

Phosphoproteomic Analysis of T Cell Receptor
and Chimeric Antigen Receptor Signalling:
Pathway Discovery and Regulatory Mechanisms

By

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B.Sc., Marlboro College, 2020

Thesis

Submitted in partial fulfillment of the requirements for the Degree of
Doctor of Philosophy in the Department of Molecular Biology, Cell
Biology, and Biochemistry at Brown University

PROVIDENCE, RHODE ISLAND

May 2026

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This dissertation by Aurora Callahan is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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- Poster **Callahan, A.**, Puterbaugh, R. Z., Ro, T., Zhang, X., Su, X., and Salomon, A. R. (6-10 December, 2025). Phosphoproteomic analysis of successive Jurkat CD19-CAR generations reveals TCR ζ -driven signalling. *Cell Bio 2025, An ASCB:EMBO Meeting*, Philadelphia, PA, USA
- Poster **Callahan, A.**, Zhang, X., Wang, A., Mojumdar, A., Zeng, L., Su, X., and Salomon, A. R. (28-31 October, 2025). CSF1R-CAR T cells induce CSF1R signaling and can promote target cell proliferation. *Immune Engineering and Cellular Immunotherapy*, Cold Spring Harbor Laboratories, NY, USA
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- Talk **Callahan, A.**, (18 Nov 2022) Understanding how Chimeric Antigen Receptors influence T cells and target cells. *Brown MCBGP Data Club*, Providence, RI, USA

Acknowledgments

I would like to thank the kitties for being fluffy and providing the emotional support I needed to not jump out the window at all times.



God (left) and Satan (right)

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CHAPTER 1

A Marriage of Technology and Discovery: Modern Mass Spectrometry and the Pursual of T cell Biology

1.1 Introduction

“Signalling by CD28 has proven to be rather enigmatic, with multiple pathways seemingly involved, including PI3K pathway and the NF- κ B pathways. Phospho-proteomic analysis performed collaboratively with the lab of the late Tony Pawson have suggested the involvement of a broader array of pathways. It’s amazing that our understanding of the molecular mechanism underlying CD28 costimulation is still rather limited nearly three decades later.” - Arthur Weiss, 2024

Understanding the biology of T cells has been a long standing biological question, with researchers as early as the 1980s and 1990s making substantial contributions to the field. Dr. Arthur Weiss is considered a founder of the T cell signalling field, providing three decades of substantial mechanistic research into the fundamental properties of T cell activation. His contributions include the characterisation of

Jurkat T cells, a fundamental research model for T cell signalling, discovering regulatory mechanisms of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) involved in T cell activation, and understanding the role of costimulation in T cell activation. Further, the rationale for chimeric antigen receptor (CAR) T cell therapy, a novel cancer immunotherapy and booming research field, began in part with Dr. Weiss' early work to understand the mechanisms involved in T cell activation. However, as he notes at the end of the above quote, it is *amazing* how little we still know about these pathways after decades of research (Weiss, 2024).

A large hurdle in our pursuit to understand T cell biology is the complexity of the human proteome. With approximately 20,000 unique proteins in the human proteome and 5,000-10,000 unique proteins identified in human T cells (Joshi et al., 2019), characterising the function of individual proteins is a herculean task. If we include the role of post translational modifications (PTMs) or protein-protein interactions (PPIs) to the T cell proteome characterisation, the difficulty rises substantially. Despite the challenge, researchers have developed techniques to understand bulk protein functions and interaction networks. The pursuit of protein mass spectrometry (proteomics) technologies have allowed researchers to comprehensively map the proteome, PTMs, and PPIs in response to single gene deletions, small molecule perturbations, or, as noted by Dr. Weiss, receptor stimulation. Here, I will discuss the symbiotic relationship between T cell biology and mass spectrometry based proteomics, highlighting key proteomics findings that have pushed our understanding of T cell biology. First, I will dive into the basics of mass spectrometry, T cell signalling, and chimeric antigen receptor T cells, before addressing specific methodologies and pivotal papers using those techniques.

1.2 The Basics of Modern Mass Spectrometry

The foundations for mass spectrometry, a magnetic cathode ray deflector, was invented incidentally by J.J. Thompson in the early 1900s while attempting to measure properties of electrons. For the next 80 years, mass spectrometry developed from a tool to understand the fundamental nature of atoms to a tool for understanding the structure of organic compounds. However, mass spectrometry-based proteomics in its current form blossomed in the 1980s with the advent of ‘electrospray ionisation’. Since the 1980s, mass spectrometry based proteomics developed into a field to serve the ever growing need of biologists for reliable, high throughput methods for discovery (Griffiths et al., 2008). Here, I will provide a brief overview of workflows (Figure 1.1) typically used for peptide mass spectrometry in the modern age, including liquid chromatography, electrospray ionisation, mass analysers, and data processing pipelines.

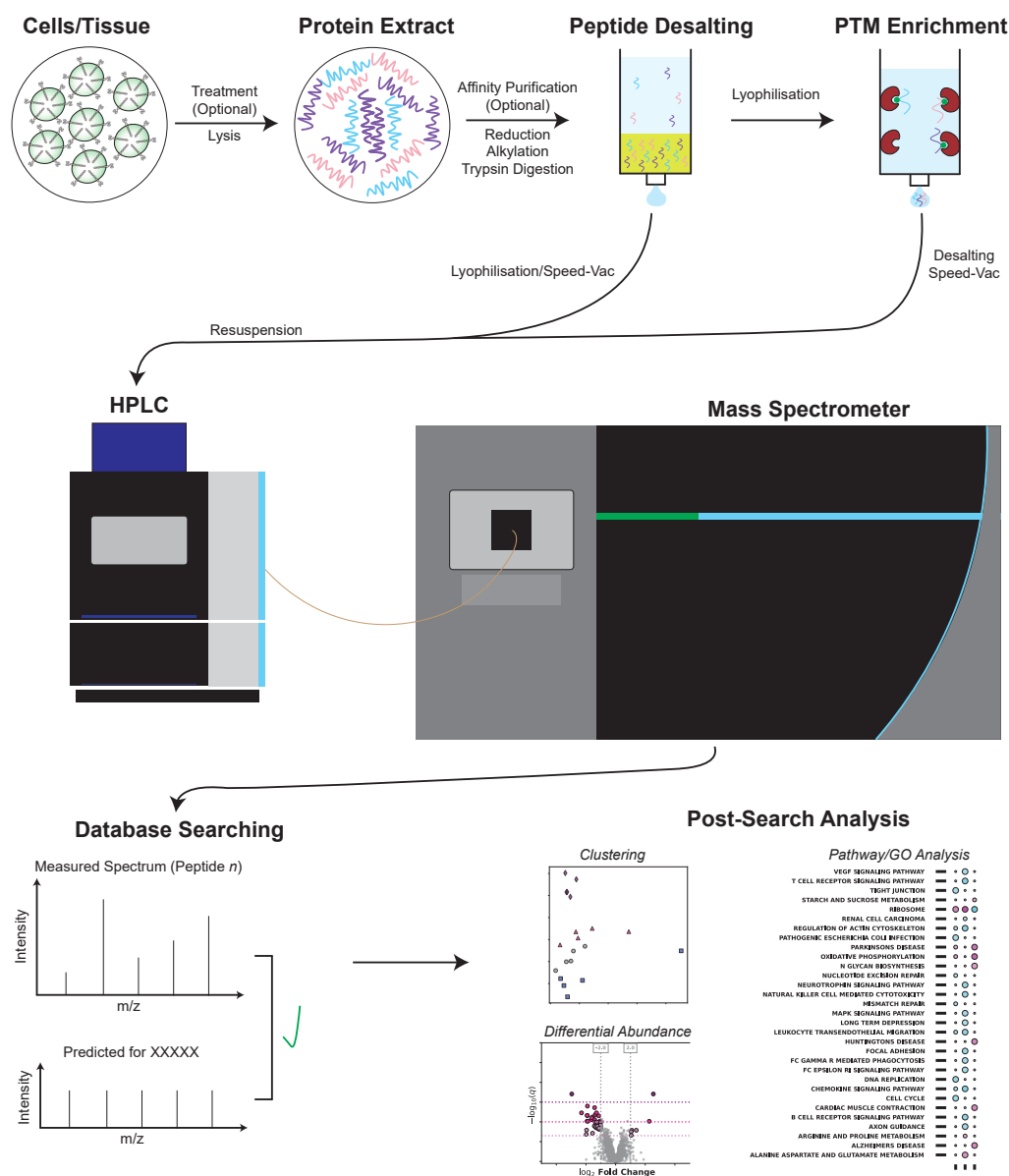


Figure 1.1: Workflow for sample preparation for modern mass spectrometry. Cells or tissues can be treated or untreated (e.g., stimulating antibodies, inhibitors) before lysis and protein extraction. Extracted proteins can then be affinity purified (optional. e.g. coimmunoprecipitation), then reduced, alkylated, and trypsinised. Salt is then removed from the peptides by binding peptides to a resin (typically reverse phase), before drying. Dried peptides can either be reconstituted and analysed on a mass spectrometer or enriched for specific post-translational modifications (PTMs) before MS analysis. After analysis on the mass spectrometer, the resulting spectra are searched against a database and assigned peptide sequences/relative abundances. Identified peptides are then subject to post-search analysis, including differential abundance analysis, clustering, and Pathway/Gene Ontology (GO) enrichment analysis.

1.2.1 Liquid Chromatography

High performance liquid chromatography (HPLC) describes the process by which compounds in a solution are separated based on some chemical properties using high pressure (200-1500 bar) in microscale capillary systems (Bittremieux et al., 2018). Modern equipment uses a cooled sample injection platform that can deliver samples (1-20 μ L) into the capillary system responsible for separation. Flow paths can vary by user/project preference, but frequently researchers use a ‘trap-and-elute’ system where peptides are first ‘trapped’ and concentrated on an in-line column (typically 2-20 cm, reverse phase) and washed with an acidic solvent (“Solvent A”; 0.1% formic acid, 0.1 M acetic acid) before elution with increasing organic solvent (“Solvent B”; 5-100% acetonitrile with Solvent A) flowing towards the analytical column (van Soest and Xiong, 2015). For bottom-up proteomics, classic analytical columns consist of a reverse phase resin (octadecyl group bound to silica gel, commonly referred to as C18) in small ($\sim 75 \mu$ m) capillaries, with pulled emitter tips designed for peptide ionisation. Analytical columns can vary greatly in length, however shorter columns (8 cm) are better for faster separation methods with lower sample complexity, whereas longer columns/separation methods (25-75 cm) more effectively separate peptides and more completely capture high complexity samples (Ahsan et al., 2017). The dominant ionisation technique used in MS-based proteomics is electrospray ionisation. For electrospray ionisation, peptides are separated in an acidic solution during separation on an analytical column, and high voltage (2-2.5 kV) is applied either to the entire analytical column or directly to the tip. As the solvent aerosolises and droplets decrease in size, electrostatic repulsion within a droplet causes droplet fracturing. This process continues until only the analyte, on which the positive charge is condensed, remains (Wilm, 2011). While other ionisation techniques exist (e.g., matrix associated laser desorption ionisation; MALDI), they are typically used for specific applications (e.g. spatial proteomics, organismal identification) on specific mass spectrometers, rather than for standardised bottom up proteomics workflows (Awad et al., 2015; Gustafsson

et al., 2011). Ions are then vacuumed into the ion source of the mass spectrometer, where they undergo isolation, quantitation, and fragmentation.

1.2.2 Mass Analysers and Modern Mass Spectrometers

The heart of a mass spectrometer are the mass analysers, as these components are responsible for separating peptide ions for capture, providing the unique input for peptide detection that is used for biological inference. Currently, four types of mass analysers are most prevalent in MS-based proteomics, which include the quadrupole, ion trap (IT), time-of-flight (TOF), and Orbitrap (Figure 1.2). Quadrupole mass analysers consist of four perpendicular rods with radio frequency (RF) and direct current (DC) voltages applied to opposing rods (Dawson, 2013, 1986). The ability of ions to overcome the force applied by the voltages depends directly on its mass-to-charge (m/z) ratio, and thus peptide ions can be selected by changing the current values applied on the rods. Due to their simplicity, quadrupoles are often coupled with other mass analysers in more complex setups requiring multiple instances of ion collection. Ion trap mass analysers work on the same principles as the quadrupoles, except rather than being linear, they can be thought of as being bent to form a ring shape with a central rod. An inert gas like Helium is often added to the IT to focus movement of the ions to the centre of the ring and reduce ion loss to collisions with the electrodes (March and Todd, 2005; Creaser and Stygall, 1998). Time-of-flight mass analysers are conceptually simple, but difficult to engineer, as they simply rely on measuring the flight time of ions through the mass spectrometer to obtain an m/z ratio for that ion species. Typically, ions enter the mass spectrometer and are pulsed into the detection tube, where their flight time is measured and converted into an m/z ratio, and improvements to this general setup (e.g., reflectors) have improved mass resolution (Guilhaus et al., 1997). Lastly, Orbitrap, or ‘Orbital Trapping’ mass analysers can be considered a modified version of ITs, and contain a central oblong rod on which constant current is applied which ions circulate around. Due to

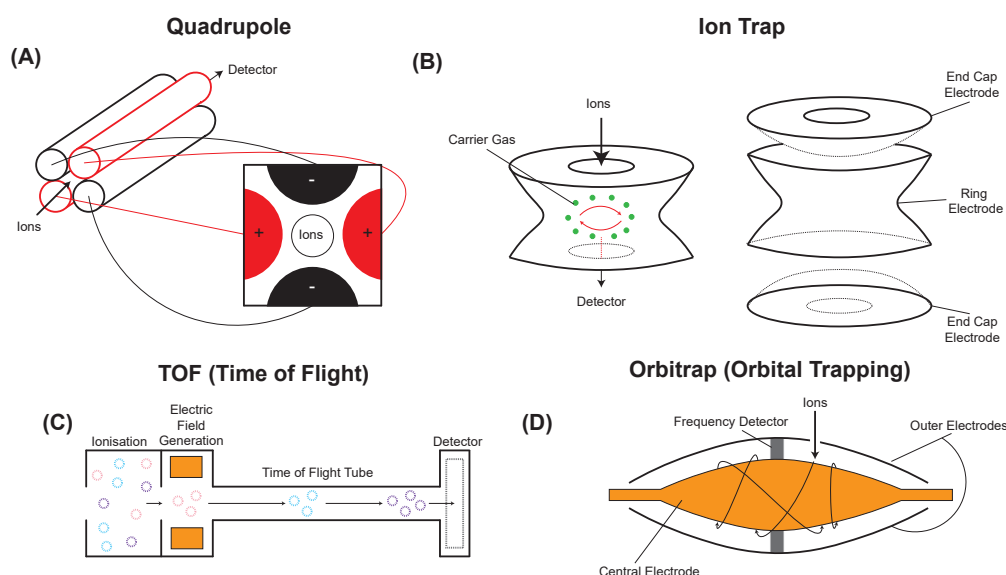


Figure 1.2: Basic diagrams of four commonly used mass analysers. (A) Quadrupole mass analyser. Four cylindrical rods with opposing rods having opposite charges, which alter the magnetic field applied to travelling ions. (B) Ion trap mass analyser. Ions are funneled into the cylinder and circulate, protected by an inert gas. After sufficient accumulation, ions are sent to the detector. (C) Time of flight mass analyser. Ions are accelerated into a flight tube and detected at the end. (D) Orbital trapping [Orbitrap] mass analyser. Ions are funneled into the analyser and circulate around the central rod. Oscillation of the ions about the rod correspond to the m/z ratio.

the shape of the central rod, ions are injected perpendicular to the rod and the Orbitrap detects m/z ratios by the oscillation of ions about the rod. The design of the Orbitrap allows for high mass resolution due to the energy independence of oscillations about the length of the central rod (Hu et al., 2005). Despite the many differences between mass analysers, they all share the same underlying goal, to measure a m/z ratio for a particular ion species, and are often used together in modern instruments.

Tandem mass spectrometry, or the coupling of various mass analysers for sequential analysis of ions, is the most common setup in modern mass spectrometry. Mass spectrometers typically may contain multiple mass analysers, however the goal is always to acquire whole peptide ions (MS1), and fragment peptide ions and analyse the fragments (MS2) (de Hoffmann, 1996; McLafferty, 1981). During MS1, whole peptide ions are acquired either in data dependent acquisition (DDA) mode,

where the top n most abundant ions at a given scan are selected for sequencing, or data independent acquisition (DIA) mode, where the mass spectrometer scans through m/z windows collecting *all* ions that are deconvoluted during data analysis. Generally, the MS2 fragment spectra is used for determining the identity of a peptide by matching these spectra to a database of predicted spectra based on all possible protein sequences, and the MS1 ion abundance value is considered the ‘relative peptide abundance’(Chapman et al., 2014). As a practical example, the Orbitrap Ascend Tribrid MS (developed by Thermo Fisher Scientific and released in 2022) uses a quadrupole for ion filtering and contains both Orbitrap and IT mass analysers, which allows the user to decide which type of mass analyser to use for MS1 and/or MS2 acquisition(He et al., 2023). Similarly, the QExactive line of mass spectrometers and advancements on this line (developed by Thermo Fisher Scientific and released in 2011) use a quadrupole to isolate peptide ions for fragmentation before acquisition of the fragment ions in an Orbitrap mass analyser(Michalski et al., 2011). Despite the 11 year difference in the Orbitrap Ascend Tribrid and the QExactive mass spectrometers, their basic construction remains the same.

1.2.3 Database Searching

After acquisition of peptide spectra and relative abundance data, database searching software is used to assign peptide sequences to each spectrum. These softwares automate the process of calculating the mass differences between adjacent N-terminal- or C-terminal-originating mass peaks in a measured mass spectrum (known as b- and y-ions, respectively), which correspond to the summed molecular weight of combinations of particular amino acids, by using a database of known protein sequences. The results, termed peptide spectrum matches (PSMs), describe the assigned peptide sequence and the charge assigned to the measured peptide. All acquired PSMs are then subject to false discovery rate (FDR) filtering. Historically, FDR filtering has been done using a reverse decoy database, where the protein

database used for searching is reversed and included in the search space. The estimated global FDR then becomes the ratio of false target PSMs identified above the determined threshold to the number of target PSMs above the threshold, and only peptides with a particular score are kept (usually FDR is set to 0.1%). Further, a database of contaminants is usually included in the search space, and potential contaminants are removed (e.g., keratins, mycoplasma). For label-free proteomics experiments, the relative abundance value for a PSM corresponds to the integrated area of a selected ion chromatogram of the m/z corresponding to that PSM across retention time, which is used for downstream statistical analysis and biological interpretation(Shuken, 2023). However, for more complex sample preparation techniques, other metrics are required to determine PSM abundance.

1.2.4 Isobaric Mass Tags

In the early 2000s, researchers began developing isobaric mass tags, chemical reagents with identical molar masses but differential fragmentation patterns, with the most notable being Tandem Mass Tags (TMT; Thompson et al. (2003)) and isobaric tags for relative and absolute quantitation (iTRAQ; Gygi et al. (1999); Ross et al. (2004); Chong et al. (2006)). Isobaric mass tags react with specific groups on peptides (e.g., primary amines), and are covalently ligated onto peptides post protein digestion during sample preparation. After labelling peptides, samples are combined and prepared together for MS analysis. Due to the identical molar mass of the tags, peptides from different samples co-elute and ionise at the same time, and are thus acquired during MS1 acquisition and fragmented for MS2 acquisition simultaneously(Dayon and Affolter, 2020). A second round of fragmentation and third round of mass analysis (MS3) is required to fragment the tags and identify which peptides contain which particular tag, thus allowing researchers to determine the sample of origin in these multiplexed samples. Frequently in isobaric tag MS, the MS2 or MS3 value is used for relative abundance quantification depending on the experimental design. However, MS2 quantitation suffers from uneven sample

load and coisolation of multiple ions, and MS3 quantitation requires extra filtering (synchronous precursor selection) for sensitive quantitation (Navarrete-Perea et al., 2018). Isobaric tags have the advantage of improving identification of low abundance peptide ions and increasing data completeness for high abundance peptide ions due to acquisition of ions from *all* samples at the same time. However, isobaric tags are limited in the number of samples available for multiplexing (currently available TMTPro kits can do 16-35 samples) and their overall cost (\$20,000 for 32-35-plex kits). Still, the sensitivity gains from using isobaric tags have birthed the field of single cell proteomics, which take advantage of multiplexing for identifying peptides at the single cell level (Petelski et al., 2021).

1.2.5 Summary

In summary, MS-based proteomics allows researchers to profile the proteome of cells or tissues and gain high-throughput biological insight regarding proteins. By exploiting the chemical properties of amino acids, HPLC separates peptides, which can then be ionised by application of voltage to the solution (electrospray). Ionised peptides are then introduced into the mass spectrometer, measured, and fragmented (and fragmented again, for MS3 quantitation). The resulting peptide spectra and accompanying ion abundances are identified using freely- or commercially-available software (e.g., MaxQuant, Spectromine/Spectronaught, Mascot, Proteome Discoverer), which produce both peptide- and protein-level relative abundance analysis. Those abundances can then be used for biological discovery, including differential abundance and pathway analyses, fueling further research on highly important biological pathways.

1.3 T cells and T cell Activation

1.3.1 The T cell Receptor and Major Histocompatibility Complex Restriction

T cells are a key part of the adaptive immune system, performing a wide range of functions from direct killing of virally infected or aberrant cells and coordinating responses of other immune cells. The function and specificity of most T cells comes from the α and β chains of the T cell receptor (TCR) signalling complex, which are unique to each T cell and directly bind antigens (small peptides and sometimes lipids)(Foster et al., 1990; von Boehmer, 1986). During T cell development in the thymus the TCR α /TCR β gene locus undergoes a recombination event, producing more than 100 million unique TCR α /TCR β pairs from about 4×10^{11} circulating T cells in humans with unique antigen-binding properties(Hedrick et al., 1985; Takeshita et al., 1989). Naive T cells undergo natural selection in the thymus, where T cells with no or excessive self-antigen recognition are cleared(Sprent et al., 1988). Defects in antigen recognition, usually through defects in TCR signalling, can alter the selection of healthy T cells and result in immune disorders(Abbas et al., 2004; Ohashi, 2002). Productive selection leads to a population of T cells with moderate self recognition, as moderate/incomplete activation is required for continued T cell persistence(Martin et al., 2006; Grossman and Paul, 2000). Thus, T cells maintain the ability to recognise self antigens through development, which is critical for their longevity.

Humans T cells have developed mechanisms to differentiate between self antigens and exogenous antigens through a process called *major histocompatibility complex (MHC) restriction*. Human T cells express the TCR coreceptors cluster of differentiation 4 (CD4) and CD8 during development, which uniquely bind to pockets on the side of MHC class II and MHC class I molecules, respectively(Zinkernagel and Doherty, 1997). Generally, MHC class I are ubiquitously expressed in human cells and present antigens originating from within the cell, leading to direct killing

of antigen presenting cells (APCs) by CD8+ T cells (or effector T cells; Agrawal and Kishore (2000)). MHC class II molecules are expressed on ‘professional’ APCs like macrophages, dendritic cells, and B cells, and are responsible for presenting antigens originating from outside the cell (Drozina et al., 2005). MHC class II-based activated CD4+ T cells (or Helper T cells) secrete small proteins called cytokines, which modulate the activity of other immune cells (Seder, 1994). While CD8+ T cells and the various CD4+ T cell subsets (T helper cells 1 [Th1], Th2, Th17, T follicular helper [Tfh], and regulatory T cells [Tregs]) each perform specific functions within the body, all T cell subsets require activation through the T cell receptor to perform their functions and persist.

1.3.2 Signalling from the T cell receptor

Binding of an antigen-loaded MHC molecule to the TCR induces a signalling cascade that primes the T cell transcriptome for activation. The TCR complex consists of the $\text{TCR}\alpha/\text{TCR}\beta$ pair and three transmembrane protein dimers; $\text{CD3}\epsilon/\text{CD3}\delta$, $\text{CD3}\epsilon/\text{CD3}\gamma$, and $\text{TCR}\zeta/\text{TCR}\zeta$ (Borst et al., 1983; Krissansen et al., 1986; Borst et al., 1984; Rutledge et al., 1992). TCR ligation promotes CD45-mediated dephosphorylation of Lck^{Y505} , which unlocks Lck from an inhibitory conformation and promotes Lck^{Y394} autophosphorylation (Ostergaard et al., 1989; D’Oro et al., 1996). Activated Lck phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) on the intracellular domains of $\text{CD3}\epsilon/\text{CD3}\delta/\text{CD3}\gamma$ (one each) and $\text{TCR}\zeta$ (3 each), which are dual YxxL motifs 7 amino acids apart (Housden et al., 2003). When phosphorylated, ITAMs act as docking sites for proximal TCR signalling proteins, particularly Zap70 which binds phosphorylated ITAMs through its dual SH3 domains. TCR-bound Zap70 is then phosphorylated at tyrosine 493 ($\text{Zap70}^{\text{Y493}}$) by Lck, which serves to activate Zap70 (Visco et al., 2000; Chan et al., 1995). Lck then bridges Zap70 to the integral scaffold protein linker of activation of T cells (LAT), bringing Lck and Zap70 in proximity to their substrates (Lo et al., 2018), activating the LAT-SLP76 signalosome and activating distal signalling

pathways. Phosphorylation of LAT^{Y132} by Zap70 is a kinetic threshold for T cell activation, as weak-binding antigens do not maintain TCR/pMHC binding long enough for sufficient LAT^{Y132} phosphorylation(Lo et al., 2019). Lck-activated Itk phosphorylates PLC γ 1^{Y783}, which controls calcium homeostasis and partially activates the RAS/MAPK cascade(Heyeck et al., 1997; Irvin et al., 2000; Weiss et al., 1991). Further, Lck phosphorylates Vav1 at multiple tyrosine residues which further feeds into the activation of p38 and Jnk, and reorganisation of the actin cytoskeleton(Crespo et al., 1997; Hehner et al., 2000). Finally, activated Itk phosphorylates GADS^{Y45}, enabling integration of costimulatory signals from CD28 which is required for complete T cell activation(Hallumi et al., 2021). While signalling from the TCR is fine-tuned to avoid aberrant activation, negative feedback to the TCR is equally necessary to avoid unnecessary and/or sustained T cell activation.

Signalling from the TCR leads to activation of transcriptional networks which regulate the expression of cytokines and/or excretion of cell lysis factors, however TCR signalling also engages negative feedback pathways. In particular, the kinetic segregation model predicts that TCR/pMHC binding and subsequent T cell signalling creates a physical barrier between Lck and CD45, ablating CD45-mediated dephosphorylation of Lck^{Y505}(Moffett et al., 2025). Further, Csk plays a positive role on Lck by phosphorylation of CD45^{Y1153}, promoting direct SH2 domain-mediated binding of Lck to CD45(Autero et al., 1994), and negative role on Lck activity through direct phosphorylation of Lck^{Y505}, promoting the inactive conformation of Lck(Bougeret et al., 1996). Four T cell protein tyrosine phosphatase (TCPTP), protein tyrosine phosphatase nonreceptor type 22 (PTPN22), SH2 domain containing protein tyrosine phosphatase 1 (SHP1), and dual specificity phosphatase 22 (DUSP22, also known as JKAP) dephosphorylate Lck^{Y394}(Gjörloff-Wingren et al., 1999; Li et al., 2014; Wiede et al., 2011; Štefanová et al., 2003; Carter et al., 1999). Multiple mechanisms have been proposed for SHP1-mediated Lck^{Y394} dephosphorylation, including inhibitory receptor (TRAIL-R) docking

and direct binding to Lck dependent on non-phosphorylated Lck^{S59}(Chyuan et al., 2024; Štefanová et al., 2003). PTPN22 is thought to be brought into proximity to the TCR along with Csk by binding PAG, or through an interaction with TRAF3 in a costimulation-dependent manner(Vang et al., 2012; Wallis et al., 2017). Constitutive myristoylation of JKAP to the plasma membrane colocalises JKAP and Lck after TCR firing(Li et al., 2014). A mechanism for TCPTP-based dephosphorylation of Lck has not been proposed, it is only known that TCPTP dephosphorylates Lck^{Y394} *in vitro* and that knockout of TCPTP in T cells elevates Lck^{Y394} abundance(Wiede et al., 2011). Protein tyrosine phosphatases are required for disassembly of the LAT/SLP76 signalosome, which spatially separates activating kinases (Lck, Zap70, and Itk) from their substrates. Further, clathrin-independent endocytosis of ligand-engaged TCRs mediated by Cbl and Cbl-b based ubiquitination of TCR ζ downregulates TCR signalling in response to effective T cell activation(Naramura et al., 2002). Together, feedback regulation is highly complex and involves both protein-protein interactions between key feedforward and feedback mechanisms, as well as spatial segregation of TCR signalling components.

