary costimulatory receptor on T cells that is essential for inducing activation of several key signaling pathways that induce transcription of effector molecules required for full T cell activity. In diseases with chronic antigen exposure such as cancer and viral infections with HBV and HCV, downregulation of CD28 is observed, both experimentally as well as clinically. T cells that have reduced CD28, the dual function adhesion molecule/costimulatory receptor CD2 has demonstrated a critical role in maintaining functionality. Majzner et al demonstrated that CD58 loss in DLBCL patients resulted in a significantly poorer response to CD19 CART therapy, further strengthening the role of the CD2/CD58 axis in T cell functionality in cancer.

CD2 can be found in two distinct regions of the IS, in the central region (cSMAC) near the TCR complex as well as in the periphery, in a zone termed the corolla. Current hypotheses link the centrally located CD2 molecules with primarily costimulatory functions mediated through interactions with the TCR complex as well as signaling molecules such as the Src family kinases Lck and Fyn. In the corolla, CD2 has been

associated with interactions with the cytoskeleton, acting to stabilize the IS by helping to compartmentalize the localization of agonistic and antagonistic molecules.

The purpose of this study is to determine the role of specific proline rich motifs in the intracellular domain of CD2 in T cell activity and exhaustion. We generated a panel of CD2 mutants bearing combinations of alanine substitutions at specific proline residues to disrupt interactions with proteins that contain SH3 binding domains, as described in Table 1, and probed their effects in increasingly biologically complex assays.

These lentiviral expression vectors for these mutants were used to transduce Jurkat-NFAT-luc and primary T cells in which CD2 had been knocked out via CRISPR. We observed surface CD2 expression in all transduced cells, but at lower levels than endogenous CD2 (Figure 1, 2; Table 2, 3). This finding may be due to the strength of the promoter used, EF1 α , which may be weaker than the native CD2 promoter in this lineage, or the codon optimization strategy used when creating the vectors. Both of these areas may need to be altered to increase expression to match endogenous levels.

Interestingly, cells with any mutations to motif 4, both the triple m1_2_4 and single m4, had the lowest expression levels in both Jurkat-NFAT-luc and primary T cells, with MFIs of 69 and 62% lower than endogenous CD2 for the Jurak and 77 and 73% in the primary T cells, respectively (Figure 3, Table 2). This suggest that the structure of this mutations to motif 4 may not be as well stable and tolerated during translation, folding and trafficking as the native form.

When we look at the effect of these mutants to binding of immobilized Lck we can see that binding appears to be driven by motifs 1 and 2, with mutations to motif 4

having minor effects in the assay used (Figure 3 and table 4). This finding aligns with the hypothesis that motifs 1 and 2 are essential for CD2 mediated signaling and motif 4 is required for interactions with the cytoskeleton, although it must not be ruled out that the mutations to motif 4 did not have the desired effect.

Further support for this hypothesis can be found in the phosphoflow and JNL assays. We see that Jurkat cells with mutations to motifs 1 and 2 had decreased signaling through SLP-76 as well as T cell activation as measured by a NFAT reporter (Figure 5 and 6). The JNL assay results are particularly striking, since there are multiple costimulatory pathways activated in this assay. Jurkat cells may express costimulatory ligands outside those of the CD2/CD58 axis and may provide overlapping stimulation that would overshadow the effects of the CD2 mutants (Skanland, 2014). The fact that effects are seen in this assay, in spite of these competing factors, demonstrates the importance of these domains in NFAT activation but that CD2 does not have a dominant role in its induction, as we observed NFAT driven reporter protein expression in CD2 KO Jurkat cells, albeit at higher concentration of BSP than CD2 WT Jurkat cells (Figure 6).

T cell activation leads to the upregulation and activation of transcription factors outside of NFAT. The most well studied are AP-1, NF- κ B, TCF1 and T-BET, each of which regulate the expression of required effector and pro-survival proteins needed for T cell activity (Conley, 2016; Kim, 2020). Evaluation of CD2 mediated activation of these pathways, and the protein interactions with CD2 that regulate this interaction is an area where research should be extended.

Evaluation of multiple other signaling pathways was undertaken, such as the MAPK, ERK1/2 and LAT/VAV/ZAP70 pathways, but with no success. Further work to

identify proper clones of detection antibodies needs to be undertaken to fully understand the effects of the mutants in activation of these pathways. Evaluation of orthogonal assays for T cell activation, such as calcium flux, which is essential for signal transduction, would provide additional insights.