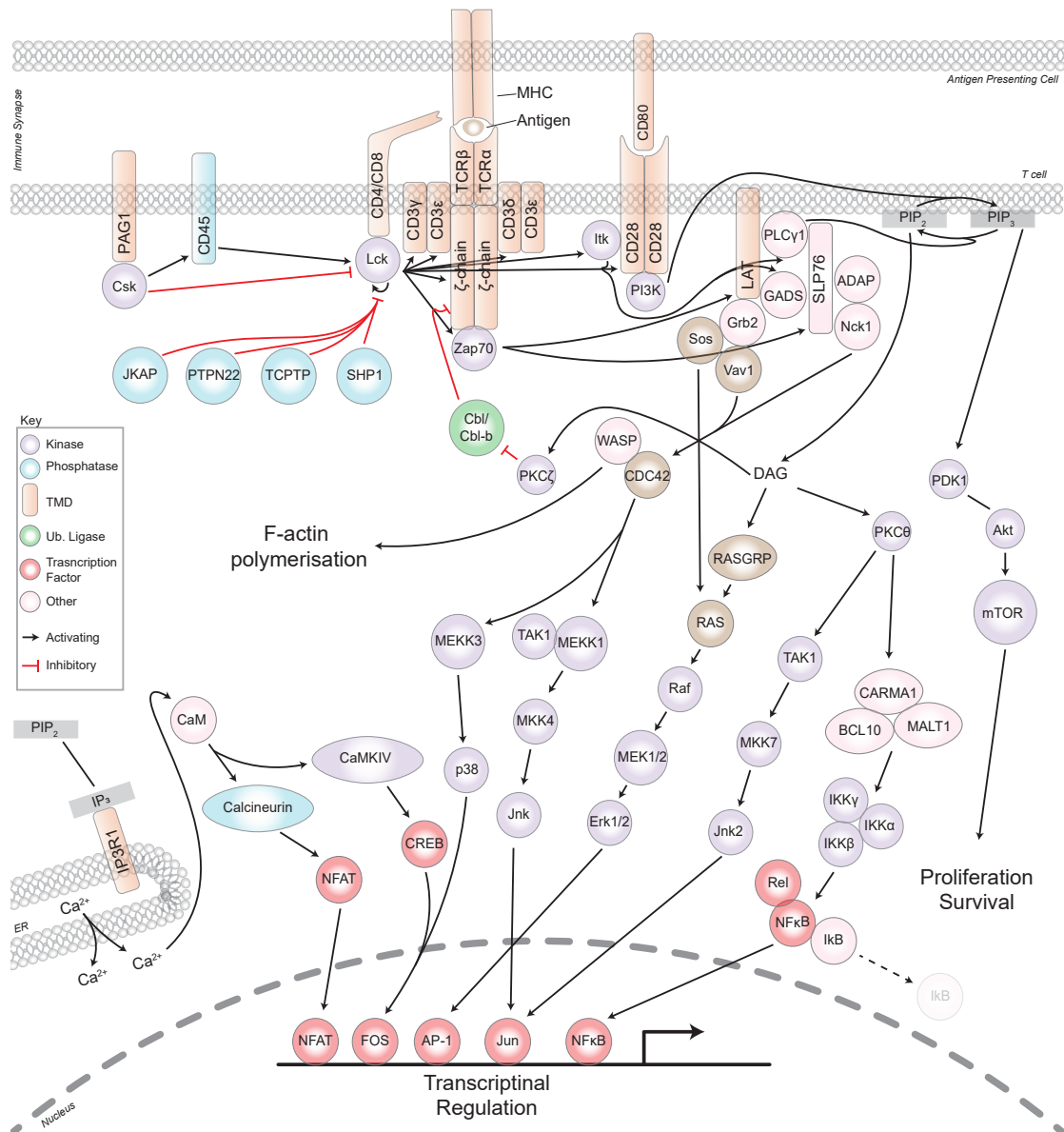


Figure 1.3: Schematic representation of signalling from the T cell receptor.

1.3.3 Mass Spectrometry and our Understanding of T cell signalling

While early studies focused on *in vitro* kinase reactions, point mutations, and functional readouts in cell lines to identify and characterise phosphorylation sites important for T cell signalling, mass spectrometry based phosphoproteomics catapulted the field forward. The first phosphoproteomic study of T cell signalling used immobilised metal affinity chromatography (IMAC), a purification technique using cationic metals to selectively trap phosphorylated peptides, identified 64

unique phosphorylated tyrosine residues on 32 proteins, many of which (e.g. ITAMs) were well characterised (Salomon et al., 2003). These sites were, as expected, responsive to CD3 ϵ /CD4 antibody crosslinking, which was and is a common method to induce T cell signalling. Later, Kim and White 2006 used this approach to map the tyrosine phosphorylation patterns after CD3 ϵ /CD28 crosslinking, which is required for complete T cell activation. Their data demonstrated that CD28 costimulation enhances phosphorylation of specific phosphotyrosine (pY) sites, namely CD28^{Y191}, Erk1^{Y204}, Erk2^{Y187}, and GADS^{Y45} by more than 50% (Kim and White, 2006). Considering that tyrosine phosphorylation makes up less than 1% of the phosphoproteome (Hunter and Sefton, 1980; Hunter, 2002, 2009), improvements in commercially available enrichment techniques (Cell Signaling Technologies monoclonal antibodies p-Tyr-100 and p-Tyr-1000, monoclonal antibody α -pY clone 4G10, and recombinant proteins like Src SH2 superbinder) have greatly improved pY peptide identification and our understanding of complex T cell signalling networks (Bergström Lind et al., 2008; van der Mijn et al., 2015; Bian et al., 2016). Locard-Paulet *et al.* 2020 used p-Tyr-1000 enrichment to profile the pY proteome of primary murine CD4⁺ T cells, identifying more than 250 unique pY sites, and providing a time-resolved dataset of primary T cell signalling events (Locard-Paulet et al., 2020). Tocheva *et al.* 2020 used p-Tyr-1000 along with antibody crosslinking to demonstrate the substantial impact of PD-1 in repressing signalling from the T cell receptor, many of which are not in the canonical TCR signalling pathway (Tocheva et al., 2020). Notably, Kaneko *et al.* 2024 used the Src SH2 Superbinder domain to enrich for pY peptides from critically ill COVID-19 patients, identifying upregulation of JAK/STAT phosphorylation sites and critical TCR signalling sites (e.g. ITAMs, immunoreceptor tyrosine based inhibitory motifs), demonstrating the immune activation stimulated by SARS-Cov-2 (Kaneko et al., 2024). Mass spectrometry based phosphoproteomics provides comprehensive profiling of phosphorylation sites in response to TCR stimulation, and has expanded our understanding of the TCR responsive phosphorylation sites during *in vitro/ex*

in vivo stimulation or disease states.

In addition to PTM-based signalling events, considerable effort has gone into mapping the interactome of various T cell signalling components. Early work evaluating components of TCR signalling pathway involved affinity purification (AP) of identified components and their binding partners, screening TCR signalling-deficient cell lines for culprit proteins, and reconstitution studies (Liao and Littman, 1995; Clipstone and Crabtree, 1992; Siliciano et al., 1992; Chan et al., 1991; Irvin et al., 2000; Walunas et al., 1994; Straus and Weiss, 1992). With advances in instrument sensitivity, coupling specific techniques like affinity purification with mass spectrometry (AP-MS) has allowed researchers to comprehensively profile the interactome of integral T cell proteins. Roncagalli *et al.* 2014 used AP-MS techniques to study the binding partners of SLP76 in activated primary murine T cells, identifying CD6 as a novel, independent hub for TCR signalling proteins like SLP76, Vav1, and Zap70 (Roncagalli et al., 2014). Ruminski *et al.* 2023 then used AP-MS to show that the adapter proteins Grb2 and GADS play major roles in constructing the SLP76 signalosome in Jurkat T cells, particularly losing recruitment of LAT (GADS $-/-$) and elevated recruitment of Vav1/CD6 (Grb2 $-/-$; Ruminski et al. (2023)). Voisinne *et al.* 2019 generated 15 mouse lines with TCR signalling proteins with terminal Twin-Strep-Tags (OSTs) and performed temporal CoIP-MS during T cell signalling, identifying 115 previously uncharacterised (162 previously known) interactions between TCR signalling proteins and discovering that TCR signal diversification happens with interactions at the plasma membrane (Voisinne et al., 2019). Similarly, Tian *et al.* 2015 combined AP-MS to evaluate the CD28-dependent signalosome. Tian *et al.* 2015 synthesised a 33 amino acid segment of the CD28 cytosolic domain with specific modifications (pY191, pY209, pY191Y209), immobilised them on beads, and captured proteins from Jurkat T cell lysates that bound to each version of the CD28 cytosolic domain, confirming the dependence on CD28 phosphorylation for PI3K recruitment and identifying novel interactions with the CD28 cytosolic domain (Tian et al., 2015).

Lastly, Luff *et al.* 2021 tagged p110 δ with BirA and performed AP-MS in naive and blasted murine T cells, showing that specific proteins like ICOS and PI3KAP1 are TCR stimulation-dependent interactors only in blasted T cells(Luff et al., 2021). Proteomic studies evaluating signalling complexes have confirmed previous, low-throughput research on TCR signalling complexes while greatly expanding the proteins involved in TCR signalling.

1.4 Chimeric Antigen Receptors and T cell Immunotherapy

1.4.1 The History of Chimeric Antigen Receptor Design

Research into chimeric receptor proteins began in the late 1980s, when researchers began studying the influence of ligand affinity on intracellular signalling properties of receptor proteins. These studies included Kuwana *et al.* 1987 showing that α -phosphorycholine antibody-derived variable regions and TCR-derived constant regions could be fused together in a gene product and maintain activity against bacteria expressing phosphorycholine(Kuwana et al., 1987). Irving and Weiss 1991 showed that a chimeric CD8 receptor with the intracellular domain of TCR ζ is sufficient for engaging the TCR signalling pathway, and later Desai *et al.* 1993 uncovered the role of dimerisation in CD45 phosphatase function by creating a chimeric receptor with the EGFR ligand binding domain and CD45 transmembrane/intracellular domains(Irving and Weiss, 1991; Desai et al., 1993). Similarly, Gross *et al.* 1989 and 1992 showed that expression of an immunoglobulin-TCR hybrid protein was possible and that they maintain signalling function(Gross et al., 1989; Gross and Eshhar, 1992). Gross *et al.* 1992 correctly predicted that chimeric receptors would one day reach the level of gene therapy for humans, as currently six gene therapy products using chimeric receptors are available for use in humans. The standard design proposed by Gross *et al.* 1992 is largely still in use today, and

serves as the functional scaffold for rational design of new CARs.

Modern chimeric antigen receptors (CARs) are single-pass transmembrane receptors consisting of five primary domains; the antigen binding domain, a hinge region, the transmembrane domain, one (or more) costimulation domains, and a primary TCR signalling domain (Figure 1.4A; Lindner et al. (2020)). Historically, binding domains have consisted for single chain variable fragments (scFvs) composed of the variable region of antibodies with high specificity for a target antigen, however recently researchers have developed CARs that use the natural ligand of cell surface receptors as their binding moieties (Gross and Eshhar, 1992; Branella and Spencer, 2022). Hinge domains play multiple roles in CAR function, including the transport efficiency of CARs to the membrane and exclusion of signalling proteins during immune synapse formation (Fujiwara et al., 2020; Xiao et al., 2022). Transmembrane domains (TMD) are typically sourced from T cell components (e.g. CD8/CD28), however the choice of TMD can have functional consequences, as Muller *et al.* 2021 show that the CD28 TMD causes heterodimerisation of CARs and native CD28 proteins (Muller et al., 2021). Considering the requirement of costimulatory receptor ligation for complete T cell activation, modern CAR design includes the intracellular domain of one or more T cell costimulatory proteins (e.g. CD28, 4-1BB, ICOS) to act as a ‘second signal’ for T cell activation (Jenkins and Johnson, 1993). In the six US FDA approved designs (Figure 1.4B), CD28 and 4-1BB intracellular domains account for all costimulation, however researchers are actively pursuing other costimulatory domains for clinical use. Finally, the intracellular signalling domain of TCR ζ is used as the primary stimulation domain for all US FDA approved CAR designs. Preclinical research has shown that other ITAM-containing domains (e.g., CD3 ϵ) are sufficient and sometimes advantageous for reducing side effects of CAR T cell therapies compared to classical designs. Despite the functional knowledge regarding CAR design, our understanding of CAR activation mechanisms and side effects are less well understood (Figure 1.4C).

1.4.2 Mechanisms of Chimeric Antigen Receptor Activation

By design, CARs activate T cells through canonical TCR signalling pathways. After engagement with a target associated antigen (TAA), CAR T cells engage an Lck/Fyn dependent phosphorylation of CAR ITAMs, allowing for recruitment of Zap70. Similarly to TCR signalling, CARs can engage the LAT/SLP76 signalosome, promoting signal diversification and transcriptional responses(Lindner et al., 2020). Tight binding of CARs to their TAA lead to exclusion of CD45 from the immune synapse which promotes CAR signalling, similarly to TCRs binding to pMHCs(Xiao et al., 2022). However, despite the design intentions of CARs, a large body of literature has shown that CARs do not engage the TCR signalling pathway similarly to native TCRs. In particular, Dong *et al.* 2020 demonstrated that CARs form unstable immunological synapses, which can impact their signalling and activation capacity(Dong et al., 2020). Barden *et al.* 2023 demonstrated that CARs and TCRs within the same cell act independently, as ITAMs on CD3 ϵ , CD3 δ , and CD3 γ are not phosphorylated in response to CAR engagement(Barden et al., 2023). Gudipati *et al.* 2020 showed that CARs, with CD28 or 4-1BB costimulation, require a higher antigen load to effectively engage CAR-TCR ζ phosphorylation and Zap70 recruitment than native TCRs(Gudipati et al., 2020). Further, Wu *et al.* 2021 found that a pMHC-targeted CAR had an unrestricted, longer duration, lower magnitude signalling responses than a native pMHC/TCR system(Wu et al., 2021). While there appear to be some universal differences between CAR and TCR signalling, researchers have observed many costimulation-dependent differences between CARs costimulated with CD28 or 4-1BB.

Phenotypically, 4-1BB containing CAR T cells are known to have higher longevity than CD28 containing CAR T cells owing to slower initial activation, however CD28 costimulation leads to more rapid and robust responses(Kawalekar et al., 2016; Zhao et al., 2015). At the level of signalling, multiple studies have

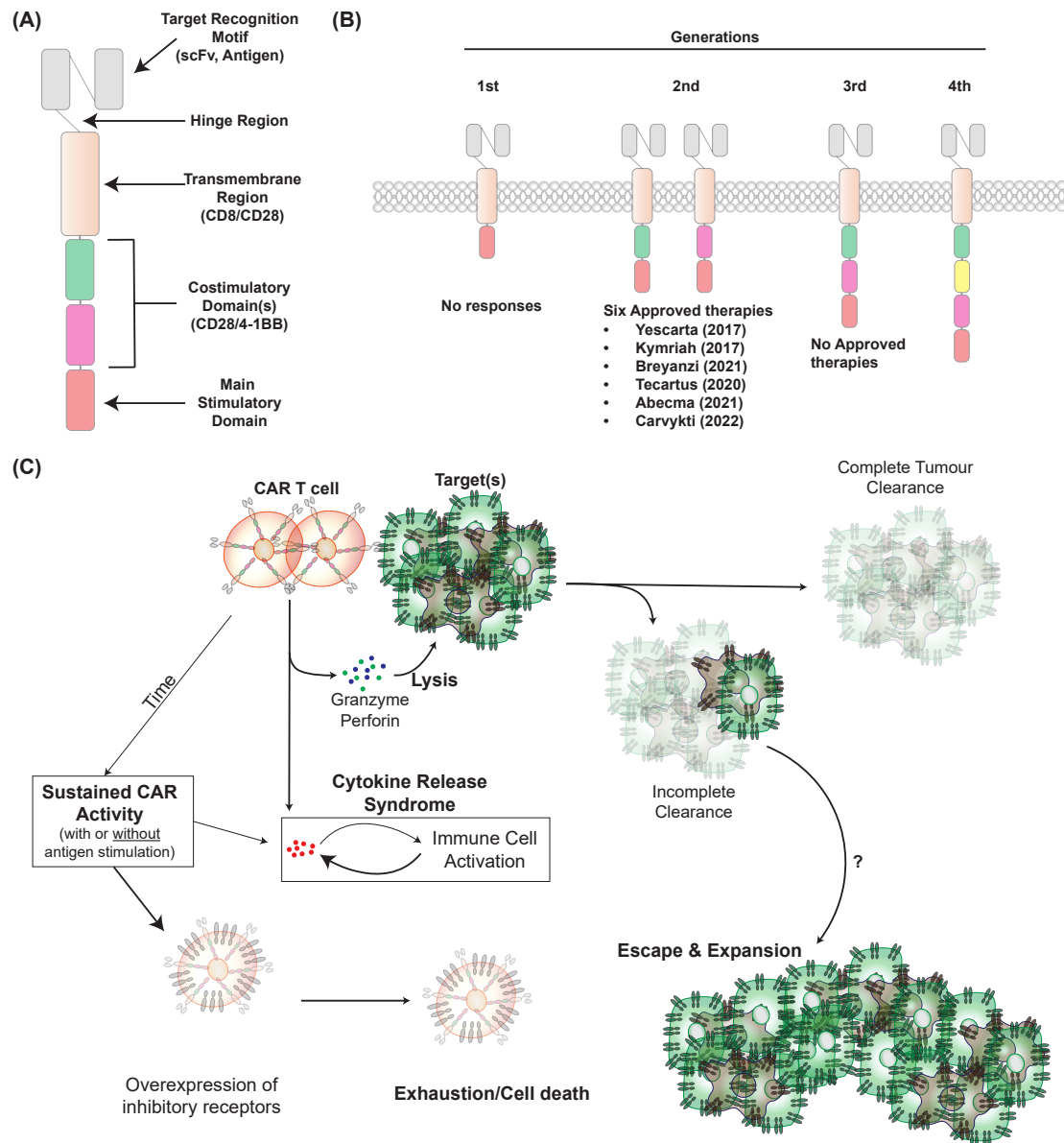


Figure 1.4: Chimeric antigen receptors are synthetic receptors designed to activate T cells and eliminate tumours. (A) Structural breakdown of CARs. (B) Cartoon representation of CAR generations. Approved therapies are indicated below where applicable. (C) Schematic representation of CAR function and side effects.

demonstrated that 4-1BB costimulation engages TRAF2 and TRAF3 leading to non-canonical NF- κ B activation(Philipson et al., 2020; Li et al., 2018). Sun *et al.* 2020 showed that a THEMIS-SHP1 complex is recruited to the 4-1BB costimulatory domain and thresholds CAR activation(Sun et al., 2020). Beyond these insights, signalling from 4-1BB costimulated CARs and the native 4-1BB receptor is not well understood. In contrast, CD28 costimulation during TCR engagement is well studied, and by extension CD28 costimulated CARs are more well understood. Researchers have shown that constitutive docking of Lck to the PYAP motif promotes ligand-independent CAR T cell signalling, and that mutation of the Itk binding site PRRP reduces overactivation and exhaustion of CAR T cells(Kofler et al., 2011; Salter et al., 2018; Boucher et al., 2021; Guedan et al., 2020). In line with native research on native CD28(Watanabe et al., 2006), CD28 costimulated CARs colocalise with GADS/SLP76 microclusters, which allow CD28 costimulated CARs to bypass the requirement of LAT for T cell activation(Dong et al., 2020). While the direct recruitment of T cell activating proteins to CD28 costimulated CARs can aid in initial CAR T cell responses, impaired negative regulation, renders CD28 CAR T cells susceptible to overactivation and exhaustion(Poorebrahim et al., 2021). Although the two prototypic costimulatory domains are used clinically, further research into the mechanism of activation for costimulation is required to effectively design new CARs for robust and safe antitumour responses.

1.4.3 Mass Spectrometry Enhances our Understanding of CAR T cell Function

Mass spectrometry based proteomics has greatly aided in our understanding of CAR T cell function. In the context of CAR T cell signalling, Salter *et al.* 2018 and Salter *et al.* 2021 have thoroughly evaluated the phosphoproteome of CAR T cells after engagement with stimulating antibody or immobilised ligand. First, Salter *et al.* 2018 employed global phosphorylation and tyrosine-specific phosphorylation enrichment methods to evaluate the phosphoproteome after engagement of CD28-

or 4-1BB-costimulated CAR T cells with activating antibodies. Salter *et al.* 2018 found that CD28 costimulated CAR T cells more robustly engaged the phosphoproteome after stimulation, which was due to constitutive Lck binding to the CD28 costimulatory domain(Salter et al., 2018). Salter *et al.* 2021 followed up on this work by comparing a CD28 costimulated CAR to native TCR/pMHC binding with CD28 costimulation. Here, Salter *et al.* 2021 used recombinant ROR1 or EBV-RAK tetramer followed by phosphotyrosine proteomics to compare CAR and TCR signalling in primary T cells. They found that key TCR signalling proteins, including the ITAMs on CD3 ϵ , CD3 γ , and CD3 δ , were only responsive to TCR stimulation, and that engineering a CAR to include CD3 ϵ and a GRB2 binding domain enhanced the antitumour efficacy of their CARs while reducing off-target effects(Salter et al., 2021). Ramello *et al.* 2019 coimmunoprecipitated CARs with their binding partners to show that 4-1BB and CD28 costimulated CARs with a CD28 transmembrane domain have distinct interactomes, however both interact with native TCR ζ and CD28 and induce phosphorylation of ITAMs on native TCR ζ (Ramello et al., 2019). Similarly, Ritmeester-Loy *et al.* 2024 identified canonical 4-1BB interactors (e.g. TRAF1, TRAF2, UBASH3A) and expected CAR interactors (e.g. Zap70) after 4-1BB costimulated CAR activation using AP-MS, and showed that these interaction networks are isolated from the TCR interactome(Ritmeester-Loy et al., 2025). In a Jurkat CAR T cell model, Teibo *et al.* 2025 used a metabolic labelling strategy to show that 4-1BB costimulated CAR T cells show robust proteomic changes only 10 minutes after engagement with a targeted tumour, leading to upregulation of the class II antigen processing helper and MIF receptor CD74(Teibo et al., 2025). Proteomic studies like these highlight the importance of mass spectrometry-based proteomics for understanding CAR function and have allowed for targeted hypotheses for improving CAR function.

Critical to the success of CAR T cell therapy is a TAA with high abundance, relatively high tumour specificity, and low potential for inducing TAA-specific responses. Early designs used scFvs targeting well characterised proteins with high

abundance on specific tumours (e.g. CD19, BCMA, HER2, EGFR, Mesothelin), however only CD19 and BCMA targeting CARs have been approved in the United States for human use (Figure 1.4). Recently, proteomic profiling of the tumour surfaceome has emerged as a leading method for identifying novel, tumour-specific antigens for targeted CAR T cell therapy. DeLucia and Lee 2019 describe an integrated transcriptomic/proteomic method for assessing the surfaceome of tumours, where cell surface proteins are biotinylated, extracted using streptavidin beads, and analysed using mass spectrometry. The data for cell surface proteins are then coupled to RNA-sequencing data, providing a complete picture of RNA transcripts and presented proteins for researchers to choose from (DeLucia and Lee, 2019). Nix *et al.* 2021 successfully used this technique to identify CD72 as a potential target for *KMT21/MLL1*-rearranged B cell acute lymphoblastic leukemia (B-ALL), a rare and frequently untreatable B-ALL subtype (Nix et al., 2021). Similar labelling techniques using various enzymatic reactions or enriching for common surfaceome PTMs (e.g., glycosylation) exist and have been used for TAA discovery (Pandey et al., 2023; Buhl et al., 2025; Di Meo et al., 2024). Using a metabolic labelling/co-culture approach, Griffith *et al.* 2022 recently demonstrated that Jurkat cells expressing a third generation scFv-based CD19 CAR effectively engaged phosphotyrosine signalling cascades, however no signalling response was observed in the CD19 expressing Raji cells (Griffith et al., 2022). Later, Callahan *et al.* 2025 used this technique to show that a CSF1R CAR targeted using CSF1, a native ligand of CSF1R, induced phosphotyrosine signalling in targeted THP-1 cells. Using primary T cells, Callahan *et al.* 2025 showed that the ligand targeted CSF1R CAR could either induce THP-1 cell proliferation at low effector-to-target (E:T) ratios or kill THP-1 cells at high E:T ratios (Callahan et al., 2025). These results demonstrate that mass spectrometry is instrumental in both identifying new TAAs for CAR T cell therapy and assessing the viability/biology of those targets in the context of CAR treatment.

1.5 Conclusions

The flexibility, accuracy, and sensitivity of modern mass spectrometers have revolutionised global protein analysis in cells. In particular, mass spectrometry-based proteomics has profoundly impacted our understanding of T cell biology and the application of T cells in cancer immunotherapy. Improvements in sensitivity and PTM purification workflows have allowed for unbiased identification of many novel T cell responsive phosphorylation sites that are still being understood. These methods have been extended to understand signalling from chimeric antigen receptor T cells, which differs both from native TCR signalling and between CAR constructs. Affinity purification mass spectrometry workflows have further expanded the interactome of TCR signalling proteins beyond the canonical, feedforward pathway proteins and of signalling from CAR T cells. Finally, mass spectrometry based proteomics has been instrumental in identifying targets for new CAR T cells using methods to evaluate the surfaceome of tumours. Overall, the field of T cell biology has benefitted massively from improvements in proteomics equipment and methodology.

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CHAPTER 2

Evaluating First-Pass, High Protein Capacity Sample Cleanup Products For Urea-based Phosphotyrosine Proteomics

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Abstract Many commercial desalting products exist for pre-MS protein/peptide cleanup, although few exist that can handle the high protein input (≥ 4 mg) required for phosphotyrosine enrichment. For these desalting products, the technical aptitude required for effective and organized desalting is often a barrier to entry for new users. Here, we evaluate four commercially available desalting techniques with varying degrees of automation, procedural difficulty, and chemistries to determine the most cost-effective, user-friendly, and sensitive technique for phosphotyrosine (pY) enrichment applications. We find that TECAN Narrow Bore Extraction (NBE) products are the most cost effective per sample and easiest to use, whereas ProtiFi's S-Trap are the most expensive per sample and Pierce C18 spin columns are the most difficult to use. ProtiFi S-Trap outperforms other preparation methods for protein profiling applications, uniquely identifying 885 unique proteins ($n \geq 3$). Consistently, ProtiFi S-Trap samples show the deepest pY site identification after Src SH2 superbinder enrichment, leading to the highest identification of significantly changing, biologically relevant pY sites in a Jurkat T cell signalling model. Our data show that ProtiFi S-Trap columns provide high quality pY site identification, improving identification of biologically meaningful and responsive pY sites.

2.1 Introduction

Mass spectrometry-based proteomics is a rapidly evolving field in the biological sciences, with a skyrocketing number of users attempting proteomics projects, with users

submitting more than 4,000 projects to the PRIDE repository per year between 2020-2023 and 3,241 in 2024(Jones et al., 2006). Improvements to MS instrumentation, methodology (bottom-up, top-down, and middle-down proteomics), and data processing techniques have increased the number of MS-based applications, including ultra deep shotgun protein profiling(Heil et al., 2023; He et al., 2023), single-cell characterisation(Ctortecka and Mechtler, 2021)., post-translational modification (PTM) enrichment(Muneer et al., 2025; Leutert et al., 2021), interactomics(Liu et al., 2026)., structural proteomics(Britt et al., 2022), spatial proteomics(Mao et al., 2021), and top-down proteomics(Roberts et al., 2024). These applications have led to a wave of discoveries concerning animal and human disease state proteomes(Guo et al., 2025) while generating new hypotheses and research aims for mechanistic biologists and translational researchers alike(Yu et al., 2022). MS-based proteomics, sample preparation, nano-LC systems, and MS instrumentation are increasingly complex, requiring expertise to understand and implement, which acts as a barrier to entry to the field. Some users elect to use MS core facilities(Bell et al., 2018; Chiva et al., 2021) or collaborate with expert laboratories, while using commercially available sample preparation and enrichment products to generate contaminant-free peptides as part of common bottom-up proteomic workflows.

For bottom-up shotgun proteomics, the most common sample preparation method involves lysis/sonication (Urea or non-ionic detergents), denaturation, reduction (e.g dithiothreitol) alkylation (e.g iodoacetamide), and enzymatic protein digestion (Trypsin or Lys-C alone or in combination), desalting using reverse phase column chromatography (typically C18), and concentrating peptides via lyophilization or chilled centrivap(Duong and Lee, 2023). Protocols for this style of sample preparation are well established and are in the process of being automated at several levels using liquid handling (LiHa) systems. These methods, while requiring some knowledge of liquid handling machines, reduce the propensity for user error in classic sample preparation techniques, such as vacuum manifold or centrifugal desalting(Vargas Medina et al., 2023; Rezvani et al., 2023; Fu et al., 2023). Despite this, many research groups and core facilities preferentially use classic sample preparation methods, particularly for the desalting step. Specific applications that require large protein input (~ 5 mg per sample), such as PTM enrichment proteomics, suffer from a general lack of commercial desalting products, with even fewer products

being automation capable.

Here, we evaluate four first pass, high input sample preparation products for use in phosphotyrosine enrichment applications using a Jurkat T cell signalling model. The products chosen (Waters Sep-Pak C18 vacuum cartridges, Pierce C18 spin columns, TECAN WWP2 Narrow Bore Extraction reverse phase columns, and ProtiFi S-Trap spin columns) are chosen based on their availability, chemistry, and methodology to represent a wide variety of commonly used sample preparation techniques. We find that Sep-Pak vacuum desalting is technically challenging, while Pierce C18 spin columns are overall the most difficult to use. ProtiFi S-Trap spin columns provide the highest overall peptide recovery ($\sim 75\%$), whereas TECAN WWP2 NBE columns provide the lowest variability yet lowest peptide recovery ($\sim 30\%$). As expected, ProtiFi S-Trap sample preparation reveals the most protein identifications (4821 total, 885 unique to S-Trap [$n \geq 3$]). Samples prepared with ProtiFi S-Trap columns show the deepest pY site identification after Src SH2 superbinder pY enrichment, identifying ~ 400 unique pY sites identified in triplicate. Finally, deep pY characterisation in ProtiFi S-Trap samples increases the identification of significantly responsive pY sites on biologically meaningful T cell signalling proteins.

2.2 Materials and Methods

2.2.1 Cell culture and stimulation

Jurkat (clone E6.1) were maintained in RPMI 1640 with 10% FBS (Cytiva #SH30910.03), 1x penicillin-streptomycin-glutamine (HyClone #SV30082.01), 2.5 $\mu\text{g}/\text{mL}$ Plasmocin (InvivoGen #ant-mpp) in a humidified incubator at 37 °C, 5% CO₂. For stimulation, 4e8 cells per replicate (4e9 cells total) were collected, washed once in 1x DPBS, then resuspended at a concentration of 2e8 cells/mL in 1x DPBS and rested for 30 minutes at 37°C. A schematic of the sample preparation process is shown in Figure 2.1. T cell receptor (TCR) stimulation was induced by the addition of α -CD3e (clone OKT3; Thermo #16-0037-85) and α -CD28 (clone CD28.6; Thermo #16-0288-81) for 30 seconds before addition of α -Mouse IgG (Jackson ImmunoResearch #115-005-062). The final concentration of primary and secondary antibodies were 2 $\mu\text{g}/\text{mL}$ and 22 $\mu\text{g}/\text{mL}$, respec-

tively. Stimulation was stopped by addition of 9 M Urea Lysis Buffer (9 M Urea, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM β -glycerophosphate in 20 mM HEPES). The ‘0 minute’ samples were generated by addition of 9 M Urea Lysis Buffer, then subsequent addition of primary and secondary antibody solutions to assure no cell stimulation occurred.

2.2.2 Proteomics Sample Processing

Proteomics sample preparation & In-solution digestion

Lysates were sonicated at 70% amplitude for 30 seconds, once on ice and centrifuged at 1,800 $\times g$ for 15 minutes before protein quantification by BCA (Thermo #23225). Western blotting samples were taken by diluting 50 μ L from each sample 1:1 in 2x Lamelli Sample Buffer (4% SDS, 125 mM TRIS-HCl pH 6.8, 20% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue). For each replicate, a solution containing approximately 4.5 mg of protein ($\sim 1e8$ cells) in 4 M urea was allotted into four tubes, then volume-normalized to 2 mL using 4 M Urea and frozen before subsequent proteomics sample preparation. This was to ensure that the input sample for each sample preparation technique was identical, allowing for accurate comparisons of each method.

For Waters Sep-Pak vacuum columns, Pierce C18 spin columns, and TECAN NBE columns, urea lysed samples were thawed and diluted in 20 mM HEPES to a volume of 4.45 mL before reduction using dithiothreitol (50 μ L of 1 M DTT, final concentration 10 mM) at 37 C for 30 minutes and alkylation using iodoacetamide (500 μ L 200 mM IAM, final concentration 20 mM) at room temperature in the dark for 30 minutes. The final concentration of Urea in each sample was less than 1.8 M, well within the functional range for Trypsin and downstream desalting. Protein was digested overnight at 37 °C in solution using a 1:100 ratio of trypsin (Promega #V5113) to protein, as previously described (Callahan et al., 2025; Griffith et al., 2022; Helou et al., 2015).

Sep-Pak C18 vacuum desalting

In the morning, samples were acidified to 1% trifluoroacetic acid (TFA) and centrifuged to remove debris before C18 vacuum desalting using 360 mg sorbent Sep-Pak C18

cartridges (Waters #020515), which is expected to bind ~3.6-18 mg of peptide per Waters FAQ webpage(Waters, 2021). Briefly, the vacuum apparatus was assembled as in Figure 2.1 and kept on except to discard waste and switch between waste tubes and collection tubes. One Waters Sep-Pak column was used per sample. Columns were equilibrated with 5 mL 100% acetonitrile (ACN) followed by two 3.5 mL washes in 0.1% TFA. Approximately 5.5 mL of sample was added to each column, allowed to pass through under vacuum, then the columns were washed in 1 mL, 5 mL, and 6 mL 0.1% TFA before elution in 7 mL of 40% ACN, 0.1% TFA (Final sample volume ~7.5 mL). After elution, 1/100 (70 μ L, diluted in 0.1% TFA) of each sample was taken for protein profiling. Samples were dried by overnight speedvac(Callahan et al., 2025; Griffith et al., 2022).

Pierce C18 spin column desalting

Overnight, in solution digestion was performed as in 2.2.1, acidified to 1% TFA, and centrifuged to remove debris the following morning. Samples were desalted using the Pierce C18 spin-columns (Thermo #89851) per the manufacturer's instructions. One column was used per sample. Briefly, the caps of each column were labelled with the sample ID (Figure 2.1), then spun at 5,000 xg for 1 minute to remove storage solution. Columns were equilibrated by centrifuging 300 μ L of 100% ACN twice followed by 300 μ L of 0.1% TFA twice at 5,000 xg for 1 minute each. Approximately 5.5 mL of sample was added to each column, allowed to flow through via centrifugation 300 μ L at a time at 3,000 xg for 1 minute each, discarding the flow through after each load (18 total loads). After sample loading, peptides were washed with 300 μ L of 0.1% TFA twice by centrifugation at 3,000 xg for 1 minute each. After washing, peptides were eluted by centrifuging 300 μ L of 50% ACN, 0.1% TFA twice at 3,000 xg for 1 minute. After elution, 1/100 (6 μ L, diluted in 0.1% TFA) of each sample was taken for protein profiling. Samples were dried by overnight speedvac.

TECAN A200 automated narrow bore extraction WWP2 reverse-phase desalting

Overnight, in solution digestion was performed as described previously for Pierce C18 and Waters products. Samples were acidified to 1% TFA, and centrifuged to remove debris the following morning. Samples were desalted using the Resolvex A200 (TECAN) positive pressure liquid handling system with TECAN Column NBE WWP2 1 mL 5.0 mg columns (TECAN #417-0051R-NBE). One column was used per sample. Narrow Bore Extraction columns were equilibrated with 900 μ L of 80% ACN, 0.1% TFA then washed with 900 μ L of 0.1% TFA using a 60 second positive pressure flash. Acidified samples were then loaded by sequential addition followed by a 45 second positive pressure flash, until the whole sample volume had passed through the column (6 loads). After loading, 900 μ L of 0.1% TFA was passed through the columns with a 60 second flash followed by a 30 second flash. Samples were eluted into a collection plate with three sequential 45 second flashes of 200 μ L 50% ACN, 0.1% TFA for a total elution volume of 600 μ L. After elution, 1/100 (6 μ L, diluted in 0.1% TFA) of each sample was aliquoted for protein profiling. Samples were dried by overnight speedvac.

ProtiFi S-Trap sample preparation

In solution reduction and alkylation were performed by addition of 20 μ L 1 M DTT (final concentration 10 mM) at 37 °C for 30 minutes, followed by 200 μ L 200 mM IAM (final concentration 20 mM) at room temperature in the dark for 30 minutes. Samples were centrifuged to remove any debris before acidification to 0.1% TFA and 12 mL of S-Trap buffer (90% Methanol, 100 mM TEAB, pH 7.1). Protein samples were bound to the ProtiFi S-Trap midi columns (C02-midi-10, one column per sample) by sequential centrifuging of 4 mL sample solution through the columns at 3,000 xg for 5 minutes (no equilibration step necessary). Columns were washed with 4 mL of S-Trap buffer three times before moving the column to a new centrifuge tube and adding digestion buffer (50 mM TEAB and 1:100 ratio of trypsin). The cap of the centrifuge tube was loosely shut, and tubes were wrapped in wet KimTech wipes before overnight trypsinisation at 37°C. The next morning, 500 μ L of 50 mM TEAB was applied to the column and the digestion solution was centrifuged out of the column at 3,000 xg for 5 minutes. Next,

500 μL of 0.2% Formic acid was centrifuged through the column, followed by 50% ACN, 0.2% Formic acid to complete elution. After elution, 1/100 (20 μL , diluted in 0.1% TFA) of each sample was taken for protein profiling. Large volume samples were dried by 36-hour lyophilisation and small volume samples were dried by overnight speedvac.

2.2.3 Phosphotyrosine enrichment with Src SH2 Superbinder

Enrichment of tyrosine phosphorylated peptides was performed as previously described (Callahan et al., 2025; Griffith et al., 2022; Callahan et al., 2024; Chua and Salomon, 2021; Chua et al., 2020; Bian et al., 2016; Tong et al., 2017; Yao et al., 2019). Briefly, ~ 4 mg of peptides were resuspended in 1.4 mL of ice cold IAP buffer (10 mM sodium phosphate monobasic monohydrate, 50 mM sodium chloride, 50 mM morpholino-propanesulfonic) for ~ 1 hour at room temperature with periodic vortexing. One hundred micrograms of Src SH2 superbinder (sSH2) conjugated agarose beads were allotted into 1.7 mL protein low-binding microcentrifuge tubes and washed 3 times in 1 mL of ice cold IAP buffer. sSH2 beads were incubated with samples for 2 hours at 4 $^{\circ}\text{C}$ on a 4 rpm rotator, and then centrifuged at 1,500 xg for 2 minutes. The supernatant was discarded, and the beads were washed 2 times in 1 mL of ice cold IAP buffer, then three times in 1 mL of ice cold HPLC grade water by centrifugation at 1,500 xg for 2 minutes at 4 $^{\circ}\text{C}$. After the last wash, all of the liquid was removed using an insulin syringe, and the peptides were eluted from the beads using 100 μL of 0.15% TFA on a 1,150 rpm shaker, twice. Peptide-containing solution was removed using an insulin syringe and desalted using Empore StageTips as described in Section 2.2.4.

2.2.4 StageTip desalting of enriched phosphopeptides

Enriched phosphopeptides were desalted using StageTips (Empore #70-2019-1001-3EA) per the manufacturer’s instructions. Tips were arranged in microcentrifuge tube adapters and activated by centrifuging 100 μL of 100% LC-MS grade methanol and 100 μL of 50% ACN, 0.1% TFA at 1,800 xg for 2 minutes. Tips were then washed by centrifuging 100 μL of 0.1% TFA twice at 1,800 xg for 2 minutes before loading samples and centrifuging at 1,800 xg for 2 minutes. Peptide-loaded tips were washed by centrifuging 100 μL of 0.1% TFA twice at 1,800xg for 2 minutes before elution by

centrifuging 100 μ L of 50% ACN, 0.1% TFA at 1,800 xg for 2 minutes, then reapplying the eluent solution to the column and centrifuging at 1,800 xg for 2 minutes. Eluent was then frozen and dried by speedvac before reconstitution in 5% Acetonitrile and 0.1% Formic acid and analysis by LC-MS.

2.2.5 Liquid chromatography tandem mass spectrometry parameters on the Orbitrap Ascend

Whole cell digest samples were reconstituted in 50 μ L with 1 μ L injected into the LC/MS system. Enriched pY samples were reconstituted in 35 μ L with 15 μ L injected. Peptides were separated using a Vanquish Neo UHPLC on a 7cm long (2cm bed length) 3 μ m particle size, 100 Å, Acclaim PepMap C18 Trap column and IonOpticks Aurora Ultimate 25cm long x 75 μ m ID, 1.7 μ m particle size analytical column throughout a 60-minute gradient held at 300 nl/min. Solvent A consisted of Water with 0.1% Formic Acid and Solvent B consisted of 80% Acetonitrile and 0.1% Formic Acid. Samples were acquired on a Thermo Fisher Orbitrap Ascend mass spectrometer.

The gradient consisted of loading/starting conditions of 5% B then to 30% B over 50 minutes followed by 95% B over 5 minutes then finishing at 99% B for the remaining 5 minutes. Combined control was used for loading and washing. The trap column was washed using 4 zebra washes, and the analytical column was washed using 3 alternating aqueous and organic cycles before being re-equilibrated for the next sample injection. Spray voltage was set to 2050V, Ion Transfer Tube Temperature was 275 °C, and a user-defined lock mass of 445.12003 was used. MS1 scans used wide quad isolation, orbitrap as the detector with a resolution of 120K and a mass range of 300-1500 m/z. A maximum injection time of 50 ms, normalized AGC target of 250% and RF Lens set to 30% was selected. Data was collected in centroid mode. MIPS (monoisotopic peak and peptide mode), Charge state (2-7), Precursor Selection Range (375-1500 m/z), Dynamic Exclusion(exclude after 1 time, 15 sec exclusion time, 10ppm low and high mass tolerance, excluding isotopes), Intensity Threshold (intensity range of 20000 (min) – 1E20 (max) with relative intensity threshold of 20%) filters were included. Data-dependent MS2 scans were acquired using cycle time (3 sec), a normalized HCD fragmentation energy of

30%, and a quadrupole isolation width of 0.7m/z. Fragments were detected using the Ion Trap with Turbo Mode at a scan range of 150-2000 m/z. A maximum injection time of 35 ms, normalized AGC target of 100%, and maximum injection time mode of dynamic was used. Data was also collected in centroid mode.

2.2.6 Database search parameters and peptide acceptance criteria

Thermo .RAW files were processed using Spectromine (Version 4.4.240326) using 1% peptide and protein FDR search constraints and all settings were system defaults, with the exception of excluding single hit peptides. The search included Trypsin enzyme specificity (maximum 2 missed cleavages), and peptide lengths between 7 and 52 amino acids. Mass tolerances at the MS1 and MS2 level were set to ‘Dynamic’, which allows Spectromine to determine the optimal mass tolerances for main searches following a first pass (rough) calibration and second pass (fine) calibration. Match Between Runs is not an option in Spectromine and therefore not implemented. A given precursor must pass identification cutoffs in each individual run where it’s quantity is reported. Regarding any alignments or calibrations during the Pulsar search step, these are not aimed at addressing missing data or carrying forward information from other runs. A given precursor must meet all identification cutoffs in that given run to be reported as identified and subsequently quantified, which is quite different from MBR. The reference Homo sapiens protein .fasta from UNIPROT (201908, 98,300 entries). Variable modifications consisted of oxidation (M), Phosphorylation (S, T, Y), and protein N-terminal Acetylation. Carbamidomethylation was the sole static modification for the Trypsin search. Identified peptides/proteins were filtered to a 1% FDR.

2.2.7 Data Analysis

Post-database searching analysis was performed as previously described(Callahan et al., 2025, 2024) using Python 3.12 with matplotlib (version 3.9.1), scipy (version 1.14.0), numpy (version 2.0.1), scikit-learn (version 1.5.1), pandas (version 2.2.2), py-venn, and a custom ‘helpers’ module.

For protein profiling samples, the Protein Groups, Peptides, and PTM reports were exported to .csv files and loaded into Python. For Protein Groups, Peptides, and PTMs, unique entries were determined by Gene Name, Stripped Sequence, or Flanking Sequence, respectively. For protein profiling PTMs, only PTMs annotated as ‘Phospho (STY)’ on the amino acid Y were kept. Potential duplicates were removed by keeping entries with the fewest missing values and highest median intensity, as previously described(Callahan et al., 2024). Sample counts were determined by counting the number of non-missing value entries in a label-free quantitation column for a particular sample. For Venn diagrams, an entry was kept for a manufacturer/group if there was at least one non-missing value in that group for that entry. For pY samples, the Peptides and PTM reports were exported to .csv files and loaded into Python. Unique entries were determined equivalently as for protein profiling samples, except PTM samples were required to have the ‘Phospho (STY)’ modification only on Y, which is conservative in that only peptides where the most probable phosphorylated amino acid is tyrosine are kept. Unique entry counting and Venn diagrams were created as described for protein profiling samples. Statistical significance between stimulated and unstimulated samples for each pY site was determined using a Welch’s T-test and global false discovery rate adjustment using the method of Storey(Welch, 1947; Storey and Tibshirani, 2003). At no point during the analysis were missing values imputed.

2.2.8 Western blotting

Western blotting was performed as previously described(Callahan et al., 2025; Griffith et al., 2022). Briefly, samples were separated on home-poured 10% polyacrylamide gels at 100 V for 120 minutes before transferring to a PVDF membrane (Millipore Sigma #IPFL00010) at 100 V for 100 minutes. Membranes were blocked using a TBS blocking buffer (LI-COR #927-60001) before overnight incubation in a primary antibody solution. The next morning, membranes were washed three times in 1X TBST before incubation in a secondary antibody solution for 60 minutes at room temperature. Finally, the membranes were washed three times in 1X TBST and twice in 1X TBS before imaging on a LI-COR Odyssey M scanner. Quantification was performed using LI-COR ImageStudio Version 5.2, and statistical analysis was performed in Python 3.12 as

previously described(Callahan et al., 2025).

Primary antibodies: pY clone 4G10 (1:2000, Cell Signalling Technologies #96215), GAPDH (1:10000, Millipore Sigma #G9545), PLC γ 1 (1:10000, Millipore Sigma #05-163), PLC γ 1Y783 (1:2000, Cell Signalling Technologies #2821), Erk1/2 (Cell Signalling Technologies #9107), ErkT202Y204 (1:2000, Cell Signalling Technologies #9101)

Secondary antibodies: IRDye 680RD Goat anti-Mouse IgG (LI-COR #926-68070) IRDye 800CW Goat anti-Mouse IgG (LI-COR #926-33210), IRDye 680RD Donkey anti Rabbit IgG (LI-COR #926-68073), IRDye 800CW Donkey anti Rabbit IgG (LI-COR #926-32213)

2.3 Results

2.3.1 Experimental Design and Rationale

To test the capability of commercially available, high protein input sample preparation products for phosphotyrosine proteomics, we used a Jurkat T cell receptor (TCR) stimulation model (Figure 2.1), which has very well described tyrosine phosphorylation and RAS/MAPK activation patterns(Abraham and Weiss, 2004; Helou et al., 2013). Five replicates of basal state (0 minute) and stimulated (2 minutes with CD3/CD28 crosslinking) were first lysed by addition of 9 M Urea, then split into 4.5 mg aliquots and volume normalised (final concentration ≤ 1.8 M Urea) for input to each sample preparation method. For sample processing and/or sample preparation applications, we used ProtiFi S-Trap, a centrifugal sample preparation column, TECAN's Narrow Bore Extraction (NBE), reverse-phase columns compatible with the Resolvex A200 liquid handling system for semi-automated desalting, Pierce C18 microcentrifuge columns, and Waters' Sep-Pak C18 vacuum cartridges (Figure 2.1, right). After trypsin digestion and desalting, 1/100 of each sample (~ 45 μ g) was taken for protein profiling (n=5), and ~ 4 mg (n=4) were used for phosphotyrosine (pY) enrichment using the Src SH2 superbinder (Figure 2.1).

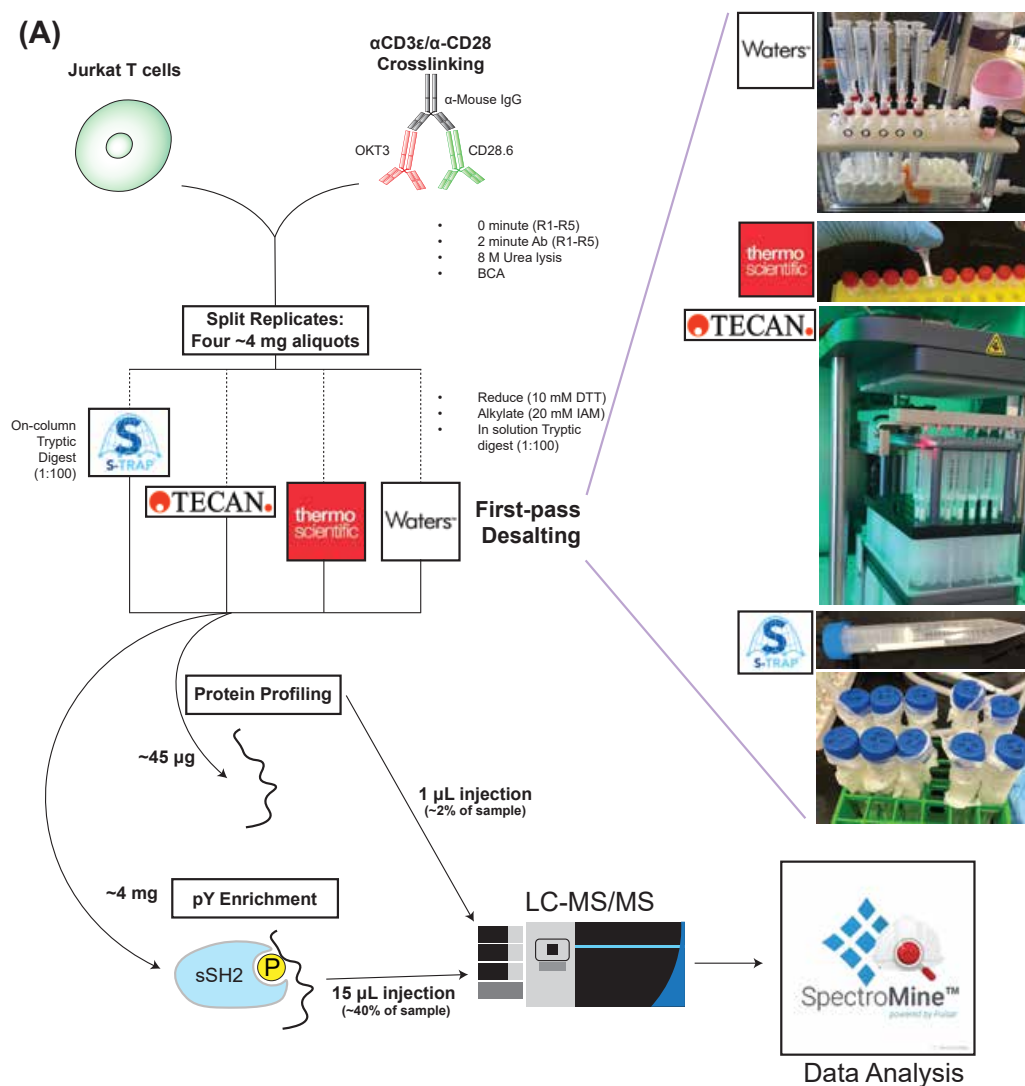


Figure 2.1: Workflow for evaluating high-protein input desalting products for proteomics applications. Schematic representation of sample preparation. Pictures depict the actual sample preparation products during use.

2.3.2 S-Trap Provides the Highest Peptide Recovery and Deepest Protein Identification

Physical setups of each digestion platform are noted in Figure 2.1 with only Waters and TECAN requiring specialized equipment to operate them (a Vacuum Manifold and Resolvex A200 positive Pressure Manifold respectively). We first sought to qualitatively evaluate the usability, cost, and effectiveness of each first-pass desalting product. As shown in Figure 2.2A TECAN NBE columns had the lowest total experimental time for any preparation method (53 minutes total desalting time, with ~ 15 minutes hands on pre and ~ 5 minutes hands on post trypsin), whereas Pierce C18 columns were the most laborious overall (2 hours and 3 minutes total for desalting, about two hours hands on time) due to the screw-cap design and the small working volumes per spin ($\sim 300 \mu\text{L}$). ProtiFi S-Trap Midi columns required more time before trypsin addition (3 hours 35 minutes total) due to sporadic column clogging during the binding buffer wash steps, however, post-trypsin only required eluting free peptides (20 minutes: about 2 hours hands on time) by centrifugation. Waters Sep-Pak C18 cartridges, the preferred desalting method in the Salomon laboratory, had a highly variable desalting time (46 minutes for 10 samples from an expert user, but ~ 2 hours for inexperienced users; about 75 minutes hands on time) and a large elution volume (7 mL), requiring 48 hours of lyophilization for complete drying. To evaluate the efficacy of each sample preparation method pre-LC-MS/MS analysis, we use “percent recovery”, defined as the measured peptide amount (μg , by A205 NanoDrop) divided by the expected peptide amount (μg , based on the BCA) for each protein profiling sample. On average, S-Trap had the highest percent recovery of any product (76.02%), followed by Pierce C18 columns (58.53%), Sep-Pak (40.52%), and NBE columns (28.06%) (Figure 2.2B). Higher recovery percentage from S-Trap came with an increased cost, as S-Trap columns are also the most expensive at $\sim \$17.50$ per sample (as of Jan. 2025) for the smallest purchasable amount (Figure 2.2C). Importantly, TECAN does not provide a peptide binding capacity for their columns, and we were only able to recover $\sim 1\text{--}1.5$ mg of peptide in the 96 well format. In summary, while TECAN NBE extraction is undoubtedly the easiest method requiring the least user interaction, ProtiFi S-Trap columns provide the highest percent recovery of any

product tested.