Taken together these data demonstrate the role of the proline rich motifs of the intracellular domain of CD2 in cell signaling and T cell activation after initial activation, but the main hypothesis of this study is the necessity of a functional fourth intracellular SH3 domain binding motif for T cell activity in chronic antigen exposure. To answer this question, a repeat challenge assay developed at NIBR was used. In this assay, primary T cells transduced with the CD2 mutants were exposed to several rounds of bispecific antibody mediated cell killing and target cell growth monitored. We found that complete loss of CD2 resulted in a reduced T cell activity, marked by loss of target cell killing, after the third challenge, and any activity was lost after subsequent rechallenges. T cells with endogenous CD2 maintained killing potential throughout the 5 rounds of challenge, demonstrating the necessity of CD2 in a model of chronic antigen exposure (Figure 7). This can be compared to the findings of Majzur and Yan (Majzur, 2020; Yan 2022), where loss of CD58 on tumor cells impacted chimeric antigen receptor T cell (CART) activity in multiple in vitro and in vivo models of activity, which itself echoes a correlation between CD58 loss in tumor cells and a poorer response to CART in DLBCL patients in a clinical trial. This study demonstrates a clear role for the 4th motif in the maintenance T cell killing during chronic antigen exposure. When this motif is either mutated or absent due to CD2 knockout, T cells lose the ability to control target cell growth after 3 to 4 challenges (Figure 7). However, these mutants do have a positive

effect on activity (Figure 7), allowing for prolonging target cell killing for another 1-2 rounds of challenge compared to the CD2 KO. This suggests that factors outside of the intracellular motifs under evaluation in this study may play a role in the durability of T cell activity during chronic antigen exposure and need to be further evaluated.

Several candidates for further experimentation may be identified from our knowledge of the structure of CD2. First, there are two additional intracellular proline rich motifs that we did not evaluate in this study. Just because we do not have an understanding of the role of these motifs at this time, further study may yield information about their interactions that may contribute to our understanding of CD2. Second, the T11.3 and CD2R epitopes of the membrane proximal extracellular domain of CD2 play a prominent role in formation of CD2 clusters in cis and subsequent localization to specific compartments in the IS and thus which functions, signaling or cytoskeletal anchoring, are favored (Li, 1996). Functional studies of how the SH3 domain binding motifs interplay with the extracellular domains regulating CD2 cis interactions are lacking and are needed to elucidate their role in localization to specific compartments of the IS.

The exact mechanism for the role of motif 4 in durable T cell activity needs further elucidation. Hutchings et al (Hutchings, 2003) postulate a model for regulation of CD2-cytoskeletal interactions driven by CIN85 and CD2BP3, where motif 4 is bound to either CIN85 or CD2BP3 when the cell is at rest and degraded upon activation. This allows CMS to bind the cytoplasmic tail of CD2 at motif 4 and subsequently bind to the actin cytoskeleton through the actin capping protein CAPZ. No other motif in the intracellular portion of CD2 has been shown to associate with CIN85, CD2BP3 or CMS,

even though they all share similar polyproline sequences. Targeted knockout of CMS in primary T cells would allow for an orthogonal approach to verify the need for CD2 anchoring to the cytoskeleton via CMS for effective target cell killing in a chronic setting.

Outside of T cell effector function, it is still unknown how CD2 signaling affects other states of the T cell, such as memory and exhaustion phenotypes. This is particularly relevant in situations such as chronic antigen exposure which places a higher burden on CD2 signaling than early stages of antigen exposure, and where specific memory lineages or exhaustion states may have a higher reliance upon CD2. For example, high CD2 expression has been associated with central memory phenotypes and may act to lower the barrier to activation after re-exposure to antigen (Binder, 2020), but this hypothesis needs to be evaluated, as well as its dependencies on particular signaling pathways or interactions with the cytoskeleton. The role of CD2 in T cells with an exhausted phenotype, which is characterized by high PD-1 expression and a reduction in effector molecule and cytokine production, has been demonstrated, but the exact mechanism has yet to be determined. Based on the preliminary results of this study, I feel this platform, with modifications, is uniquely poised to help address many of these questions.

The first improvement would be to find a more suitable promoter than the current EF-1 α . We see dramatically lower expression in both primary T cells and Jurkat cells across the entire panel of mutants compared to endogenous CD2 in the parental cells. Other promoters, such as the CD2 promoter or hPGK may drive higher levels of expression and counter any effects due to lower protein levels. An additional benefit of

changing to the CD2 promoter would be the potential to have expression level driven by activation of the T cells that normally lead to CD2 upregulation.