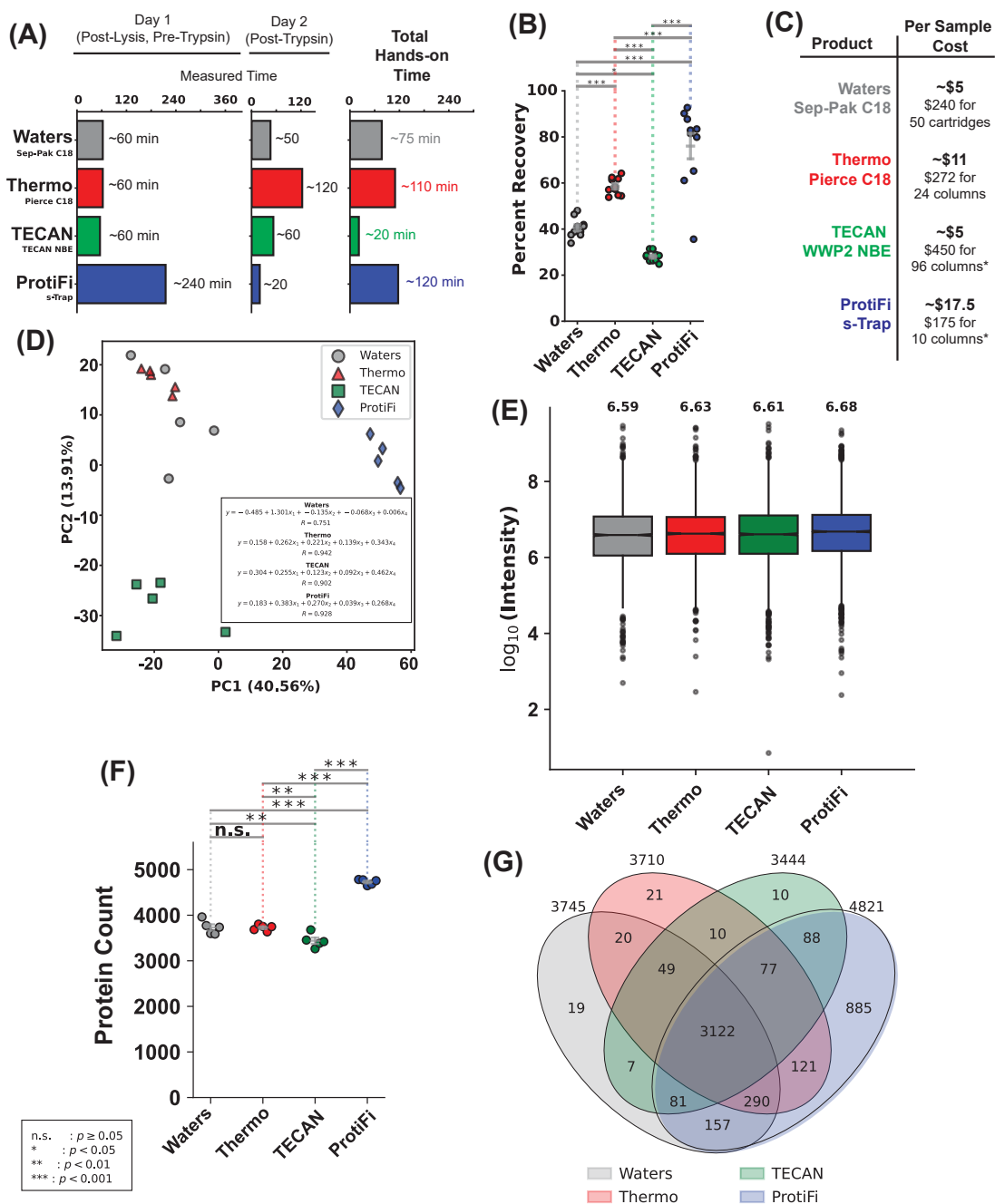


Figure 2.2: (Caption on next page)

Figure 2.2: ProtiFi S-Trap has the highest recovery of all tested products and repeatedly identifies the most protein groups in whole cell digest samples. (A) Measured time required to process 10 samples in parallel for each sample preparation product. (B) Percent recovery for each tested sample preparation product. Calculated as the ratio of the measured peptide content divided by the expected protein input for each WCD sample. (C) Table displaying the cost for each sample preparation product. (D) Principal component analysis of unique protein groups identified in WCD samples. (E) Distribution of intensities for unique protein groups identified in WCD samples ($n \geq 3$ per protein group). (F) Graph showing sample-specific counts of unique protein groups identified in WCD samples. FWER adjusted p-values are determined by Fisher's LSD corrected using the method of Holm and Sidak. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. (G) Venn-diagrams showing overlap of unique protein groups identified in WCD samples ($n \geq 3$ per protein group).

We found that ProtiFi S-Trap columns provided the highest maximum total ion current (TIC) with an average TIC max of 3.48E10, compared with 2.27E10 for Waters Sep-Pak, 2.31E10 for Pierce Spin Columns, and 2.32E10 for TECAN NBE columns (Figure 2.3). While TIC does not accurately predict peptide identifications, it is worth noting that the elution profiles for each sample preparation product are different, with TECAN WWP2 NBE prepared samples showing a late increase in TIC intensity (Figure 2.3). Generally, the median intensity of proteins identified in at least triplicate using S-Trap is slightly higher than other products (Figure 2.2E), a trend more pronounced when evaluating proteins identified in at least one sample (Figure 2.4A-B). Principal component analysis of unique protein groups showed replicate clustering for each product with Waters Sep-Pak and Pierce columns clustering together. Multiple regression indicated that TECAN NBE columns had the highest reproducibility, with a correlation coefficient of $R=0.928$ (Figure 2.2D), and similar coefficients of variation (CV) for protein groups identified using each product with Pierce C18 columns having the lowest median CV% (medians ~20-25%, Figure 2.4C). ProtiFi S-Trap columns had significantly higher protein group identification of all the products, whereas TECAN NBE columns had the lowest (Figure 2.2F). Of the 4821 unique protein groups identified in at least 3 S-Trap samples, 3122 protein groups were observed using all products and 885 are uniquely identified using S-Trap (Figure 2.2G). Evaluating stripped peptide sequences showed similar results, as ProtiFi S-Trap preparation repeatedly identified the most stripped peptide sequences (Figure 2.5). In agreement with enhanced protein identification, S-Trap samples also showed markedly improved identification of phosphopeptides (pS, pT, pY) in whole cell digest samples, identifying about 2-fold more phosphorylation sites per replicate with moderate reproducibility and identifying 273 unique phosphorylation sites compared with the other products (Figure 2.6). Together, our data confirm that, when using a standardized, urea-based workflow for protein profiling, ProtiFi S-Trap columns provide superior proteome characterization in Jurkat T cells.

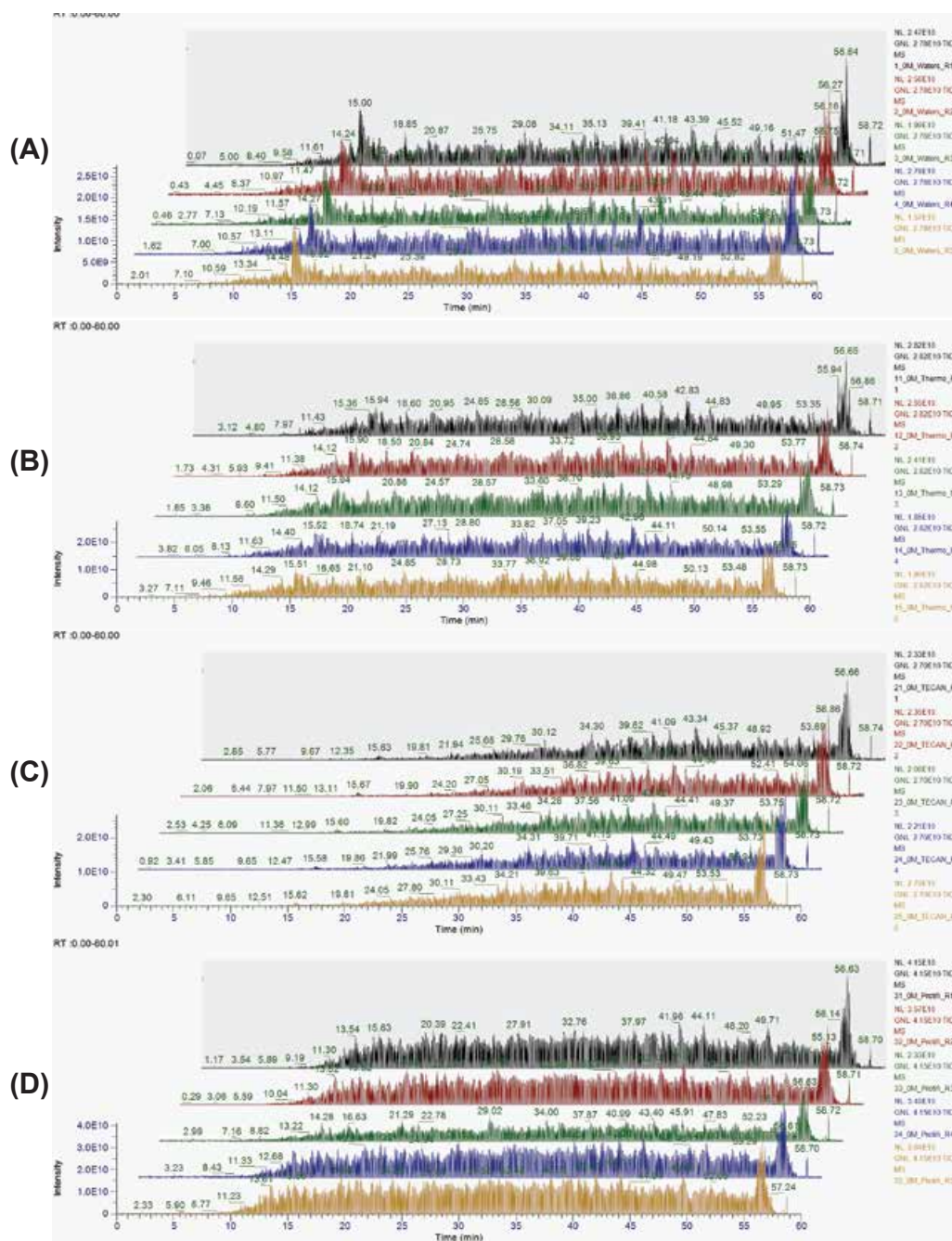


Figure 2.3: Total ion current for all whole cell digest samples from (A) Waters Sep-Pak vacuum columns, (B) Pierce C18 spin columns, (C) TECAN WWP2 Narrow Bore Extraction columns, or (D) ProtiFi s-Trap spin columns.

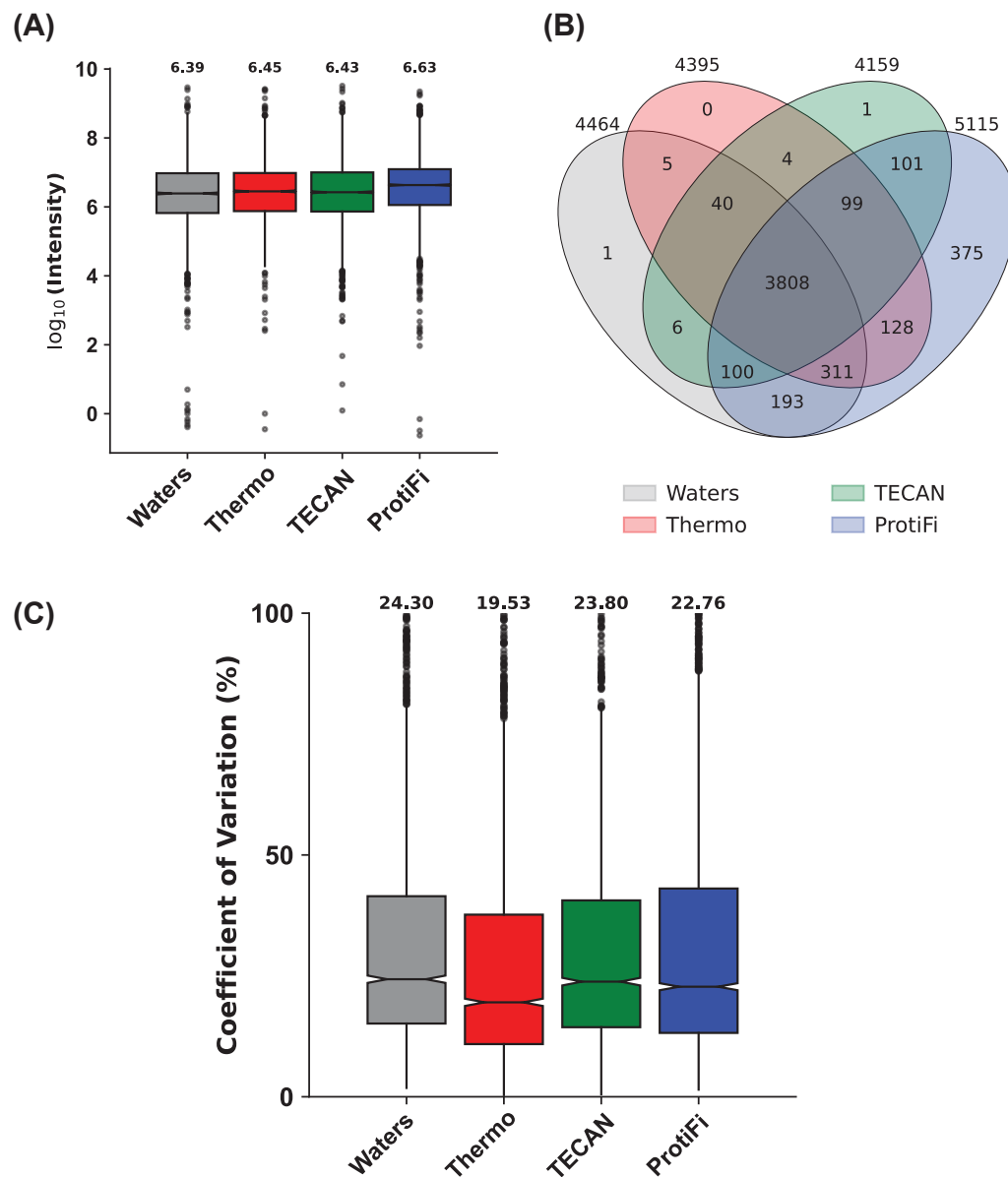


Figure 2.4: For non-repeatably identifiable proteins, ProtiFi s-Trap has the highest identification number and intensity distribution. (A) Distribution of the mean intensity for protein groups identified in at least one WCD sample. (B) Venn-diagram showing the overlap between different sample preparation methods for protein groups identified in at least one WCD sample. (C) Box-and-whisker plot showing coefficient of variation percentages for protein groups identified in at least 3 samples. Median CV% shown above each box.

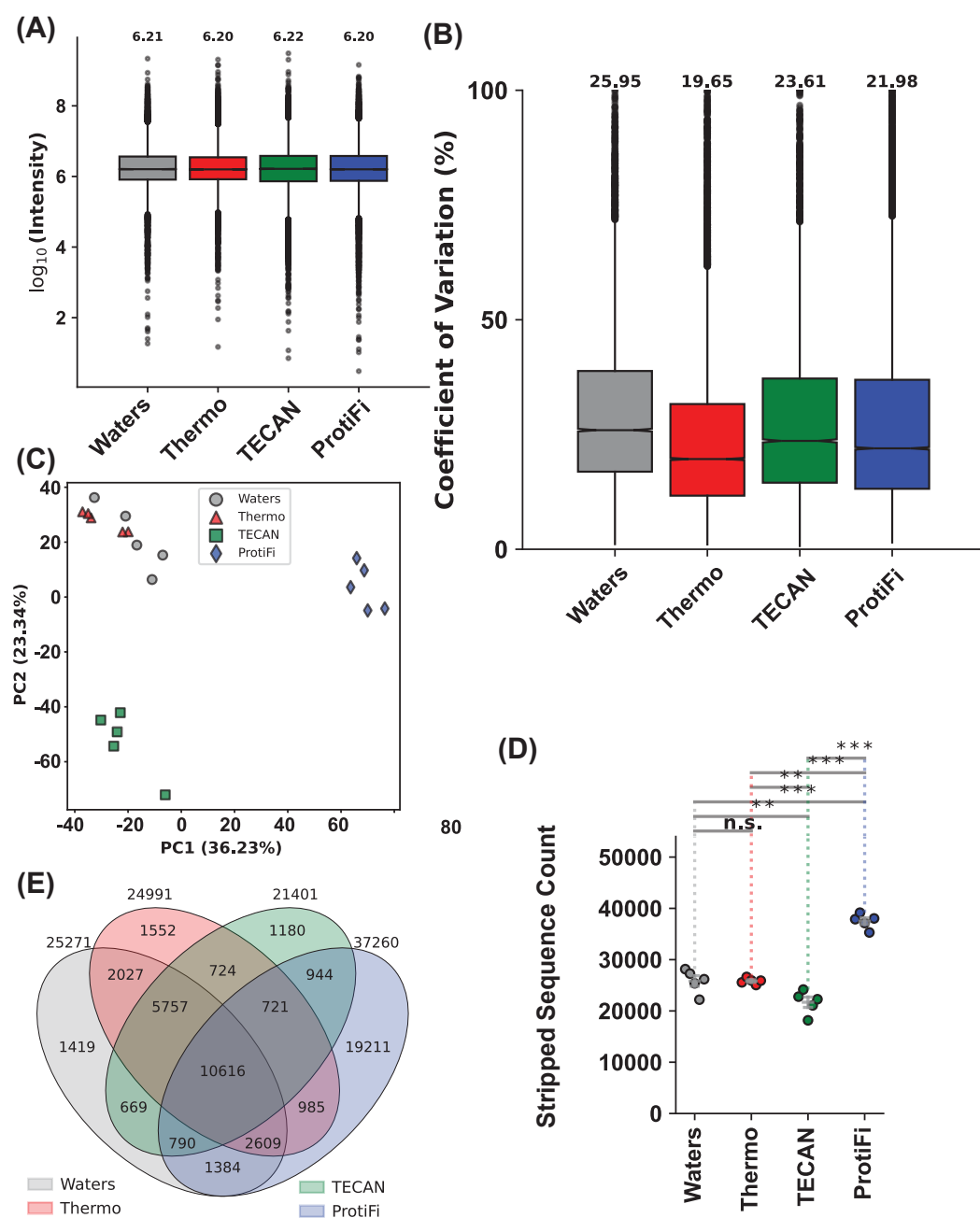


Figure 2.5: Protifi s-Trap identifies the most stripped peptide sequences compared with any other sample preparation product. (A) Distribution of intensities for unique stripped sequences identified in WCD samples ($n \geq 3$ per stripped sequence). (B) Box-and-whisker plot showing coefficient of variation percentages for stripped sequences identified in at least 3 samples. Median CV% shown above each box. (C) Principal component analysis of unique stripped sequences identified in WCD samples. (D) Graph showing sample-specific counts of unique protein groups identified in WCD samples. FWER adjusted p-values are determined by Fisher's LSD corrected using the method of Holm and Sidak. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. (E) Venn-diagrams showing overlap of unique protein groups identified in WCD samples ($n \geq 3$ per stripped sequence).

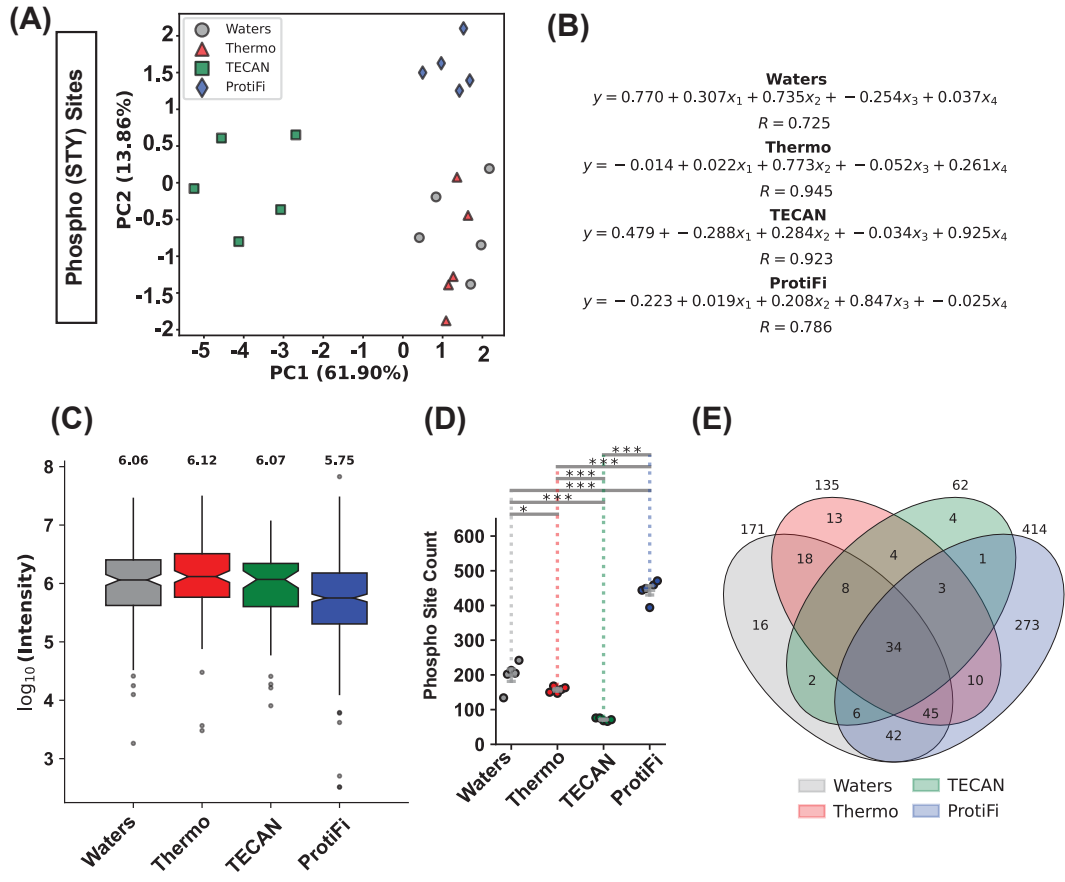


Figure 2.6: Without enrichment, ProtiFi s-Trap identifies the highest number of phosphorylation sites. (A) Principal component analysis of unique phosphorylation sites from WCD samples. (B) Line of best fit and correlation coefficient for unique phosphorylation sites from WCD samples as determined by multiple linear regression. (C) Distribution of the mean intensity for phosphorylation sites identified in at least one WCD sample. (D) Graph showing sample-specific counts of unique phosphorylation sites. FWER adjusted p-values are determined by Fisher's LSD corrected using the method of Holm and Sidak. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. (E) Venn-diagram showing the overlap in unique phosphorylation site identification between sample preparation methods ($n \geq 3$).

2.3.3 ProtiFi S-Trap Provides Superior pY site Identification

We next sought to determine whether specific first-pass sample preparation products improved the efficacy of a well-characterised pY enrichment protocol whereby phosphotyrosine samples are acquired by performing Src SH2 superbinder pY enrichments. After generating lyophilized enriched samples, peptides were reconstituted in 35 μ L of 5% Acetonitrile and 0.1% Formic Acid, and injecting 15 μ L (~43% of the total sample) due to the low abundance of tyrosine phosphorylation in cells (Hunter and Sefton, 1980; Hunter, 2002, 2009). The TIC shape for pY samples appeared distinct for samples prepared using each product, with Waters Sep-Pak samples showing a very intense peak around 22 minutes and ProtiFi S-Trap having relatively consistent intensities across the gradient (Figure 2.7). For pY sites measured in at least triplicate, there were no appreciable difference between average pY site intensity for stimulated and unstimulated samples. pY sites from Waters Sep-Pak, however, had slightly higher average intensities compared to other products (Figure 2.8C). Unique pY sites identified in all 0- and 2-minute samples were highly reproducible irrespective of the product used, with replicates generally clustering by PCA and correlation coefficients > 0.9 (Figures 2.8A-B, 2.9), however the median CV% of pY samples were high ($>25\%$) particularly in TECAN NBE prepared samples (123% and 87% for pY sites in unstimulated and stimulated samples, respectively; Figure 2.9C). Further, sSH2 pY enrichment was highly specific irrespective of the sample preparation method, with between 2- and 4-fold more pY sites identified than pS and pT sites (Figure 2.9D). Variability notwithstanding, ProtiFi S-Trap outperformed all other products in pY site identification generating between 300 and 600 pY sites per replicate (Figure 2.8D-E). Of the unique pY sites identified in S-Trap, some were specific to the S-Trap workflow, with >200 unique pY sites identified exclusively in S-Trap samples ($n \geq 3$; Figure 2.8F-G). Samples prepared using S-Trap also had the highest unphosphorylated peptide identification among the tested products and Waters Sep-Pak samples have the lowest (Figure 2.10). Together, these results show that samples prepared using ProtiFi S-Trap before pY enrichment had the highest pY site identification compared with Waters Sep-Pak C18 cartridges, Pierce C18 spin columns,

or TECAN NBE columns.

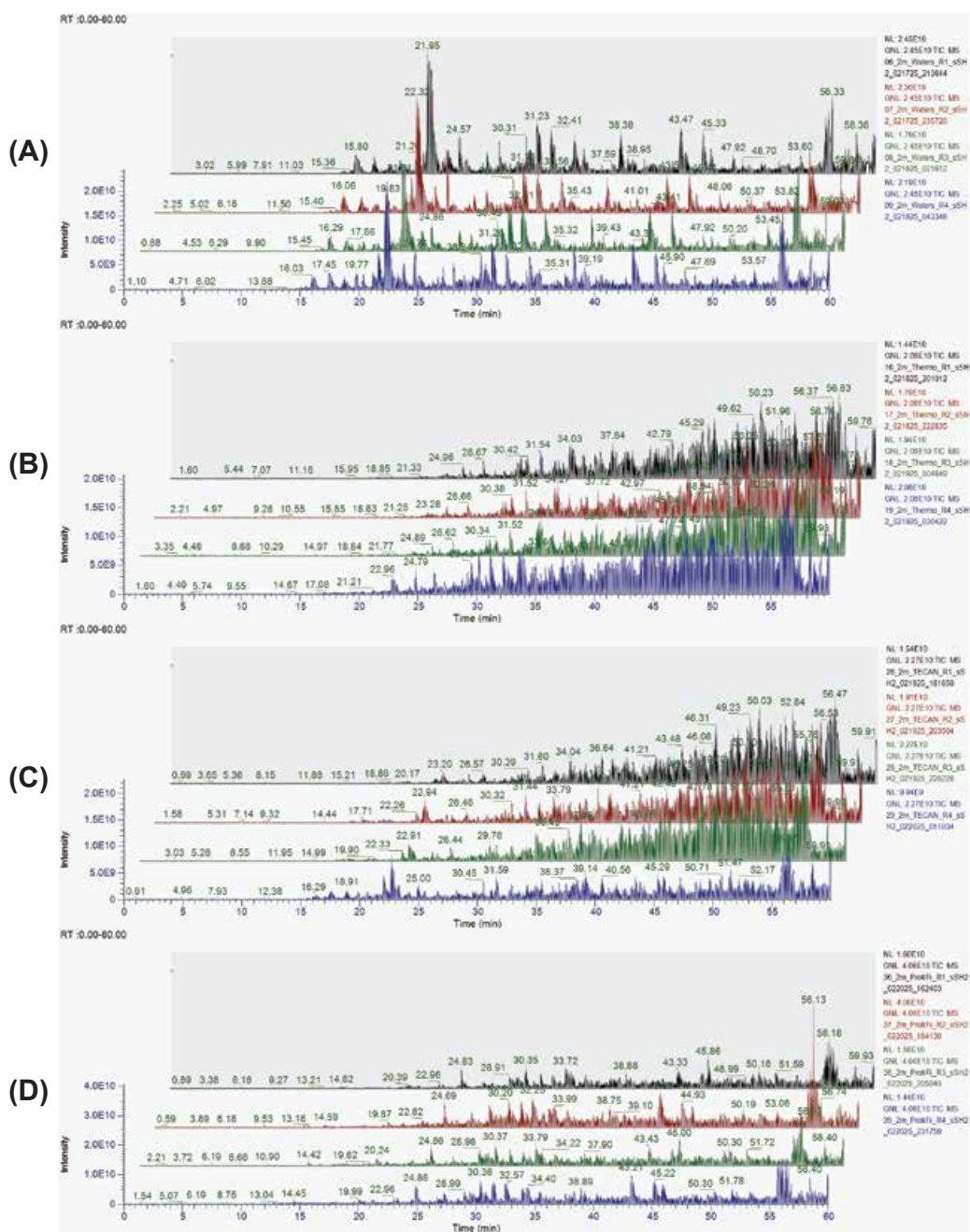


Figure 2.7: Total ion current for all 0m pY samples from (A) Waters Sep-Pak vacuum columns, (B) Pierce C18 spin columns, (C) TECAN WWP2 Narrow Bore Extraction columns, or (D) ProtiFi s-Trap spin columns.

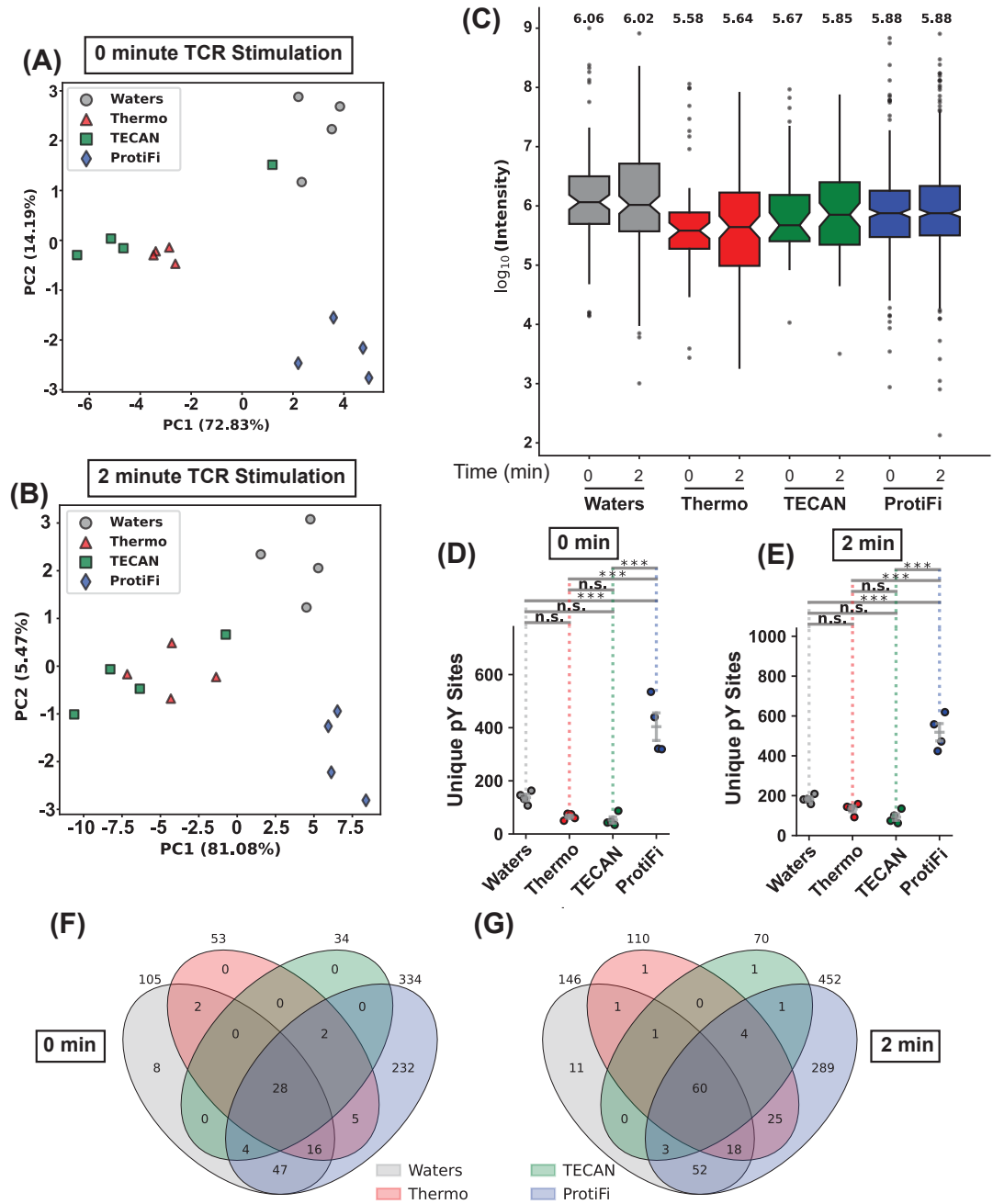


Figure 2.8: ProtiFi S-Trap sample preparation reproducibly permits the highest identification of the pY proteome in stimulated and unstimulated Jurkat T cells. (A-B) Principal component analysis of unique pY sites identified in 0- and 2-minute stimulation sSH2 enrichment samples, respectively. (C) Distribution of intensities for unique pY sites identified in sSH2 enrichment samples ($n \geq 3$ per pY site). (D-E) Graph showing sample-specific counts of unique pY sites in 0- and 2-minute stimulation sSH2 enrichment samples, respectively. FWER adjusted p-values are determined by Fisher's LSD corrected using the method of Holm and Sidak. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. (F-G) Venn-diagrams showing overlap of unique pY sites from 0- and 2-minute stimulation sSH2 enrichment samples, respectively ($n \geq 3$ per pY site).

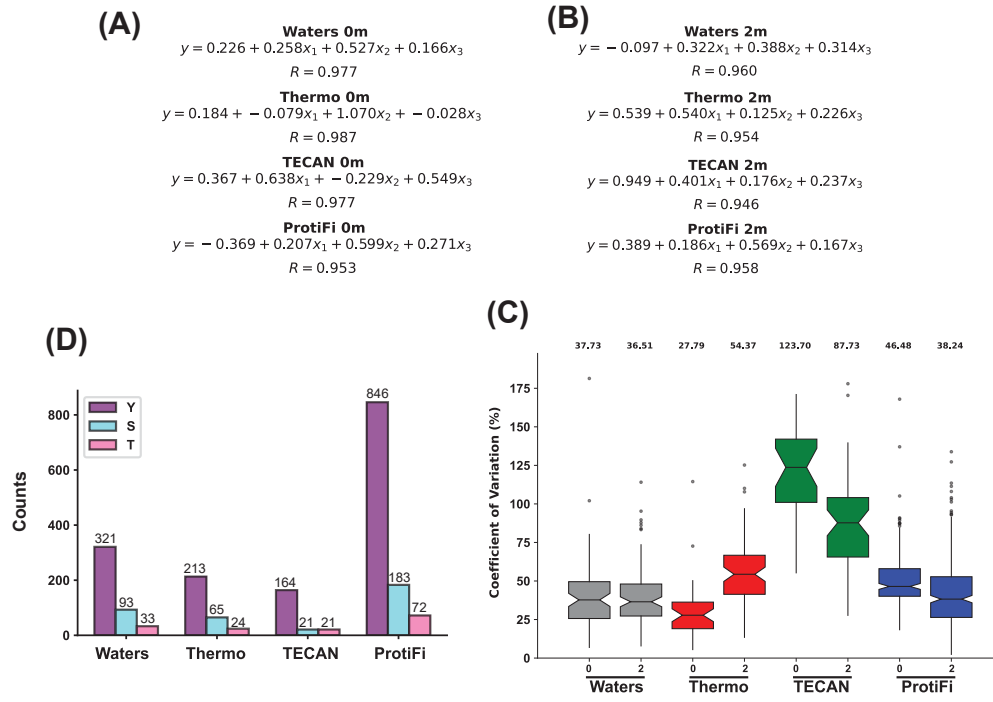


Figure 2.9: Reproducibility and specificity of sSH2 enrichments. Replicate reproducibility as measured by Multiple Linear Regression for (A) 0 minute and (B) 2 minute α -CD3 ϵ / α -CD28 stimulation samples. (C) Box-and-whisker plot showing coefficient of variation percentages for unique pY sites identified in at least 3 samples. Median CV% shown above each box. (D) Bar chart showing the number of pY, pS, and pT sites identified in at least one sample when using each sample preparation method.

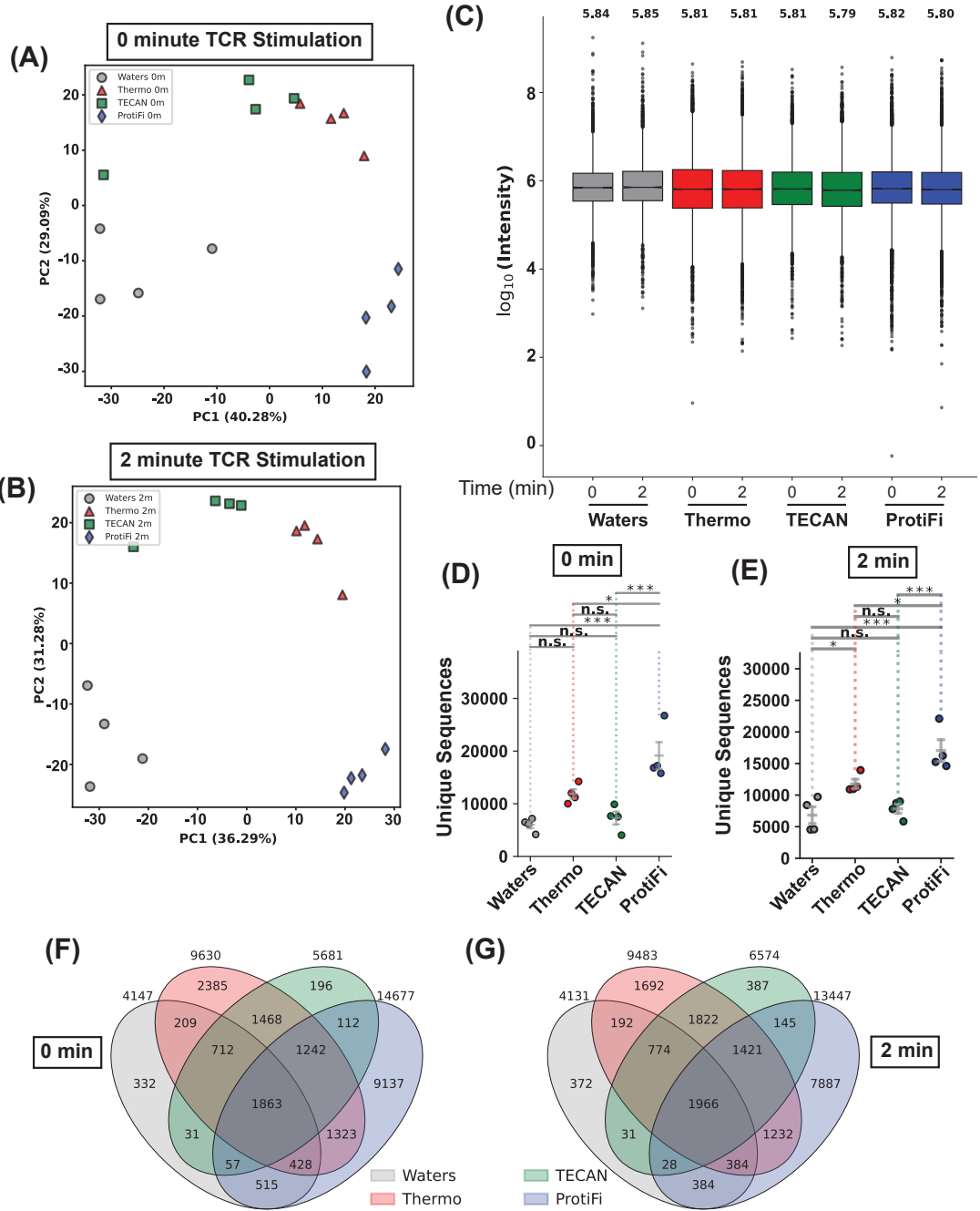


Figure 2.10: Analysis of non-pY peptides observed in sSH2 enrichments correlates with percent recovery after first-pass sample preparation. (A-B) Principal component analysis of non-pY peptides identified in 0- and 2-minute stimulation sSH2 enrichment samples, respectively. (C) Distribution of intensities for non-pY peptides identified in sSH2 enrichment samples ($n \geq 3$). (D-E) Graph showing sample-specific counts of unique stripped peptide sequences in 0- and 2-minute stimulation sSH2 enrichment samples, respectively. FWER adjusted p-values are determined by Fisher's LSD corrected using the method of Holm and Sidak. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. (F-G) Venn-diagrams showing overlap of non-pY peptides from 0- and 2-minute stimulation sSH2 enrichment samples, respectively ($n \geq 3$).

2.3.4 ProtiFi S-Trap Provides Superior pY site Changes Amongst Desalting Products

To evaluate whether the increase in pY site identification corresponded to an increase in biologically meaningful information, we compared the 0-minute and 2-minute α -CD3 ϵ / α -CD28 stimulated samples and evaluated the phosphorylation of key T cell receptor signalling proteins (Figure 2.11A). When comparing all sample preparation methods, we observed an increase in pY abundance with Waters Sep-Pak, Pierce C18, and ProtiFi S-Trap showing 14, 10, and 30, significantly changing unique pY sites respectively, ($q < 0.05$; Figure 2.11C, 2.11D, 2.11F, 2.11G). Samples prepared using TECAN WWP2 NBE columns showed a single significantly changing pY site, although other sites were increased albeit not significantly (Figure 2.11E, 2.11G). By Western blot analysis, we observed a significant increase in PLC γ 1^{Y783}, the activation site on PLC γ 1 and a substrate of interleukin-2 inducible T cell kinase (Itk)(Wilcox and Berg, 2003), Erk1^{T202Y204}/Erk2^{T185Y187}, the activation sites for Erk1/2, and global pY abundance, which supported the increase in pY abundance we observed using mass spectrometry (Figures 2.11B, 2.11H, Figure 2.12). Samples prepared using ProtiFi S-Trap columns showed significant induction of many known T cell signalling phosphorylation sites, including the immunotyrosine based activation motif (ITAM) sites CD3 ϵ ^{Y188} and CD3 ϵ ^{Y199}, Nck^{Y105}, PAG1^{Y417}, PLC γ 1^{Y771}, PLC γ 1^{Y783}, Zap70^{Y292}, and Zap70^{Y492}, all of which were also seen significantly changing when using at least one other product. Some important phosphorylation sites, including the immunotyrosine based activation motif CD3 δ ^{Y149}, the activation site Itk^{Y512}, the inhibitor site PLC γ 1^{Y775}, and the regulatory sites PLC γ 1^{Y1253} and PKC δ ^{Y313} were significantly increased exclusively when using ProtiFi S-Trap for sample preparation. Many other phosphorylation sites were also observed trending towards significance ($0.05 \leq q < 0.1$) including ADAP^{Y571}, SHP-2^{Y62}, SHP-2^{Y584} and Tyk2^{Y107} in Pierce C18 preparations (8 total), PAG1^{Y227} and Tyk2^{Y107} in Waters preparations (7 total), and PAG1^{Y163} and PAG1^{Y227} in ProtiFi preparations (15 total; Figures 2.11C-H). In summary, our data show that increased pY site identification corresponds to an increase in unique, significantly changing, and biologically meaningful pY sites observed by LC-MS.

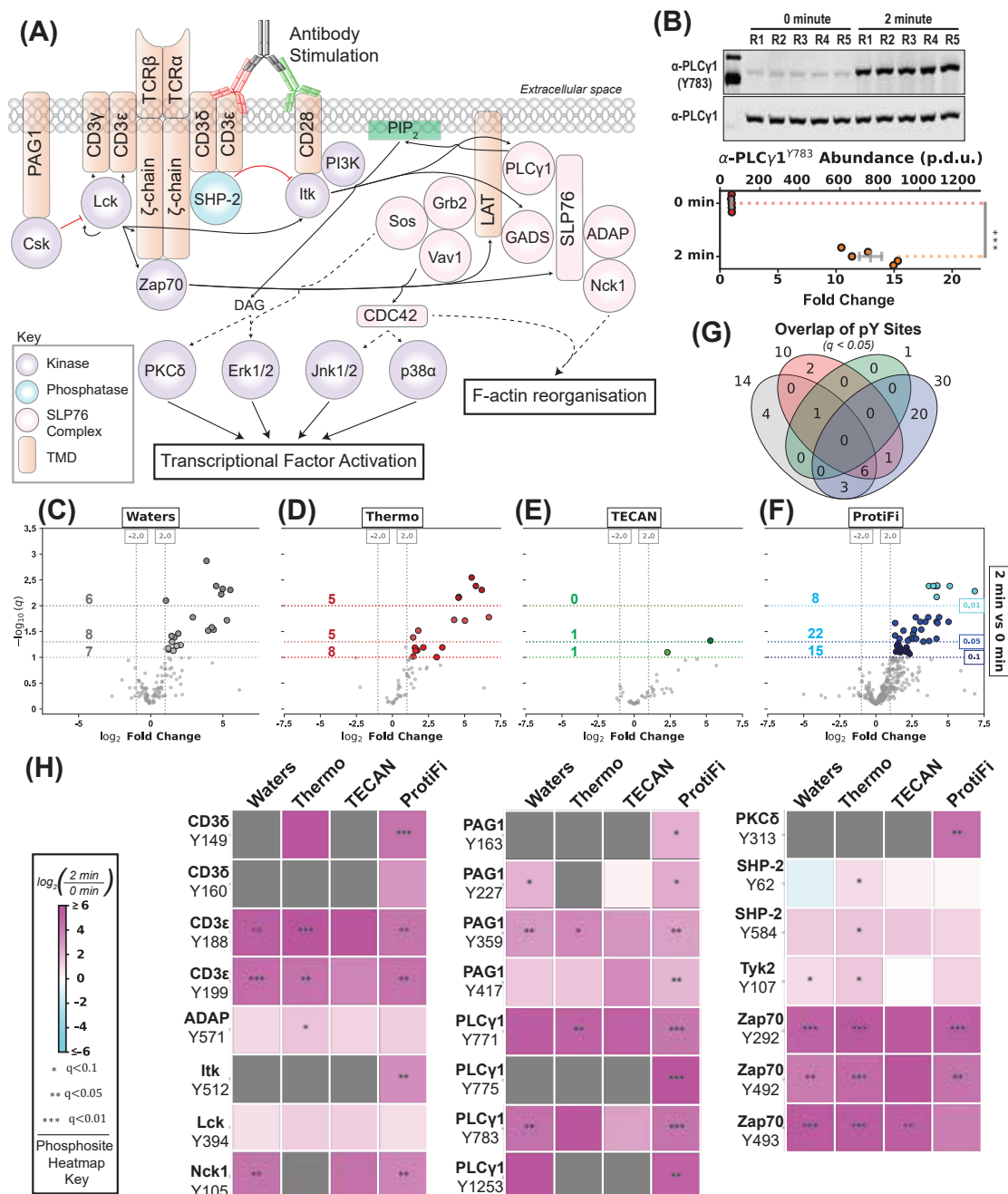


Figure 2.11: (Caption on next page)

Figure 2.11: Samples prepared using ProtiFi S-Trap show superior identification of biologically relevant, T cell receptor responsive pY sites. (A) Simplified schematic of T cell receptor signalling. (B-E) Volcano plot analysis comparing pY site abundance between 2 minutes and 0 minutes of stimulation for samples prepared using Waters Sep-Pak C18 columns, Pierce C18 spin columns, TECAN WWP2 NBE columns, and ProtiFi S-Trap spin columns, respectively. Values to the left indicate the number of unique pY sites in a given threshold ($0.1 > q \geq 0.05$, $0.05 > q \geq 0.01$, $0.01 > q$ from bottom to top). (F) Western blot analysis of PLC γ 1^{Y783} with accompanying quantification. *** indicates $p < 0.001$ by a Welch's T-test. (G) Venn-diagram showing the overlap of unique, significantly changing pY sites identified using each sample preparation method. (H) Heatmaps comparing 2 minutes and 0 minutes of T cell receptor stimulation for unique pY sites. Grey squares indicate $n \leq 3$ replicates for a given pY site in either 2- or 0-minute groups. * indicates $0.1 > q \geq 0.05$, ** indicates $0.05 > q \geq 0.01$, *** indicates $0.01 > q$.

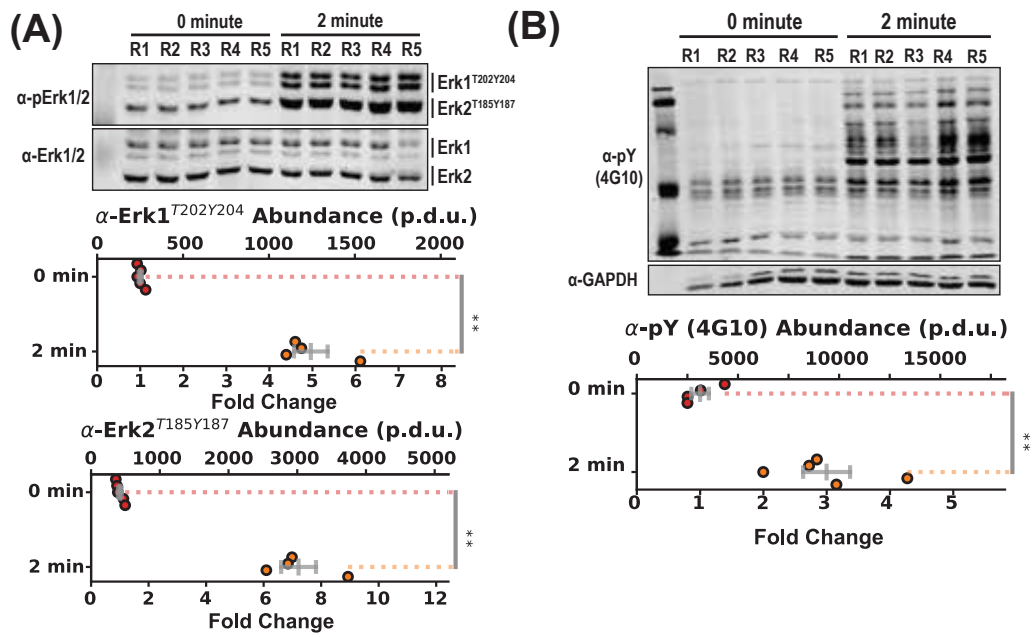


Figure 2.12: Stimulating antibodies lead to (A) phosphorylation of Erk1^{T202Y204} and Erk2^{T185Y187} and (B) global pY induction. ** indicates $p < 0.01$ by Welch's T-test.

2.4 Discussion

The sensitivity of modern mass spectrometers allows for the quantification and unique identification of thousands of peptides, with applications spanning from identifying proteins within single cells, non-denatured or intact proteins for structure-based analysis, and post-translational modification mapping(Heil et al., 2023; He et al., 2023; Ctorteka and Mechtler, 2021; Muneer et al., 2025; Leutert et al., 2021; Roberts et al., 2024; Guo et al., 2025). Further, automation using liquid handling systems has profoundly improved reproducibility of proteomics sample analysis and lowered the barrier to entry for new proteomics users(Vargas Medina et al., 2023; Rezvani et al., 2023; Fu et al., 2023). Salt removal is critical for the preparation of all proteomics samples, as residual salts and detergents interfere with peptide ionization and ultimately detection by MS(Duong and Lee, 2023). Research laboratories and core facilities have an array of commercial sample preparation products to choose from, and frequently choose a product based on their training, specific applications, or automation readiness. While evaluations of low protein input desalting products exist(Duong and Lee, 2023; Jehmlich et al., 2014), currently no work evaluating high protein input sample preparation products for applications to pY proteomics exists to our knowledge.

Here, we provide an evaluation of high protein input desalting methods for use in pY proteomics applications. Using four commercially available desalting products: Waters Sep-Pak C18 vacuum cartridges, Pierce C18 spin columns, TECAN WWP2 narrow bore extraction columns, and ProtiFi S-Trap spin columns, we evaluated the usability, recovery, peptide/protein profiling, and pY site identification using the Jurkat T cell receptor signalling model (Figure 2.1), which has very well-established tyrosine phosphorylation patterns(Abraham and Weiss, 2004). Utilizing Waters Sep-Pak columns with a low vacuum manifold required patience, focus, and good organizational skills, but were overall not very time consuming (Figure 2.1). Pierce spin columns were the most laborious to use due to their low volume format ($\sim 300 \mu\text{L}$) and the relatively high volumes (3-10 mL) required for dissolving and digesting 4 mg of protein (Figure 2.1). TECAN NBE columns, which may be used for on column digestion (not evaluated here), had nearly no hands-on time for desalting, was procedurally very simple once a

method is developed, and was very reproducible (Figure 2.1). ProtiFi’s S-Trap which required manual operation and a centrifuge, is unique in comparison to other products evaluated here in that the platform performs both desalting and digestion in suspension on the column. While this increased the hands-on time for pre-digestion processing, post-digestion time was substantially reduced (Figure 2.2A). Of all the desalting methods, ProtiFi S-Trap had the highest recovery variability and yet highest overall percent recovery ($\sim 75\%$ on average), while TECAN NBE columns had the lowest recovery ($\sim 30\%$) yet highest reproducibility (Figure 2.2B). Sample loss due to surface absorption is known to affect protein group identification of low input samples, and various methods have been proposed to mitigate sample loss (Nie et al., 2022; Kassem et al., 2021; Martin et al., 2020). For high protein input samples surface absorption is not a likely cause of loss, as the high protein concentrations likely saturate binding to plastics during preparation. However, the differing internal geometries of desalting columns and elution conditions (spin, vacuum, positive pressure) may lead to incomplete sample elution from columns after binding. Here, we try to match the manufacturer recommended protocols to provide a standardised picture of sample preparation using these techniques. Our results are in agreement with previous work, as Uslan et al. (2025) demonstrate that S-Trap has superior peptide recovery when compared to traditional in-solution based Urea/C18 sample preparation, although our C18 cleanup recovery is lower than what they report. Specifically, Uslan et al. (2025) prepared 100 μg digests and used GLScience’s MonoSpin C18 spin columns, which use smaller volumes and have less room for error compared with our 4.5 mg preparations (Uslan et al., 2025). Therefore, a Resolvex A200 compatible column format that combines the benefits of automation and reproducibility desired for high-throughput proteomics workflows, with ProtiFi S-Trap spin column chemistry may provide the most complete peptide recovery for high protein input digestion.

We find that higher peptide recovery translates to increased protein group and pY site identification using a standardized LC-MS workflow. While Waters Sep-Pak and Pierce spin columns had similar recovery ($\sim 40\text{-}60\%$) and protein identifications ($\sim 3,600$ proteins), TECAN WWP2 NBE columns had fewer identifications (3,400 proteins) attributable to the reduction in recovery. Notably, the binding capacity of TECAN WWP2 NBE columns are not provided, however we were only able to recover ~ 1 mg in

the 96 well format. ProtiFi S-Trap, in contrast, vastly outperformed all other desalting methods, identifying ~4,800 protein groups and 885 uniquely identified proteins (Figure 2.2D-G). Our results both support and contradict existing literature, as Uslan et al. (2025) report that S-Trap underperforms in protein group identification compared to Urea/C18 sample preparation and Varnavides et al. (2022) report that S-Trap slightly outperforms Urea/C18 sample preparation (Varnavides et al., 2022). However, both Uslan et al. (2025) and Varnavides et al. (2022) normalize the amount of peptide injected (2 μ g and 500 ng, respectively), a step that we refrain from performing. In commercial protocols for pY sample preparation (e.g. pTyr-1000) it is assumed that there is no (or minimal) peptide loss during initial sample cleanup, meaning the user runs blind to peptide loss before pY enrichment. Further, many core facilities may spot-check peptide concentrations before injection, but often rely on pre-digestion protein concentrations to estimate their peptide injection amount. The results we provide demonstrate that the choice of product for high protein input sample preparation can dramatically alter recovery and protein group identification, which is likely unknown to researchers performing PTM enrichments and may alter the resulting enrichment results.

Notably, the increase in identification using ProtiFi S-Trap for first-pass sample preparation corresponded to an increase in repeatable pY site identification, identifying about 250 unique pY sites per replicate and identifying more than 400 unique pY sites. Despite Pierce C18 spin columns displaying the second best peptide recovery overall, pY samples prepared using Pierce C18 columns had demonstrably worse pY site identification than Waters Sep-Pak or S-Trap and only marginally outperformed TECAN WWP2 NBE columns (Figure 2.8). The amount of input protein varies widely with regards to pY signalling studies due to cell type and pY stoichiometry (Callahan et al., 2025; Griffith et al., 2022; Labots et al., 2017; Haake et al., 2016; Locard-Paulet et al., 2020), however most protocols for label free pY proteomics of T cells require at least 100 million cells, or around 5 mg of input protein, which is not feasible for some applications (e.g., primary cells). Our data demonstrating that Waters Sep-Pak may suffer losses during sample processing and supporting that S-Trap reproducibly recovers peptides leading to high pY identification suggests that protein input for pY studies may be able to be lowered, potentially allowing pY enrichment in primary cells

without the need for tandem mass tags(Callahan et al., 2024). Principal component analysis showed a clear shift from protein group to pY site clustering where the Pierce changes from clustering with Sep-Pak to TECAN, respectively (Figures 2.2, 2.8). This suggests that the small gains in protein group identification we find using Pierce C18 spin columns versus TECAN WWP2 NBE columns does not consist of many pY containing peptides, whereas Sep-Pak and S-Trap retain pY peptides better (Figures 2.2, 2.8). Despite clustering of Pierce and TECAN pY samples by PCA, Pierce samples did display significant upregulation of 10 pY sites after TCR stimulation whereas TECAN pY samples only show 1 significantly upregulated site (Zap70^{Y493}). The increase in unique pY sites observed using ProtiFi S-Trap columns corresponded to an increase in TCR responsive pY sites, including many biologically meaningful pY sites on key TCR signalling proteins including CD3 δ ^{Y149}, CD3 ϵ ^{Y188}, CD3 ϵ ^{Y199}, Itk^{Y512}, PLC γ 1^{Y775}, and PKC δ ^{Y313} (Figure 2.11). The most notable modification is we did not solubilize S-Trap input proteins in SDS as required by the manufacturer(HaileMariam et al., 2018). Instead, to minimize variables in comparing these products, we simply ran Urea-lysates through the S-Trap columns which is not recommended by the manufacturer but has been successfully performed in another evaluation of common digestion and desalting techniques(Ludwig et al., 2018). Surprisingly, the S-Trap columns still outperformed the traditional Urea/C18 sample preparation methods which are recommended in pY enrichment protocols.

With an increasing interest in proteomics applications and the astounding variability in sample preparation techniques, having automatable, reproducible workflows is necessary to meet demand and expectations. The TECAN Resolvex A200 provides a reliable, easy-to-use liquid handling system suitable for high protein input desalting applications, however ProtiFi S-Trap chemistry provides superior recovery leading to superior identifications by LC-MS. A low-protein input (<300 μ g) format is offered by ProtiFi which is compatible with the Resolvex A200 liquid handling system, and our results indicate that a similar high-protein input product would be ideal for pY proteomics applications that require high protein input amounts.