Second, changing from a lentivirus to a γ Retrovirus could lead to higher transduction efficiency (Irving, 2020) , and thus fewer primary t cells would need to be infected to generate the appropriate number of transduced cells needed for experimentation. With the current approach, low levels of transduction (<5%) were observed, thus a large number of primary T cells were required to generate enough transduced cells for experiments and potentially introducing a potential bias towards specific T cell subsets that were more amenable to transduction.

Third, using methods such as surface expression of the Low Affinity Nerve Growth Factor (LNGFR) rather than expression of a antibiotic resistance gene, for selection of transduced clones is a widely used technique in the manufacture of CART cells (Casucci, 2018). It allows for rapid cell isolation that has less cell loss and stress compared to standard flow cytometry, which was found to have nearly 50% cell loss and a decrease in viability after sorting. Adopting this technology into the vector would once again increase the yield of transduced cells, requiring fewer cells at the start.

Adopting these changes to the vector would improve the quality of data, producing larger numbers of transduced cells with CD2 expression in line with endogenous CD2, in a shorter time and requiring fewer cells at the start.

Furthermore, the panel of experiments would also need to be expanded to help address the remaining questions. Flow cytometry based immunophenotyping of T cells during the repeat challenge assay would track changes to the expression of memory markers, costimulatory molecules, immune checkpoint inhibitors, effector molecules and

transcription factors. It has been shown by other groups that in the CD28- setting, indicative of T cell exhaustion, that CD2 signaling can maintain the ability to produce effector molecules such as IL-2 and IFN γ (Demetriou, 2020; Leitner, 2014), but the interactions that drive that rescue are not understood. This platform, in particular the repeat challenge assay, would produce the raw materials to help answer this question.

Alternative technologies to quantify differences between the CD2 mutants, such as single cell RNAseq offers the ability to track transcriptional changes at a level of resolution beyond other technologies. This would allow for identification of pathways or proteins that may be associated with engagement of particular motifs with their associated partners where we do not have the ability to measure, due to lack of viable reagents, poor resolution of the existing technologies or non-obvious interactions that would preclude selection of the correct analyte. Additionally, the method of T cell:target cell engagement used in this study, a bispecific antibody, may not completely recapitulate natural TCR:pMHC interactions. Use of TCR:pMHC systems such as NY-ESO-1 specific TCR 1G4 or MART-1 TCR would give a natural architecture of T cell and APC cell interactions, and simultaneously giving the CD2 mutants the most natural interaction with its ligands on the APC. (Moon, 2016)

Finally, development of an imaging component to the platform would allow for visualization of how mutations to the motifs affect T cell activity. This can be beneficial to the understanding of the IS is formed, maintained and eventually degraded, and how intracellular interactions with CD2 intracellular domains drives each of these components of T cell activity. As the saying goes, “A picture is worth a thousand words”.

This understanding of the role of CD2 in T cell function has many real-world impacts. In diseases such as cancer and chronic viral infection, the CD2/CD58 axis has an outsized role in maintaining T cell activity compared to other costimulatory pathways (Leitner, 2015), and its loss leads to a poorer prognosis in multiple cancers.

Understanding the molecular interactions that drive this effect by CD2, as well as what inhibitory signals that are occurring and where they arise, could lead to therapeutic interventions that improve the function of T cells. This would lead to significant impacts to patients, where we could see complete responses with minimal residual disease due to improved T cell function.

Conversely, in diseases where we want to downregulate T cell activity, in autoimmune diseases such as IBD, understanding the role of CD2 interactions would allow for therapeutic interventions that decrease T cell function. In particular, the higher level of CD2 expression observed in memory T cells make CD2 an attractive target for intervention. In fact, an anti-CD2 monoclonal antibody, sipilizumab, is currently in Phase II trials for transplant rejection (Binder, 2020). However, this antibody blocks all CD2 interactions, both interactions with CD48 and CD58, and may have a narrow therapeutic index between excessive immune suppression and inhibition of desired T or NK cell subsets. Design of a CD2 inhibitor specific to alloreactive or graft reactive T cells would prevent this global inhibition and widen the difference between efficacious and toxic doses of the inhibitor. Data from this platform would let us know what interactions with particular domains are essential for the undesired activity and are suitable for intervention.

The data from this study demonstrates that the 4th intracellular polyproline motif of CD2 is important for effective T cell function after chronic antigen exposure, but the mechanism of action needs to be determined, as well as what role signaling through the first and second motifs play in maintenance of T cell activity.

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