2.5 Associated data

All code, graphs, and original, unprocessed Spectromine output files are available on GitHub (<https://github.com/Aurdeegz/Desalting-Comparison/tree/master>). Raw files are available from the ProteomeXchange Consortium via the Pride partner repository (Dataset ID: PXD064575).

Acknowledgements

This research is based in part upon work conducted using the Proteomics Core Facility, which was supported in part by the National Institutes of Health Grant No. 1S10OD036295 (Ascend Tribrid, FAIMS, Vanquish Neo) and the Division of Biology and Medicine, Brown University. We would also like to acknowledge NIH grant P01AI091580 and T32 GM007601.

Conflict of Interest Statement

TECAN provided the A200 unit and NBE columns for Arthur Salomon to evaluate, however TECAN had no role in the preparation of this manuscript. Aurora Callahan, Aisharja Mojumdar, and Nicholas DaSilva declare no competing interests.

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CHAPTER 3

Deep phosphotyrosine characterisation of primary murine T cells using broad spectrum optimisation of selective triggering

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Abstract Sequencing the tyrosine phosphoproteome using MS-based proteomics is challenging due to the low abundance of tyrosine phosphorylation in cells, a challenge compounded in scarce samples like primary cells or clinical samples. The broad-spectrum optimisation of selective triggering (BOOST) method was recently developed to increase phosphotyrosine sequencing in low protein input samples by leveraging tandem mass tags (TMT), phosphotyrosine enrichment, and a phosphotyrosine-loaded carrier channel. Here, we demonstrate the viability of BOOST in T cell receptor (TCR)-stimulated primary murine T cells by benchmarking the accuracy and precision of the BOOST method and discerning significant alterations in the phosphoproteome associated with receptor stimulation. Using 1 mg of protein input (about 20 million cells) and BOOST, we identify and precisely quantify more than 2000 unique pY sites compared to about 300 unique pY sites in non-BOOST control samples. We show that although replicate variation increases when using the BOOST method, BOOST does not jeopardise quantitative precision or the ability to determine statistical significance for peptides measured in triplicate. Many pY previously uncharacterised sites on important T cell signalling proteins are quantified using BOOST, and we identify new TCR responsive pY sites observable only with BOOST. Finally, we determine that the phase-spectrum deconvolution method on Orbitrap instruments can impair pY quantitation in BOOST experiments.

3.1 Introduction

Identification of tyrosine phosphorylation events by LC-MS/MS remains difficult due to the low abundance of phosphorylated tyrosine (pY) in the proteome (Hunter, 2002, 2009; Hunter and Sefton, 1980) despite the advent of robust pY capture methods, like the Src SH2 superbinder (sSH2) domain and pY-specific antibodies (Tong et al., 2017; Yao et al., 2018, 2019; Kaneko et al., 2012). Robust pY capture requires inordinate protein inputs, typically more than 5 mg (Abe et al., 2017; Belmont et al., 2017; Ahsan and Salomon, 2017; Johnson and White, 2012; Bian et al., 2016), hindering applications of pY proteomics in biologically relevant samples that are inherently limited in quantity, like primary cells or patient tissue samples (Johnson and White, 2012; Locard-Paulet et al., 2020; Gajadhar et al., 2015). Even when used successfully, the performance of pY capture methods in primary cells or patient tissue samples is suboptimal when compared to similar model systems. For example, carefully performed pY proteomics studies in primary T cells can expect a total of 200–400 unique pY sites across all conditions (Locard-Paulet et al., 2020) while similar studies in the Jurkat model system can expect 500–1600 unique pY sites (Belmont et al., 2017; Griffith et al., 2022; Helou et al., 2015). A swath of recent literature is unravelling the importance of uncharacterised pY sites on T cell proteins, including Zap70, Lck, Itk, GADS, Cbl-b and THEMIS, as well as pY sites on proteins closely related to T cell signalling proteins (Kästle et al., 2022; Barreira et al., 2018; Courtney et al., 2019; Gallagher et al., 2021; Hallumi et al., 2021; Huszenicza et al., 2024; Kagohashi et al., 2024; Kumar et al., 2018; Kumaradevan et al., 2018; Lazer et al., 2010; Lo et al., 2018, 2019; Robles-Valero et al., 2021; Saitoh et al., 2022; Sridaran et al., 2022; Strazza et al., 2021; Sun and Ayrappetov, 2023; Tawaratsumida et al., 2022; Voisinne et al., 2022; Wada et al., 2022; Wang et al., 2021; Yang et al., 2020; Zhang et al., 2021, 2024). To provide a more comprehensive understanding of signalling networks and reliable hypotheses regarding pY site function in a biologically relevant context, reliable and effective pY proteomics methods are required. Additional experimental and computational approaches are currently being developed to increase the speed, reproducibility, and depth of peptide sequencing on state-of-the-art mass spectrometers. Isobaric labelling reagents like tandem mass tags (TMTs) have allowed for post-lysis sample multiplexing, increasing sample reproducibility and accuracy of

quantitation(Thompson et al., 2003, 2019). Improvements to Fourier-transform (FT) MS instruments, particularly the development of the Orbitrap Tribrid instruments, have improved resolution of complex, isobaric-labelled sample mixtures and increased the quantitative depth in these samples(Yu et al., 2020; Noor et al., 2020). The Orbitrap Eclipse Tribrid comes with settings to optimise acquisition of multiplexed TMT samples like real-time peptide searches and TurboTMT(Yu et al., 2020; Furtwängler et al., 2022; Robitaille et al., 2020). Notably, TurboTMT implements the phase-constrained spectrum deconvolution method (Φ SDM) to efficiently deconvolve TMT reporter ion spectra, reducing acquisition time and increasing the spectral quality of TMT samples(Grinfeld et al., 2017; Kelstrup et al., 2018). While the benefits of using these experimental and computational methods are attractive for researchers, each technique has its own unique downfalls and should be optimised for the research question being addressed.

To improve the quantitation depth of pY proteomics studies with reduced protein input, we recently developed the broad-spectrum optimisation of the selective triggering (BOOST) method. By combining TMT labelling, sSH2 pY enrichment, and a carrier channel with abundant pY-containing peptides, the BOOST method increases the pY quantitation depth in experimental samples by increasing selective MS/MS triggering with the pY carrier channel(Chua et al., 2020). Importantly, increased MS/MS triggering by the pY carrier channel increases sequencing of low abundance peptides in experimental samples, reducing the minimum amount of peptide required for quantitation. Here, we report the first pY proteomics study in TCR-stimulated primary T cells from mice using the BOOST method. We benchmark the BOOST method using predetermined protein inputs and show that BOOST reproducibly increases the sequencing depth in low abundance samples, yielding more than 2000 unique pY sites in experimental samples. We show that BOOST selectively increases sequencing of low abundance peptides in experimental samples and increases detection of significant phosphoproteome changes despite a slight increase in sample variance. Importantly, the BOOST method outperforms previous pY proteomics studies and improves pY sequence coverage for T cell signalling proteins. Our data show that the BOOST method is a viable option for pY proteomics in primary cells and that BOOST uncovers significantly altered pY sites that are otherwise unobservable. We also show that acquisition settings on the mass

spectrometer, namely Φ SDM, requires further investigation for use with carrier channel proteomics experiments.

3.2 Methods

3.2.1 Stimulation of mice primary T cells

Splenic CD8+ T cells from B6 mice were isolated using the EasySep Mouse CD8+ T cell Isolation Kit (StemCell, cat. # 19853), primed with plate-bound 1 μ g/mL of α -CD3 (eBioscience, clone 145-2C11, cat. # 14-0031-86) and 0.5 μ g/mL α -CD28 (eBioscience, clone 37.51, cat. # 553294) for 2 days, then expanded using 50 U/mL of IL-2 for 4 days. Cells were rested in 1% BSA T cell media for 2 h at 2×10^6 cells/mL prior to stimulation. To initiate T cell stimulation, 25 μ g/mL of biotin-labelled α -CD3 antibody (eBioscience, clone 145-2C11, cat. # 14-0031-86) and 25 μ g/mL streptavidin were added to the cells resuspended at 1×10^8 cells/mL for 5 min at 37 C. After 5 min, cells were lysed with 1% (w/v) sodium dodecyl sulphate (SDS) in 100 mM Tris-HCl (pH 7.7). Pervanadate (PV) treatment for the carrier channel sample was performed by incubating cells with 500 μ M PV (prepared by mixing equal volume of 1 mM sodium orthovanadate and 1 mM hydrogen peroxide) for 20 min at 37 C. For experiments requiring stimulated and unstimulated cells, 2×10^7 cells (1 mg of protein) were used per replicate.

3.2.2 Sample Processing

Lysate was applied through QIAshredder Mini Spin Column by centrifugation at 20,000 $\times$ g at 37 C for 5 min. Protein concentration was determined using Pierce BCA Protein Assay (Thermo Fisher Scientific, cat. # 23225), after which it was reduced by 100 mM dithiothreitol at room temperature for 30 min. Lysate was subsequently processed and digested using the filter-aided sample preparation (FASP) method as described previously (Wiśniewski et al., 2009). Digested peptides were collected and acidified by trifluoroacetic acid and desalted using Sep-Pak C18 Cartridge (Waters cat. # WAT020515) as described previously (Ahsan and Salomon, 2017). Desalted peptides were labelled using a Tandem Mass Tag as described previously (Chua et al., 2020, 2021).

TMT-labelled peptides were mixed and purified for phosphotyrosine peptides using the Src SH2 domain superbinder as described previously(Chua et al., 2020, 2021).

3.2.3 Liquid chromatography tandem mass spectrometry

Offline basic (pH 10) fractionation of peptides was performed on a 100 mm $\times$ 1 mm Acquity BEH C18 column (Waters) using an UltiMate 3000 UHPLC system (ThermoFisher Scientific) with a 40-min gradient from 1% to 40% Buffer B_{basic} into 36 fractions. The 36 fractions were subsequently consolidated into 12 super-fractions (Buffer A_{basic} = 10 mM ammonium hydroxide in 99.5% (v/v) HPLC-grade water, 0.5% (v/v) HPLC-grade acetonitrile; Buffer B_{basic} = 10 mM ammonium hydroxide in 100% HPLC-grade acetonitrile), which were further separated on an in-line 150 mm $\times$ 75 μ m reversed phase analytical column packed in-house with XSelect CSH C18 2.5 μ m resin (Waters) using an UltiMate 3000 RSLCnano system (ThermoFisher Scientific) at a flow rate of 300 nL/min. Peptides were then eluted using a 65-min gradient from 5% to 30% Buffer B_{acidic}, followed by a 6-min gradient 30% to 90% Buffer B_{acidic} (Buffer A_{acidic} = 0.1% (v/v) formic acid in 99.4% (v/v) HPLC-grade water, 0.5% (v/v) HPLC-grade acetonitrile; Buffer B_{acidic} = 0.1% (v/v) formic acid in 99.9% (v/v) HPLC-grade acetonitrile). Data were acquired in data-dependent acquisition (DDA) mode on a Orbitrap Eclipse Tribrid mass spectrometer (ThermoFisher Scientific) with a positive spray voltage of 2.25 kV, mult notch TMT-MS3 settings(McAlister et al., 2014), and a cycle time was set at 2.5 s. At the MS1 level, precursor ions with charge states from 2 to 5 were acquired on the Orbitrap detector with a scan range of 400–1600 m/z, 120,000 resolution, maximum injection time of 50 ms, automatic gain control (AGC) target of 800,000, and a dynamic exclusion time of 15 s. An isolation window of 0.7 was used to isolate MS1 precursor ions on the quadrupole for MS2 scans. MS2 scans were acquired in centroid mode on the ion trap detector on a scan range of 400–1400 m/z via higher-energy dissociation (HCD, 33% energy) activation with an AGC target of 5000, maximum injection time of 75 ms. Using synchronous precursor selection (SPS)(McAlister et al., 2014), 10 notches were further isolated on the quadrupole using an MS2 isolation window of 3 m/z for MS3 scans, which are acquired on the Orbitrap detector on a scan range of 100–500 m/z in a mass resolution of 50,000 via HCD activation (55% energy) with a AGC target of

250,000 and maximum injection time of 150 ms in centroid mode.

3.2.4 Database search parameters and acceptance criteria for identifications

Raw files were processed in MaxQuant(Cox and Mann, 2008) version 1.6.17.0 using the integrated peptide search engine Andromeda(Cox et al., 2011). Data from Locard-Paulet et al. (2020) was downloaded from the ProteomeXchange using the identifier PXD014225 and before analysis with MaxQuant(Locard-Paulet et al., 2020). MS/MS spectra were searched against a mouse UniProt database (Mus musculus, last modified 12/01/2019) comprised of 55,412 forward protein sequences. False discovery rate (FDR) for peptide spectrum matches (PSM) was set at 1% using a reverse decoy database approach. Carbamidomethylation (cysteine) was set as fixed modification, whereas oxidation (methionine), acetylation (protein N-termini) and phosphorylation (serine, threonine, tyrosine) were set as variable modifications. Trypsin enzyme specificity was used with up to two missed cleavages. Main search peptide tolerance was set as 5 ppm, while FTMS and ITMS MS/MS match tolerances were set as 20 ppm and 0.5 Da, respectively. MS3 reporter ion mass tolerance was set at 3 mDa, using isotopic correction factors provided by the manufacturer (Lot UK291564, Lot UH283151). The search parameter files (mqpar.xml) are provided in the ProteomeXchange deposit.

3.2.5 Data analysis and code availability

All analysis and data visualisation were performed on Ubuntu 20.04 LTS in the Windows Subsystem for Linux version 2 using Python 3.8.10 with the packages “Matplotlib” (Version 3.3.2), “SciPy” (Version 1.7.3), “pandas” (Version 1.2.3), “NumPy” (Version 1.19.2), “Biopython” (version 1.78) and “matplotlib-venn” (version 0.11.6). Analysis of unique PSMs was performed using the MaxQuant output file “evidence.txt.” Unique PSMs were defined by a non-redundant amino acid sequence (including posttranslational modifications), the charge state of the peptide, and the least number of missing values across all TMT channels. In the cases where redundancy was still present, we kept the peptide with the highest median reporter intensity. For assigning flanking

sequences to each peptide and generating Figure 3.2, the MaxQuant output file “Phospho (STY)Sites.txt” was used. For determining previously annotated pY sites we used the PhosphoSitePlus (www.phosphosite.org) 55 posttranslational modification database file “Phosphorylation_site_dataset.txt” (Supporting Folder). Before analysis, all peptides at a 1% FDR flagged as “potential contaminants” or “reverse hits” were removed. Statistical significance between the mean corrected reporter intensities for 1.0, 0.3, and 0.1 mg protein input samples were determined using unpaired Student’s t-tests (Student, 1908) to calculate q-values before correcting for multiple hypotheses (generating q-values) using the method of Storey (Storey and Tibshirani, 2003; Benjamini and Hochberg, 1995). For all comparisons, statistical significance was only attained for peptides where reporter intensity values were present for at least three replicates. In line with the previous literature, we did not impute or interpolate missing values at any point during data analysis (Chua et al., 2020, 2021; Stopfer et al., 2021). For all volcano plots, we plotted $-\log_{10}(\text{q-value})$ as a function of $\log_{10}(\text{Ratio of Mean pY Intensities})$ for comparisons between 1.0 and 0.3 mg, 1.0 and 0.1 mg, and 0.3 and 0.1 mg of protein input for each TMT mix in contrived ratio experiments and between 5 and 0 min groups for stimulation experiments. For BOOST Factor plots, only pY peptides with at least one reporter ion value were used, and we used the following equation to calculate BOOST Factor for each pY peptide:

$$\begin{aligned} \text{BOOST Factor} &= \frac{\text{Total reporter ion current}_{\text{BOOST}}}{\text{Total reporter ion current}_{\text{Experimental}}} \\ &\approx \frac{1.0 \text{ mg PV intensity}}{\text{Sum}(1 \text{ mg}_{\text{R1-R3}} + 0.3 \text{ mg}_{\text{R1-R3}} + 0.1 \text{ mg}_{\text{R1-R3}})} \end{aligned}$$

3.3 Results

3.3.1 Experimental design and rationale

We evaluate the performance of the BOOST method for primary murine T cells using a similar approach as previously described (Chua et al., 2020, 2021) by performing an 11-plex TMT experiment (Figure 3.1A) with one PV BOOST or 1 mg Control sample with contrived protein ratios in triplicate (Figure 3.1B). Protein samples are derived from

CD8+ T cells from mice stimulated by an α -CD3 monoclonal antibody for 5 min, which represents the peak of TCR signalling. All samples are labelled with their respective amine-reactive isobaric tag before sSH2 enrichment, acquisition on an Orbitrap Eclipse Tribrid MS with multinode SPS-MS3 quantitation to minimise ratio distortion, and analysis with MaxQuant (Figure 3.1A). Before acquisition, samples are split and acquired with or without Φ SDM to determine whether Φ SDM influences pY quantitation. It is well established that reporter ions from carrier boosted samples can increase ratio distortion and false-positive peptide identifications due to TMT reagent impurities (Stopfer et al., 2021). We previously found that false-positive peptide identifications due to the BOOST sample in channel TMT126 were exclusive to channel TMT127C (Chua et al., 2021). Therefore, no samples were labelled with TMT127N to prevent false-positive peptide identifications and ratio distortion (Figure 3.1B,C). From all four contrived ratio TMT experiments, most phosphorylation sites are localised to tyrosine residues (70%) with 94.1% of pY sites being assigned with probability > 0.75 (Figure 3.2).

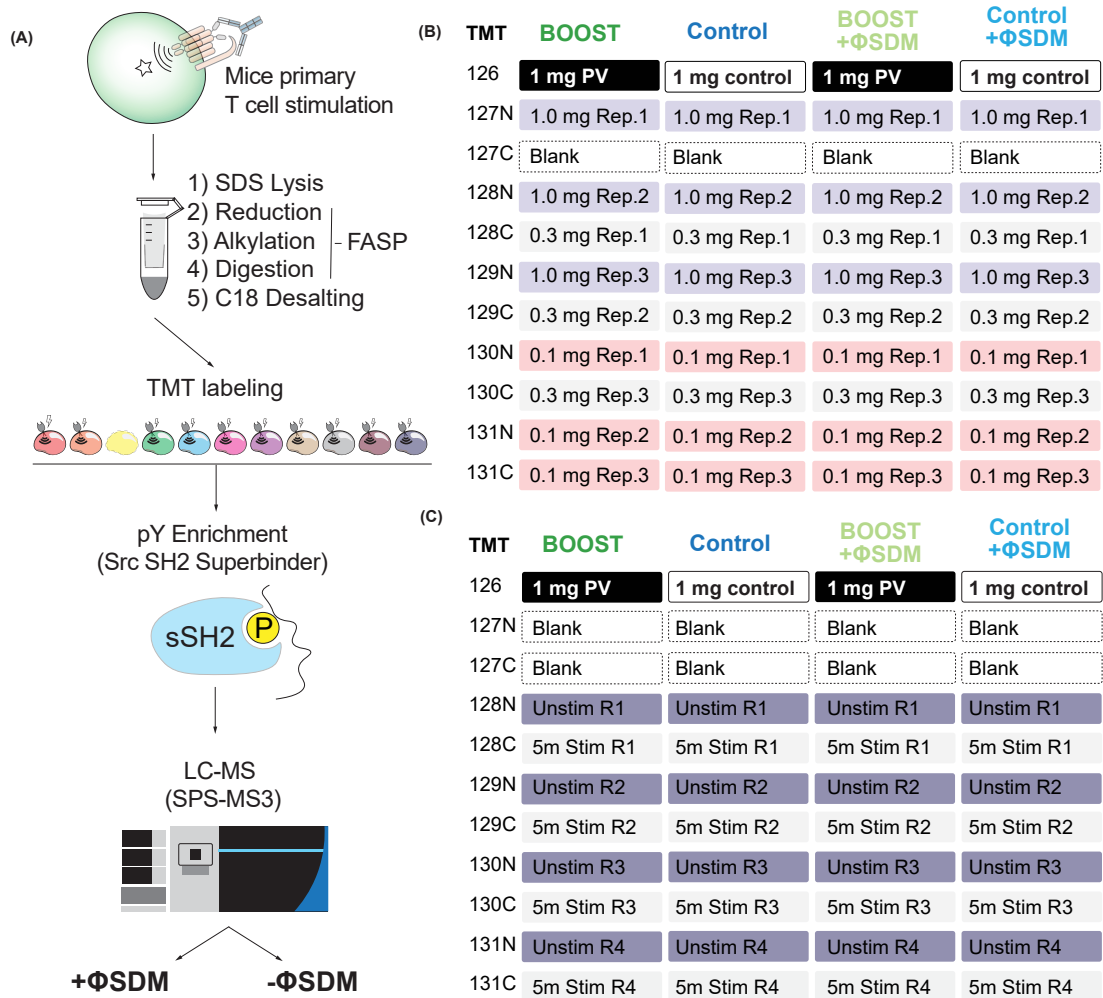


Figure 3.1: Schematic representation of (A) the experimental workflow, (B) the TMT mix and channel design for contrived ratio experiments, and (C) the TMT mix and channel design for TCR stimulation experiments. Pervanadate (PV), Phase-spectrum deconvolution (Φ SDM).

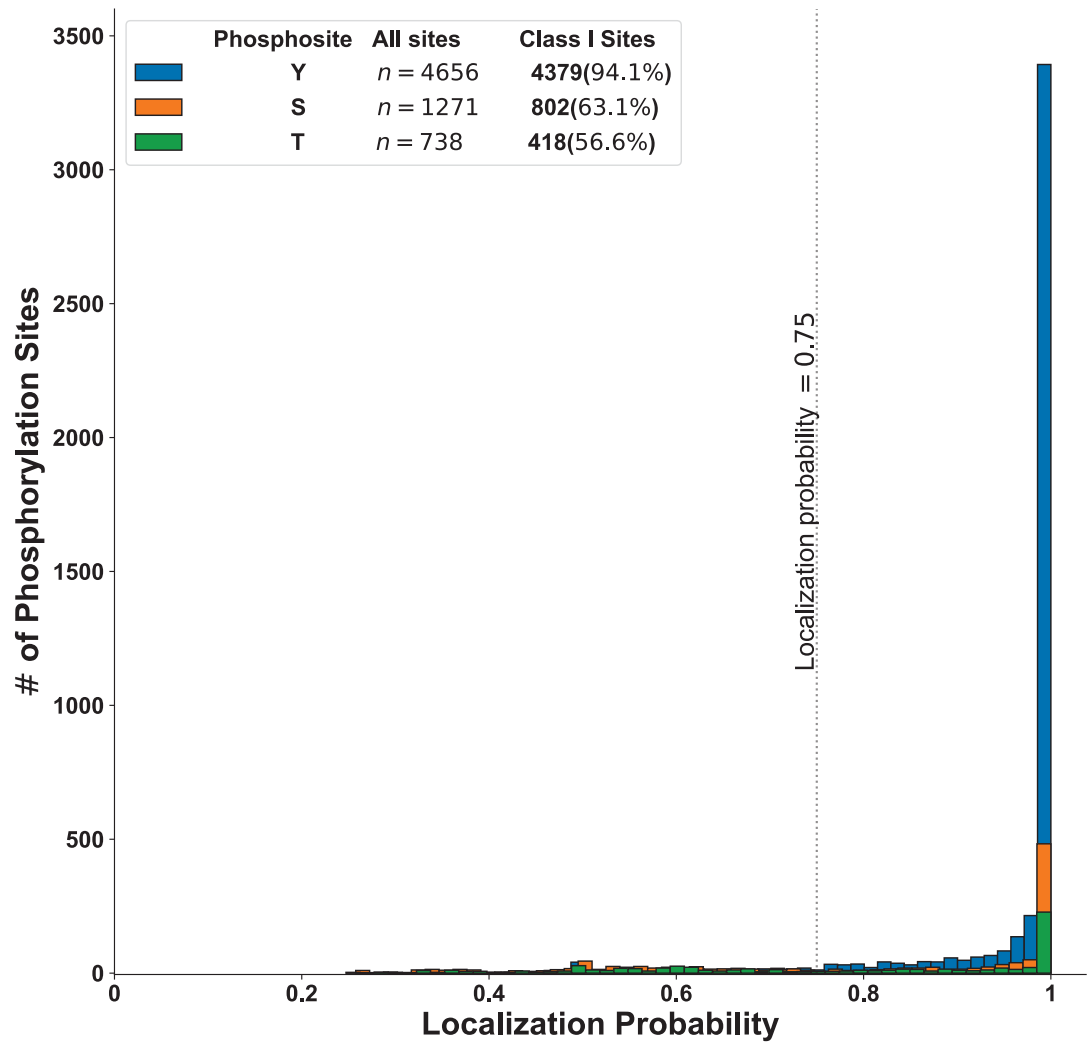


Figure 3.2: A histogram with depicting all PSMs from all experiments containing at least one phosphorylated serine (S), threonine (T), or tyrosine (Y) amino acid as a function of localization probability ($n_{\text{bins}} = 75$). The total number and number of Class I (localization probability > 0.75) phosphorylation sites for each amino acid are noted in the Figure Legend.

3.3.2 BOOST accurately increases acquisition of low abundance pY peptides

The BOOST method is designed to increase the quantification of peptides that are naturally low abundance in each sample, thereby increasing the potential discovery of biologically relevant pY sites(Chua et al., 2021; Specht and Slavov, 2020). We observe that BOOST improves sequencing depth by 3- to 7- fold compared to the control experiment (Figure 6.3A) but is accompanied by increased within-group variability (Figure 6.3B). The increased variation is likely due to acquisition of low abundance pY peptides, as the 0.1 mg control group has similar variation to the BOOST groups. As seen previously, we observe a decrease in the median reporter ion intensity in BOOST samples (Figure 3.4A), which is likely a result of reaching the AGC target during MS1 precursor ion selection and decreased sampling of experimental peptides(Specht and Slavov, 2020). The decrease in median reporter ion intensity is only observed for pY peptides, as non-pY peptides (background binding to the sSH2 beads) show greater reporter ion intensity and a similar number of unique pY PSMs in BOOST than control-TMT samples (Figure 3.53). Notably, including the pY carrier channel does not influence the expected ratios for sequenced peptides (Figure 3.4B), and the BOOST method increases the number of statistically significant pY PSM ratios in all comparisons (Figure 3.3C). We observe 3006 pY peptide PSMs that are quantified in the BOOST channel (BOOST-gained) and experimental channel(s) compared to 87 peptides unique to the control (Figure 3.3D). To quantify the ability of the BOOST method to increase acquisition of low abundance pY peptides in experimental samples as intended, we use the BOOST factor, a previously characterised metric defined as the ratio of the total reporter ion current in the BOOST channel to the sum of the total ion current in all experimental channels(Chua et al., 2020, 2021). We find that 98% of BOOST-gained peptides have a BOOST factor greater than 1 (median 42.34; Figure 3.3E,F) and that 90% of significantly changing ratios have a BOOST factor between 1 and 20 (Figure 3.3F), which agrees with previous literature evaluating the effectiveness of carrier channels in single cell proteomics experiments(Cheung et al., 2021; Petelski et al., 2021; Vistain and Tay, 2021). When acquiring these data using Φ SDM under the same conditions, we observe a

universal degradation of the data, particularly in the BOOST experiment. Using Φ SDM, per-sample pY PSM yield and statistically significant ratios are halved, variation doubles in the 0.1 mg samples, there is a uniform lowering of BOOST factors (median 21.75), and the majority of BOOST factors for significant ratios lay between 1 and 10 despite little change to per-sample intensity distributions or contrived ratios, with similar trends in non-pY PSMs as observed without SDM (Figures 3.6, 3.7, 3.8). These data show that the BOOST method improves sequencing of low abundance pY peptides in primary T cells from mice, as expected.

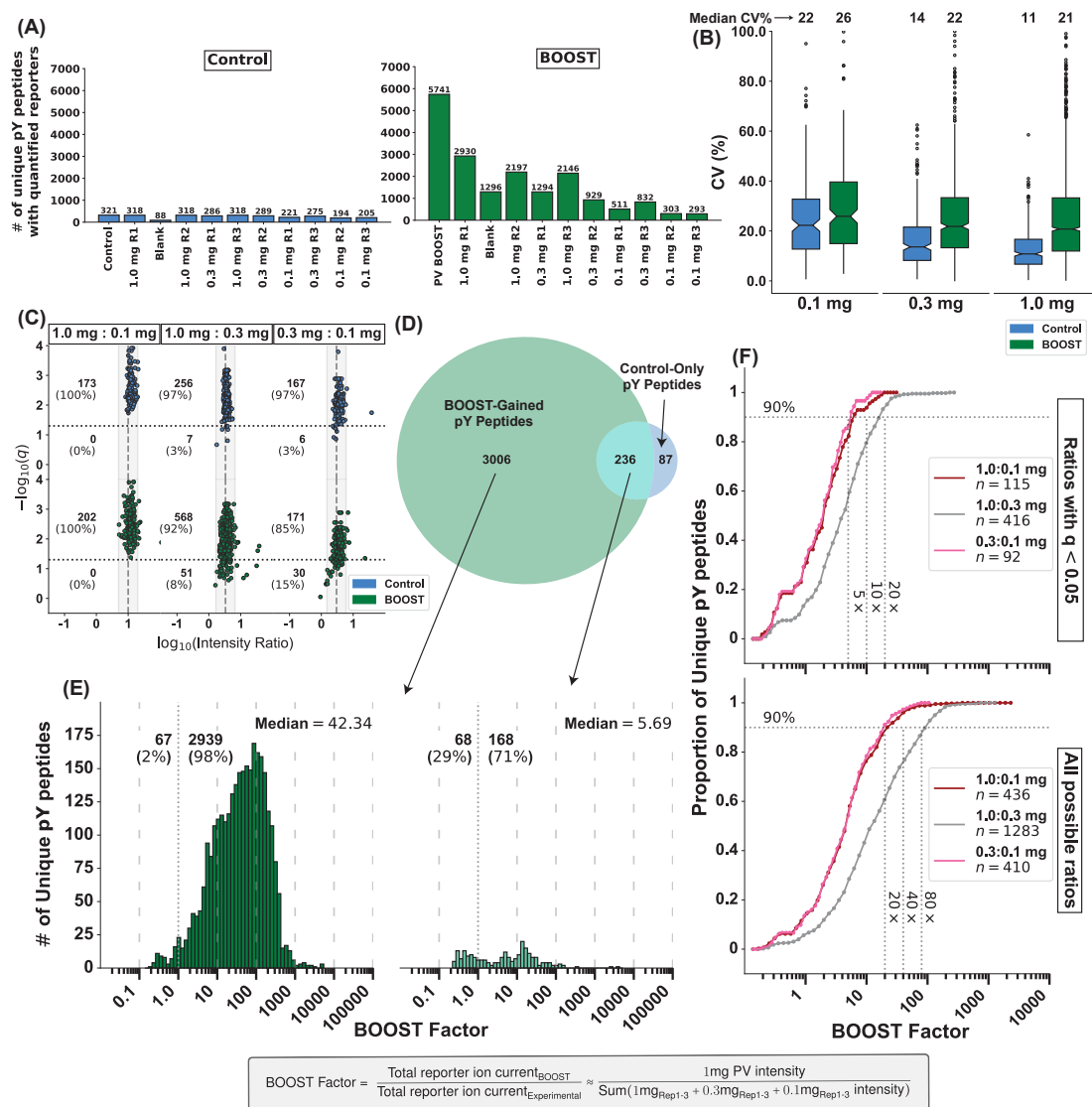


Figure 3.3: The BOOST method improves quantitation of low abundance pY peptides while maintaining accuracy. (A) Number of unique pY peptide PSMs identified in the control (left) and BOOST (right) TMT mixes. Samples are ordered as in Figure 3.1B. Numbers above each bar represent the number of unique pY peptides for that sample. (B) Box-and-whisker plots showing the coefficient of variation percentages (CV%) for unique pY peptide PSMs measured in triplicate in the BOOST and control experimental samples for each protein input amount. Median CV% is shown above each sample. (C) Volcano plots comparing pY peptide PSMs between different protein input amounts. The vertical grey dashed line and the grey shaded area indicate the theoretically expected ratio and two-fold above or below, respectively. The horizontal dashed line represents a q-value of 0.05, with the numbers above and below showing the number of significant ratios with a q-value greater than or less than 0.05. (D) Venn diagram showing the overlap of unique pY peptide PSMs between the BOOST and control samples. (E) Histogram of BOOST factor values for BOOST-gained pY peptide PSMs and overlapping pY peptide PSMs. Values to the right or left of 1 indicate the number of pY peptide PSMs greater than or less than 1. (F) Empirical BOOST factor distribution for pY peptide PSMs with significant ratios (top) and for all possible ratios (bottom).

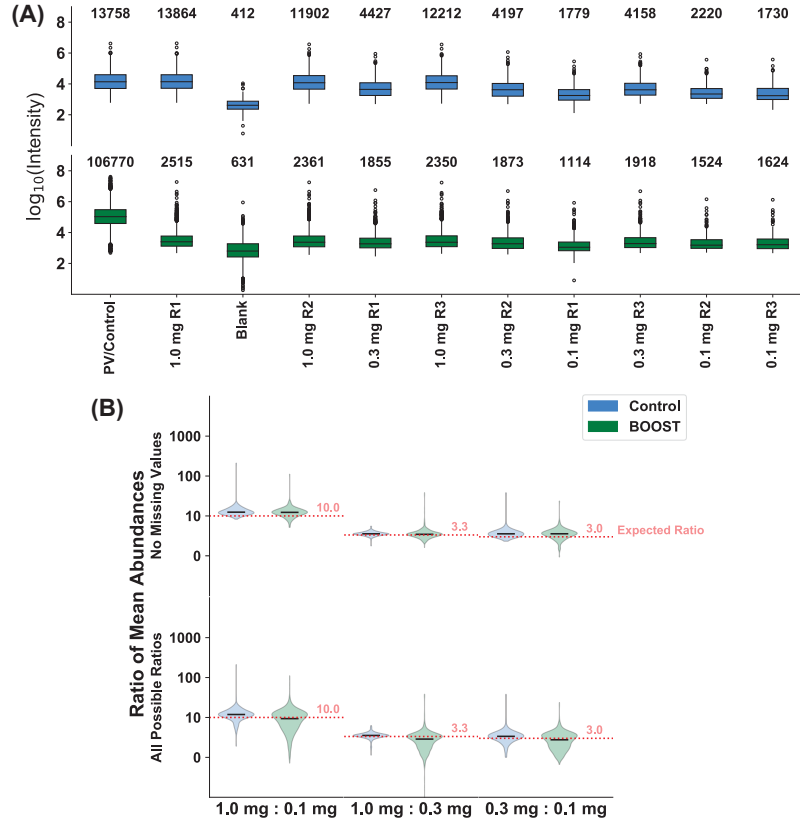


Figure 3.4: The BOOST method reduces the median reporter ion intensity in a protein-dependent manner without influencing the expected abundance ratios. (A) Box-and-whisker plots showing the per-sample $\log_{10}$ transformed peptide abundance distributions. Numbers above each box indicate the raw median reporter ion intensity. (B) Violin plots showing the distribution of ratios for 1.0 mg to 0.1 mg (left), 1.0 mg to 0.3 mg (center), and 0.3 mg to 0.1 mg (right) for peptides with a measurement in *any* sample (bottom row) and peptides measured in triplicate (top row). Red dotted lines indicated the expected ratio based on protein input amounts for each comparison.

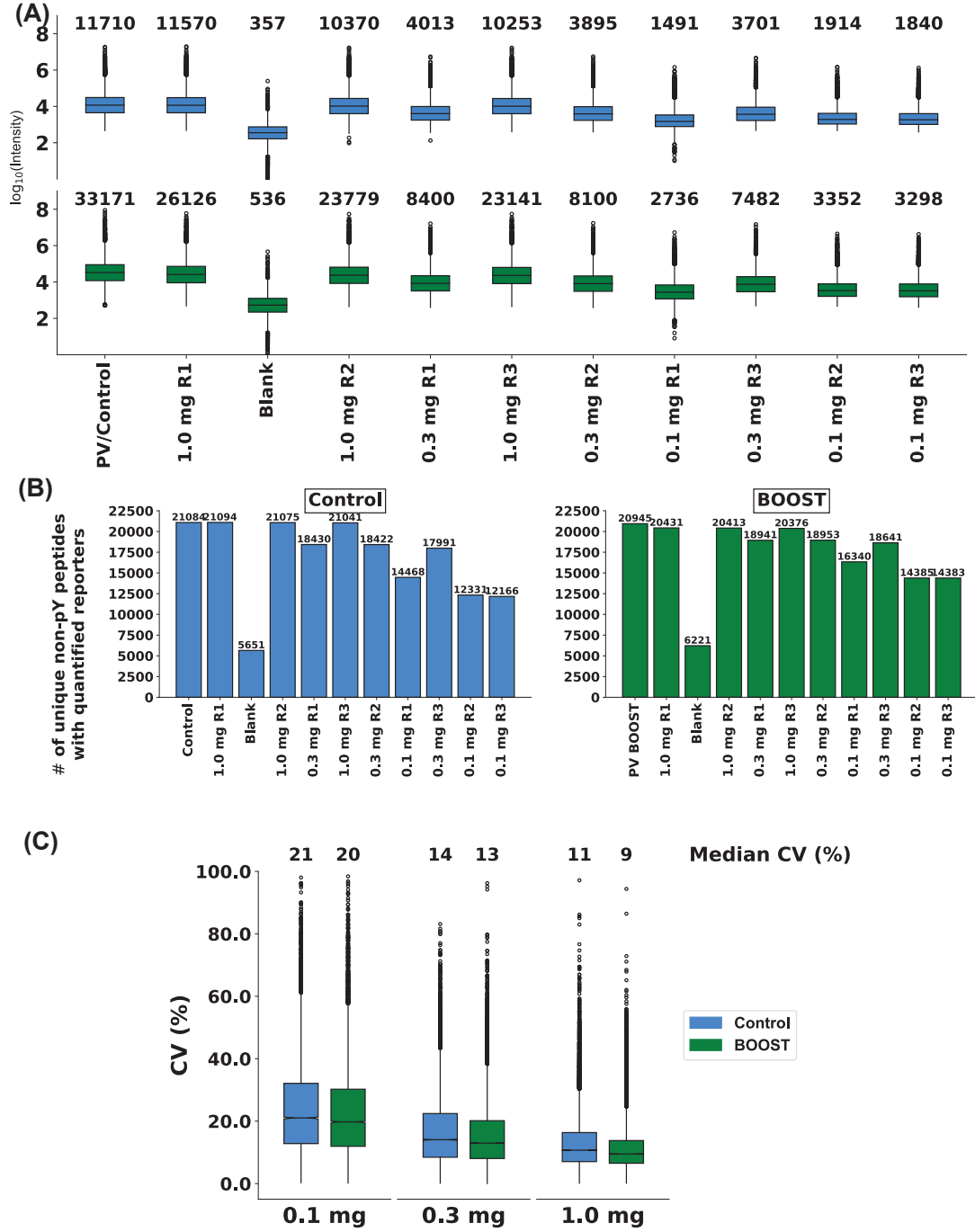


Figure 3.5: Non-pY PSMs in BOOST show higher median intensities despite similar PSM yield between BOOST and control with Φ SDM disabled. (A) As in Figure 3.4, except for non-pY PSMs. (B) As in Figure 3.3A, except for non-pY PSMs. (C) As in Figure 3.3B, except for non-pY PSMs.

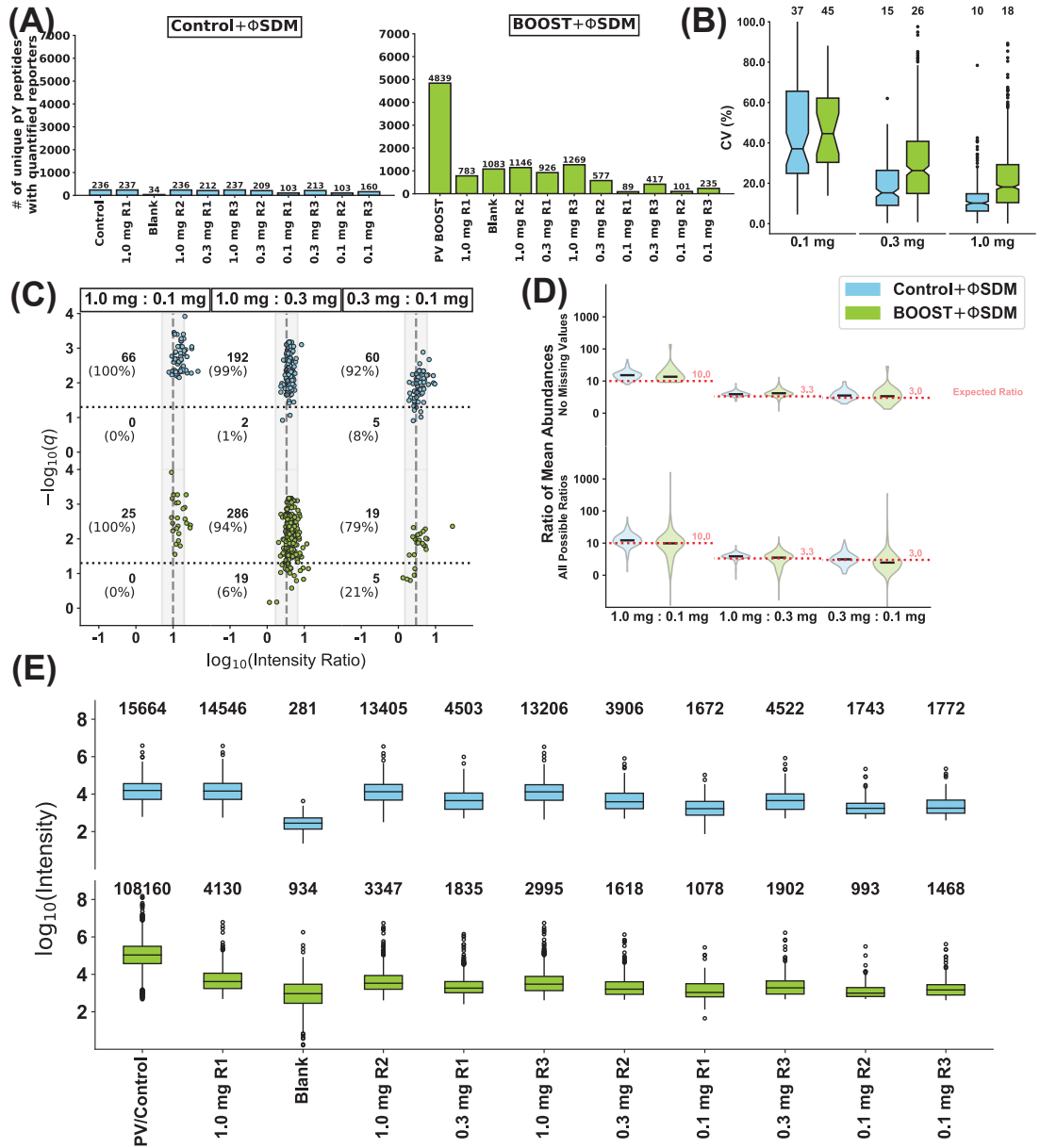


Figure 3.6: Φ SDM impairs quantitation of and severely impairs pY quantitation in the BOOST experiment. (A) Number of unique pY peptide PSMs sequenced in the control experiment (left) and BOOST experiment (right) with Φ SDM enabled. (B) Box-and-whisker plots showing the CV% for unique pY peptides measured in triplicate for each protein input amount. Numbers above the plot indicate the median CV%. (C) Volcano plots comparing pY peptide PSMs at between different protein input amounts. The vertical grey dashed line and the grey shaded area indicate the theoretically expected ratio and two-fold above or below, respectively. (D) and (E) As in Figures 3.4B and 3.4A, respectively, except for samples acquired with Φ SDM.

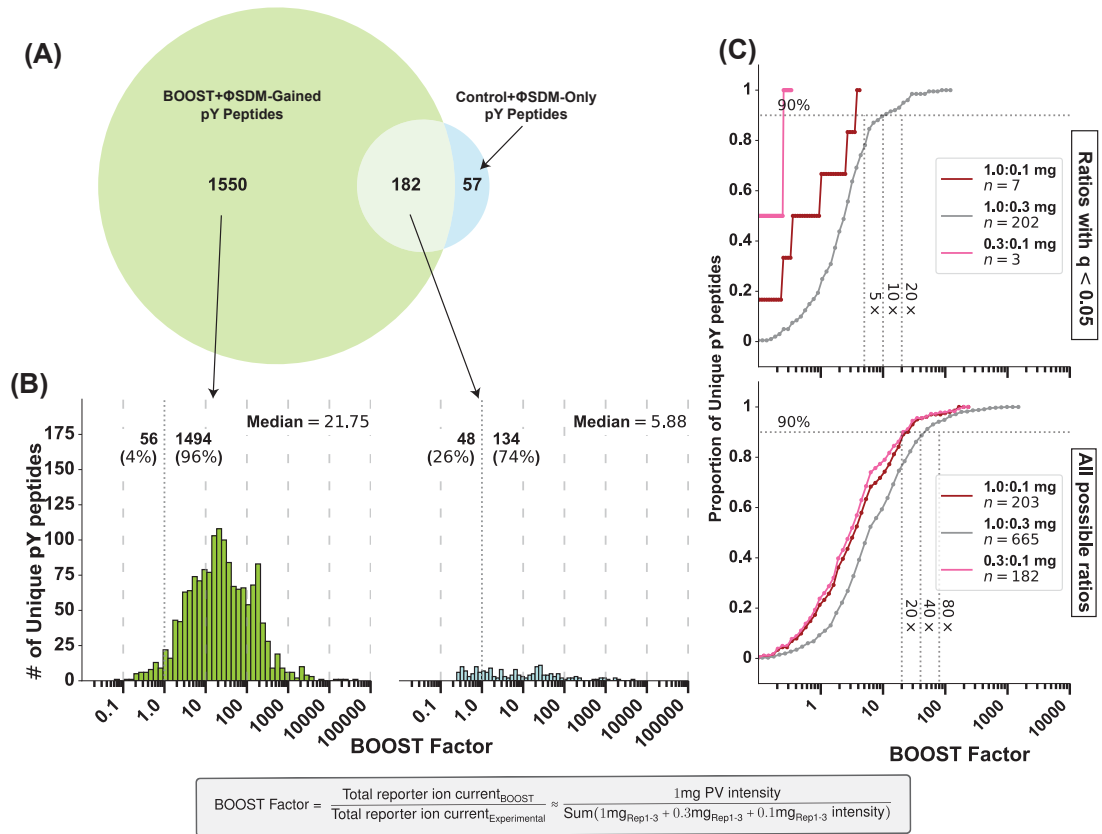


Figure 3.7: Yield of BOOST gained pY peptides, acquisition of low abundance peptides, and significance of ratios are impaired when using ΦSDM. (A) Venn diagram showing the overlap of unique pY peptide PSMs between the BOOST and control samples. (B) Histogram of BOOST factor values for BOOST-gained pY peptide PSMs and overlapping pY peptide PSMs when using ΦSDM. (C) Empirical BOOST factor distribution for pY peptide PSMs with significant ratios (top) and for all possible ratios (bottom).

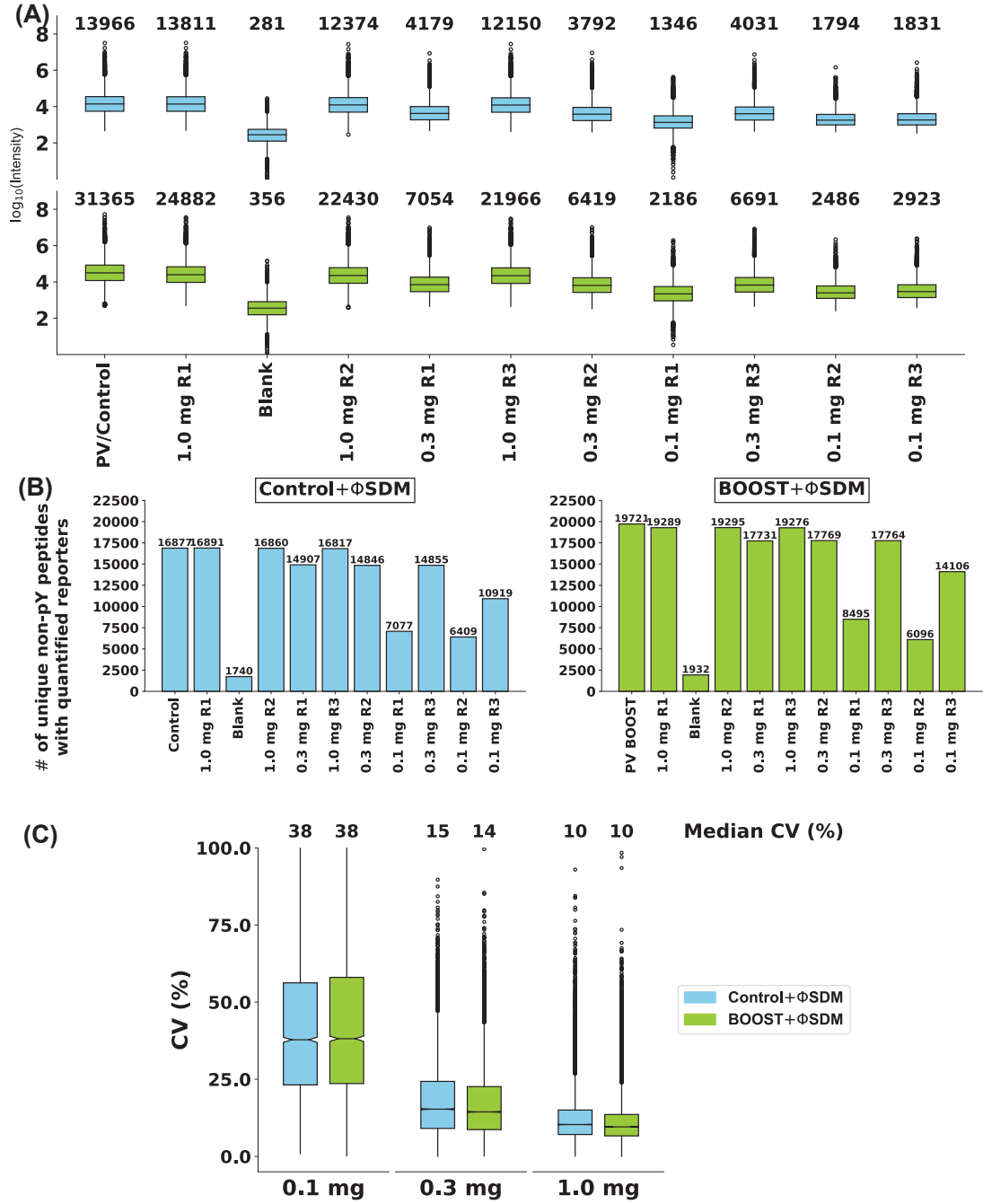


Figure 3.8: Non-pY PSMs in BOOST show higher median intensities despite similar PSM yield between BOOST and control with Φ SDM enabled. (A) As in Supporting Figure 3.4, except for non-pY PSMs. (B) As in Figure 3.3, except for non-pY PSMs. (C) As in Figure 3.3B, except for non-pY PSMs.

3.3.3 BOOST increases sequencing of T cell signalling pY peptides compared to label-free experiments

To evaluate the sequencing capacity of the BOOST method, we compare our 1.0 mg contrived ratio data (about 2×10^7 cells per replicate) to our previous BOOST experiment in Jurkat T cells (Chua et al., 2020), a label-free global phosphorylation dataset using a TiO2 enrichment strategy from murine CD4+ T cells (2×10^7 cells per replicate) 13, and a label-free pY dataset using the P-Tyr-1000 enrichment strategy from murine CD4+ T cells (1×10^8 cells per replicate) (Locard-Paulet et al., 2020). For comparison, raw files from Locard-Paulet et al. (2020) were processed through MaxQuant using the same search parameters as our data and those from Chua et al. (2020). As expected, we see very low pY sequencing in the CD4+ T cell global phosphorylation dataset (49 total sites) and pY dataset (218 total sites) compared to the Jurkat BOOST (665 PhosphoSitePlus mouse equivalent sites from 2597 total human sites) and CD8+ T cell BOOST (2402 total sites; Figure 3.9A). When evaluating only pY peptides from proteins annotated in the WikiPathways T cell signalling pathway, the lack of sequencing is even more apparent. CD4+ T cell global phosphorylation enrichment under-sequences pY, showing 14 pY sites that are observed in every dataset, and CD4+ T cell pY enrichment contains only 44 pY sites and only five unique sites (Figure 3.9B). Of the common sites observed, most are immunotyrosine based activation motifs (ITAMs: CD3 ϵ ^{Y170/Y181}, CD3 γ ^{Y160/Y171}, CD3 δ ^{Y149/Y160}, ζ -chain^{Y72/Y83}, ζ -chain^{Y111/Y123}, ζ -chain^{Y142/Y153}) and other well-defined pY sites like Lck^{Y192}, Erk1^{Y205}, Erk2^{Y185}, ITK^{Y517} and ZAP70^{Y290} (Figure 3.9C, Figure 3.10) (Kästle et al., 2022; Love and Hayes, 2010; Kortum et al., 2013; Wilcox and Berg, 2003; Ko et al., 2021). In contrast, the CD8+ BOOST dataset contains 123 pY sites, 45 of which are unique to the BOOST experiment (Figure 3.9B). These include some pY sites of known function, like Fyn^{Y28}, PD-1^{Y225}, SHP-1^{Y301}, PAK2^{Y139}, ZAP70^{Y178} and ZAP70^{Y596Y597} (Hansen et al., 1997; Palacios and Weiss, 2004; Chemnitz et al., 2004; Minoo et al., 2004; Renkema et al., 2002; Zeitlmann et al., 1998), and sites on important T cell signalling proteins with unknown function, like LAT^{Y45}, PLC γ 1^{Y210}, PLC γ 1^{Y472}, ZAP70^{Y198}, SHP-1^{Y61}, SHP-1^{Y64}, SHP-1^{Y374}, SHP-1^{Y377}, DLG1^{Y399}, PAK6^{Y366}, CD45^{Y631}, CD45^{Y678}, CD45^{Y680}, CD45^{Y754}, CD45^{Y852} and

CD45^{Y969} (Figure 3.9C, Figure 3.10)(Gaud et al., 2018). Notably, many of these pY sites lay in important active domains or regulatory domains of these proteins, suggesting they may play a functional role for each protein. Overall, this comparison demonstrates how the BOOST method promotes superior pY sequencing depth compared to traditional phosphoenrichment LC-MS/MS experimental methods with reduced protein input.

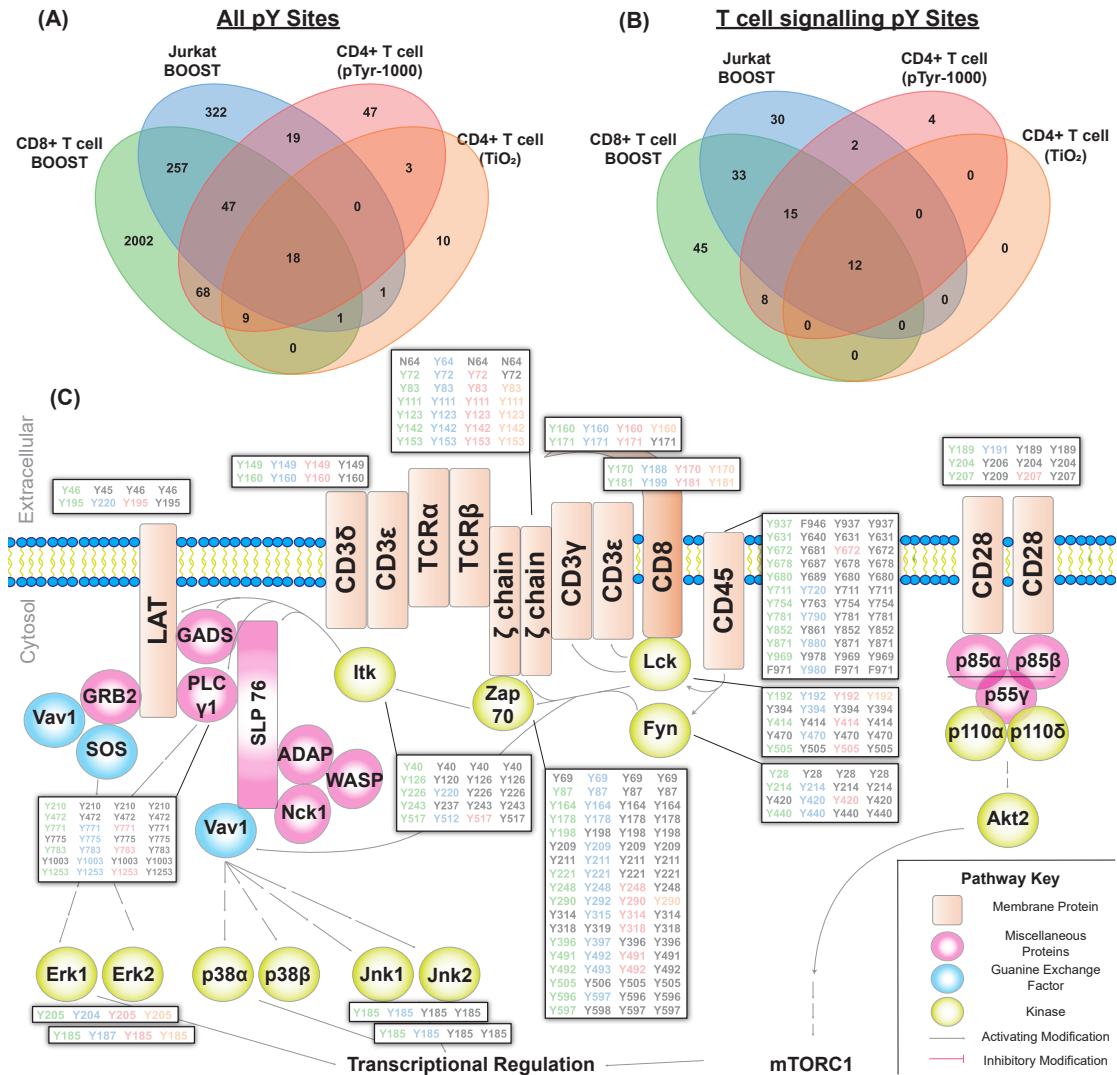


Figure 3.9: Using BOOST increases pY site yield compared with traditional label-free LC-MS/MS experiments. Jurkat BOOST data are from Chua et al. (2020), and CD4+ T cell data are from Locard-Paulet et al. (2020). (A) Venn-diagram showing overlap between pY sites observed in this study, in Jurkat T cells using BOOST, in CD4+ T cells without BOOST, and CD4+ T cells using global pY enrichment by titanium dioxide. (B) As in (A), except for peptides on proteins from the WikiPathways T cell signalling pathway. (C) Overlay of select sites on a schematic representation of the T cell signalling pathway. For all pY sites on T cell signalling proteins, refer to Figure 3.10.

Protein	Our Study	Chua 2020	Locard-Paulet 2020 pY	Locard-Paulet 2020 pSTY	Protein	Our Study	Chua 2020	Locard-Paulet 2020 pY	Locard-Paulet 2020 pSTY
Ak2	Y122	Y122	Y122	Y122	PD-1	Y225	Y225	Y225	Y225
CARD11	Y489	Y489	Y489	Y489	p110 α	Y317	Y317	Y317	Y317
Cbl-b	Y360	Y360	Y360	Y360	p110 δ	Y523	Y524	Y523	Y523
	Y363	Y363	Y363	Y363	p85 α	Y416	Y416	Y416	Y416
	Y708	Y709	Y708	Y708		Y76	Y76	Y76	Y76
	Y763	Y763	Y763	Y763		Y452	Y452	Y452	Y452
TCR ζ	N64	Y64	N64	N64		Y467	Y467	Y467	Y467
	Y72	Y72	Y72	Y72		Y470	Y470	Y470	Y470
	Y83	Y83	Y83	Y83		Y688	Y688	Y688	Y688
	Y111	Y111	Y111	Y111	p85 β	Y458	Y464	Y458	Y458
	Y123	Y123	Y123	Y123	p55 γ	Y202	Y202	Y202	Y202
	Y142	Y142	Y142	Y142	PLCy1	Y210	Y210	Y210	Y210
	Y153	Y153	Y153	Y153		Y472	Y472	Y472	Y472
CD28	Y189	Y191	Y189	Y189		Y771	Y771	Y771	Y771
	Y204	Y206	Y204	Y204		Y775	Y775	Y775	Y775
	Y207	Y209	Y207	Y207		Y783	Y783	Y783	Y783
CD3 δ	Y149	Y149	Y149	Y149		Y1003	Y1003	Y1003	Y1003
	Y160	Y160	Y160	Y160		Y1253	Y1253	Y1253	Y1253
CD3 ϵ	Y170	Y188	Y170	Y170	PKC θ	Y28	Y28	Y28	Y28
	Y181	Y198	Y181	Y181		Y545	Y545	Y545	Y545
CD3 γ	Y160	Y160	Y160	Y160		Y61	Y61	Y61	Y61
	Y171	Y171	Y171	Y171	SHP-1	Y64	Y64	Y64	Y64
CDC42	Y64	Y64	Y64	Y64		Y213	Y213	Y213	Y213
CDK4	Y17	Y17	Y17	Y17		Y214	Y214	Y214	Y214
CTLA-4	Y201	Y201	Y201	Y201		Y276	Y276	Y276	Y276
DLG1	Y399	Y399	Y399	Y399		Y301	Y301	Y301	Y301
	Y761	Y760	Y761	Y761		Y306	Y306	Y306	Y306
	Y785	Y784	Y785	Y785		Y374	Y374	Y374	Y374
Fyn	Y28	Y28	Y28	Y28		Y377	Y377	Y377	Y377
	Y214	Y214	Y214	Y214		Y536	Y536	Y536	Y536
	Y420	Y420	Y420	Y420		Y541	Y541	Y541	Y541
	Y440	Y440	Y440	Y440		Y564	Y564	Y564	Y564
GADS	Y45	Y45	Y45	Y45	CD45	Y937	Y946	Y937	Y937
	Y218	Y222	Y218	Y218		Y631	Y640	Y631	Y631
Grb2	Y209	Y209	Y209	Y209		Y672	Y681	Y672	Y672
GSK3 β	Y216	Y216	Y216	Y216		Y678	Y687	Y678	Y678
Itk	Y40	Y40	Y40	Y40		Y680	Y689	Y680	Y680
	Y126	Y120	Y126	Y126		Y711	Y720	Y711	Y711
	Y226	Y220	Y226	Y226		Y754	Y763	Y754	Y754
	Y243	Y237	Y243	Y243		Y781	Y790	Y781	Y781
	Y517	Y512	Y517	Y517		Y852	Y861	Y852	Y852
LAT	Y46	Y45	Y46	Y46		Y871	Y880	Y871	Y871
	Y195	Y220	Y195	Y195		Y969	Y978	Y969	Y969
Lck	Y192	Y192	Y192	Y192		F971	Y980	F971	F971
	Y394	Y394	Y394	Y394	RHOA	Y66	Y66	Y66	Y66
	Y414	Y414	Y414	Y414	Tec	Y205	Y206	Y205	Y205
	Y470	Y470	Y470	Y470		Y227	Y228	Y227	Y227
	Y505	Y505	Y505	Y505		Y280	Y281	Y280	Y280
SLP76	Y173	Y173	Y173	Y173		Y518	Y519	Y518	Y518
	Y483	Y483	Y483	Y483	Vav1	Y110	Y110	Y110	Y110
TAK1	Y558	Y585	Y558	Y558		Y192	Y192	Y192	Y192
Erk2	Y185	Y187	Y185	Y185		Y541	Y541	Y541	Y541
Jnk3	Y223	Y223	Y223	Y223		Y791	Y791	Y791	Y791
p38 β	Y182	Y182	Y182	Y182		Y826	Y826	Y826	Y826
p38 γ	Y185	Y185	Y185	Y185	Vav2	Y142	Y142	Y142	Y142
p38 α	Y182	Y182	Y182	Y182	Vav3	Y141	Y141	Y141	Y141
Erk1	Y205	Y204	Y205	Y205		Y217	Y217	Y217	Y217
Jnk1	Y185	Y185	Y185	Y185	Zap70	Y69	Y69	Y69	Y69
Jnk2	Y185	Y185	Y185	Y185		Y87	Y87	Y87	Y87
NCK1	Y13	Y13	Y13	Y13		Y164	Y164	Y164	Y164
	Y55	Y55	Y55	Y55		Y178	Y178	Y178	Y178
	Y105	Y105	Y105	Y105		Y198	Y198	Y198	Y198
NCK2	Y110	Y110	Y110	Y110		Y209	Y209	Y209	Y209
	Y342	Y342	Y342	Y342		Y211	Y211	Y211	Y211
NFAT1	Y754	Y752	Y754	Y754		Y221	Y221	Y221	Y221
NFAT4	Y86	Y86	Y86	Y86		Y248	Y248	Y248	Y248
	Y150	Y150	Y150	Y150		Y290	Y292	Y290	Y290
NF κ B-p105	Y238	Y240	Y238	Y238		Y314	Y315	Y314	Y314
PAK1	Y142	Y142	Y142	Y142		Y318	Y319	Y318	Y318
	Y153	Y153	Y153	Y153		Y396	Y397	Y396	Y396
	Y474	Y474	Y474	Y474		Y491	Y492	Y491	Y491
PAK2	Y130	Y130	Y130	Y130		Y492	Y493	Y492	Y492
	Y139	Y139	Y139	Y139		Y505	Y506	Y505	Y505
	Y453	Y453	Y453	Y453		Y596	Y597	Y596	Y596
PAK6	Y366	Y365	Y366	Y366		Y597	Y598	Y597	Y597

Figure 3.10: The BOOST method increases sequencing of pY sites on T cell signalling proteins compared with conventional label-free pY and pSTY experiments. pY and pSTY data from Locard-Paulet et al. (2020) are CD4+ T cells that were TCR stimulated using soluble antibodies for 5 minutes, starting with ~ 20 mg of protein (Locard-Paulet et al., 2020).

3.3.4 BOOST increases sequencing of T cell signalling pY peptides compared to label-free experiments

To evaluate the potential for new biological discovery using the BOOST method, we compare the pY proteome of primary T cells before and after TCR stimulation with or without using BOOST (Figure 3.1C). In line with our previous results, using the BOOST method leads to a universal drop in pY peptide intensity, an increase in replicate variability, and an increase in sequencing depth in experimental samples (Figures 3.11A,B and 3.12A,B), due to the acquisition of low abundance pY peptides (Figure 3.12C,D). When comparing the stimulated to unstimulated states, using the BOOST method slightly increases the number of significantly changing pY peptides, most of which lay in the range of $0.01 < q < 0.05$ (Figure 3.11C,D). In total, 43 unique pY peptides (45 unique pY sites) are significantly changing in both BOOST and non-BOOST stimulation experiments, while 87 unique pY peptides (60 unique pY sites) are significantly changing in the BOOST experiment alone (Figure 3.11E,F). While the control experiment largely shows sequencing of expected pY sites (Figure 3.11G, right), including ITAMs and activation sites on non-receptor tyrosine kinases, BOOST uncovers a previously uncharacterised set of TCR responsive pY sites. Notably, SLP76^{Y483}, CSK^{Y184}, and Cblb^{Y763} are TCR responsive pY sites with unknown function on critical regulators of TCR signalling (Figure 3.11G, left). Importantly, the pY sites observed in both the control and BOOST experiments show similar fold-change and significance trends (Figure 3.11G, centre), suggesting the pY sites seen in the BOOST experiments are as reliably acquired as the control. We also find that using Φ SDM during data acquisition again severely reduces the quantity and quality of pY PSMs acquired in the BOOST experiment, with only a modest reduction in pY peptide yield in control samples (Figures 3.13 and 3.14). These data show that BOOST is a reliable way to extract novel, differential pY sites from stimulated murine T cells, and the most comprehensive view of pY signalling from the TCR comes from a combination of BOOST and non-BOOST TMT experiments.

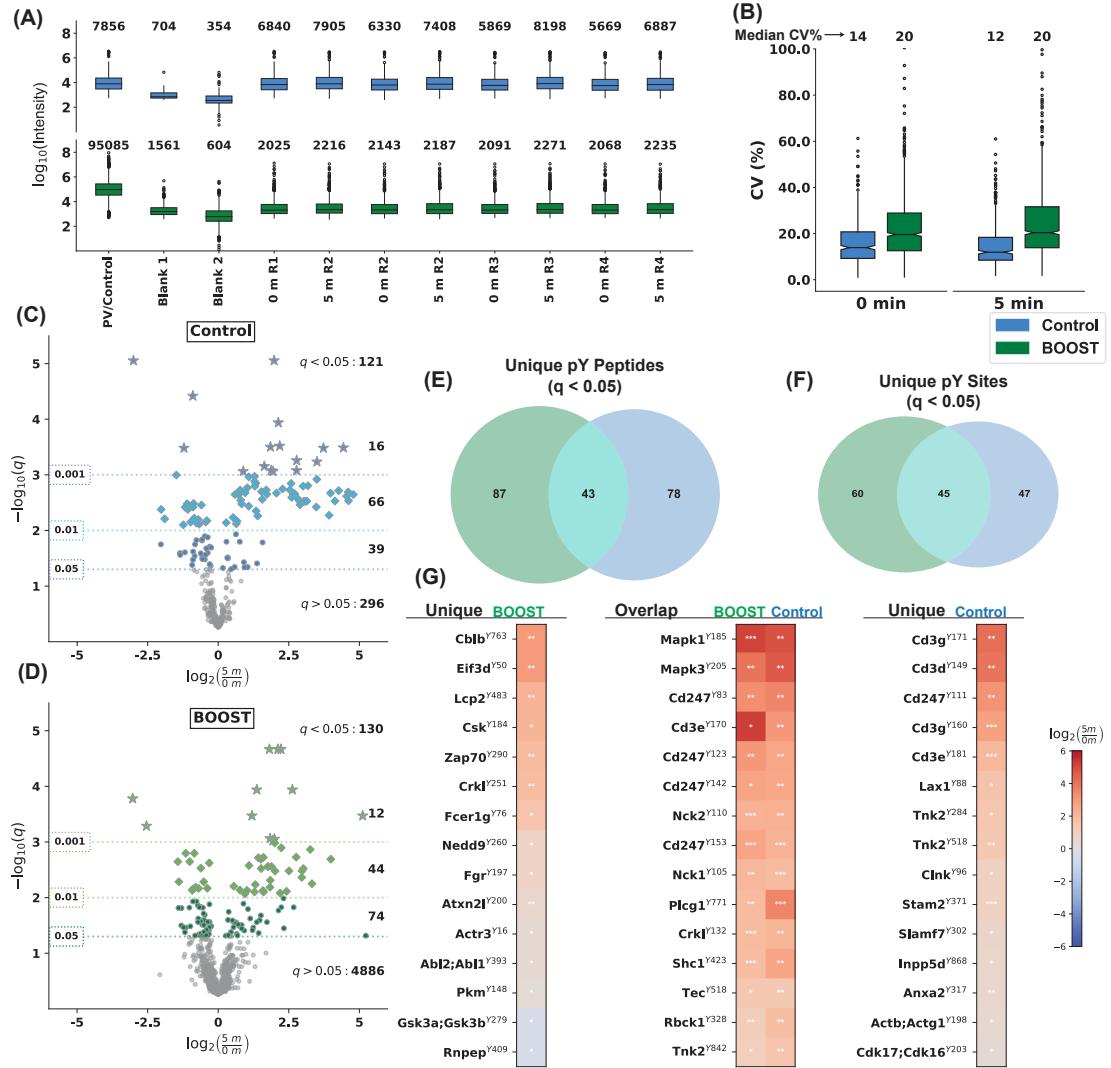


Figure 3.11: BOOST allows for quantitation of T cell responsive pY sites that are not observed in traditional TMT experiments. (A) $\log_{10}$ transformed reporter intensities for control and BOOST samples. (B) As in Figure 3.3B, except for 0 and 5 min samples. (C) Volcano plot of 5 min versus 0 min of TCR stimulation for control samples. Numbers on the right indicate the number of points in between the q -values on the left, with the total significant points denoted at the top. (D) As in (C), except for BOOST samples. (E) Venn diagram showing unique pY PSMs with $q < 0.05$ after 5 min of stimulation. (F) As in (E), except for unique pY sites. (G) Heatmaps showing a selection of significantly changing pY sites observed in the BOOST (left), BOOST and control (middle), and control (right) experiments.

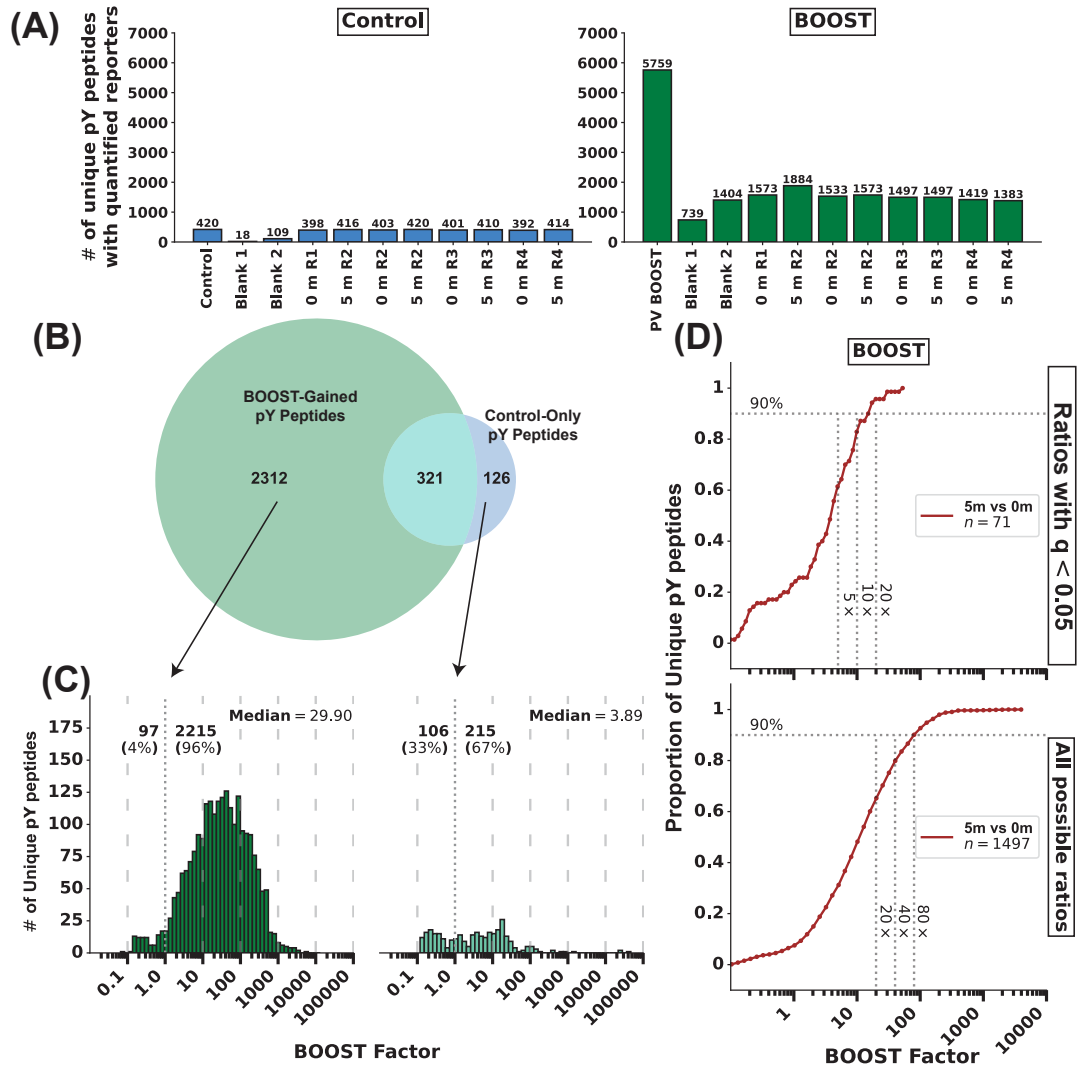


Figure 3.12: Data quality verification on the time course TCR stimulation BOOST experiment. (A), (B), (C), (D) As in Figures 3.3A, 3.3D, 3.3C, and 3.3F, respectively, for the TCR stimulation experiment.

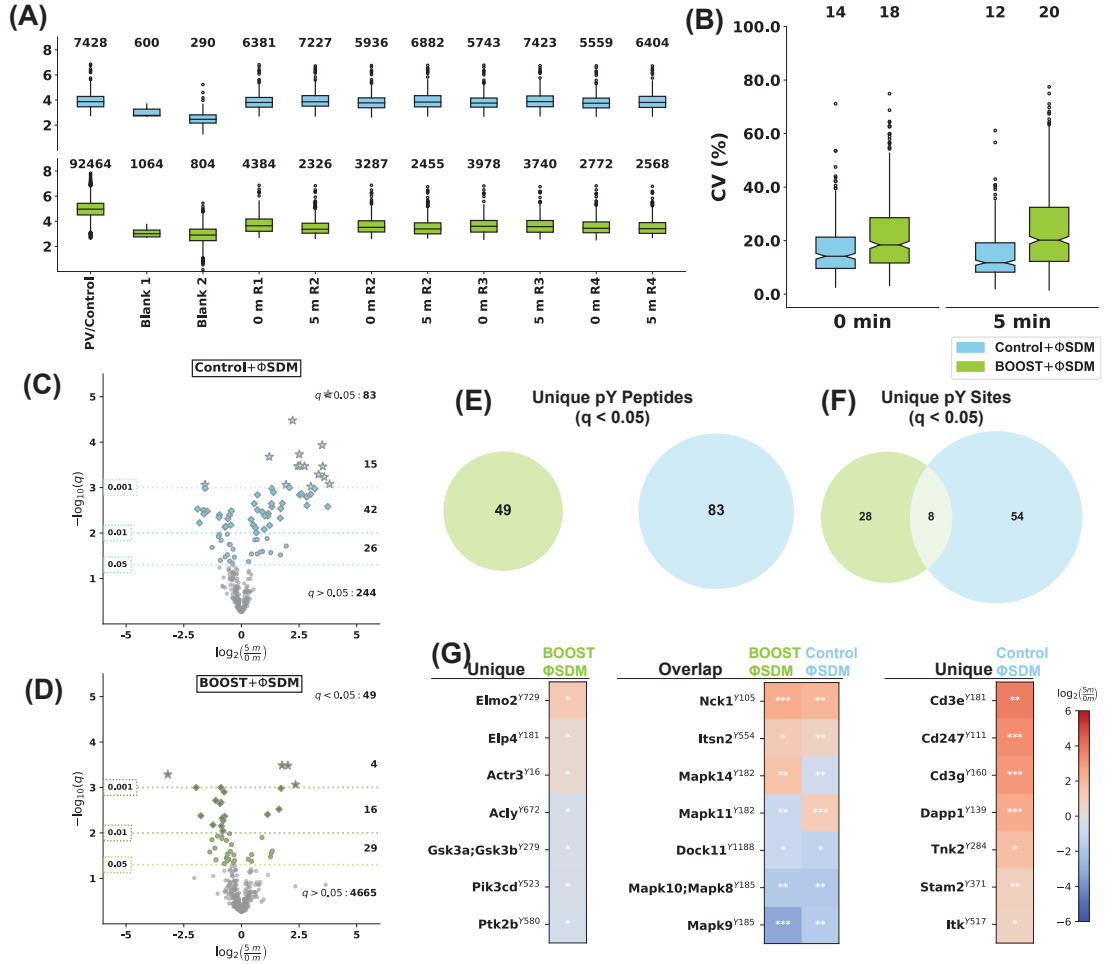


Figure 3.13: Φ SDM degrades the yield of TCR responsive pY sites in the BOOST experiment, with only minimal degradation to the control experiment. (A) and (B) As in Figures 3.4A and 3.5C, respectively, except for the time course TCR stimulation samples acquired with Φ SDM. (C) and (D) Volcano plots comparing pY peptide abundance between TCR stimulated to unstimulated for control and BOOST samples, respectively, acquired with Φ SDM. (E) and (F) overlap between significantly changing unique pY peptides and pY sites, respectively. (G) Heatmaps showing a selection of significantly changing pY sites observed in the BOOST (left), BOOST and control (middle), and control (right) experiments acquired with Φ SDM.

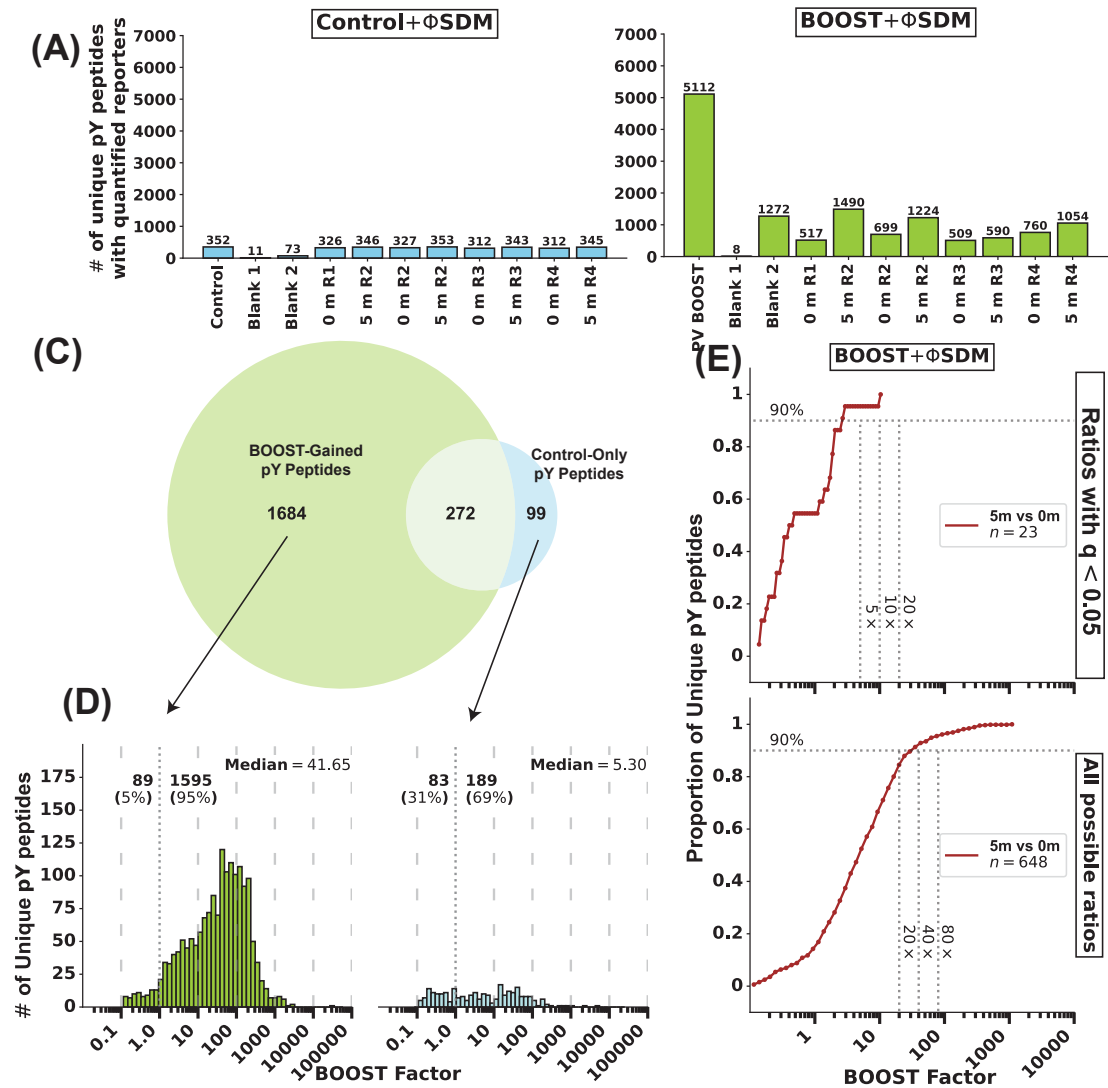


Figure 3.14: Data quality verification on the time course TCR stimulation BOOST experiment. (A), (B), (C), (D) As in Figures 3.3A, 3.3D, 3.3E, and 3.3F, respectively, for the TCR stimulation experiment acquired with Φ SDM.

3.4 Discussion

Improving our understanding of the tyrosine phosphorylation events involved in diseases and their treatments requires accurate and reliable methods for deep profiling of the pY proteome. Phosphotyrosine profiling methods would ideally require as little sample as possible as the most biologically relevant samples, including primary cell experiments or clinical samples, are limited in supply(Engholm-Keller and Larsen, 2013). Although LC-MS/MS techniques are desirable for wide-scale analysis of pY events, the low abundance of pY in the proteome makes pY proteomics studies challenging or impossible in the most relevant samples. While recent advances in TMT multiplexing capacity(Thompson et al., 2019; Robitaille et al., 2020; McAlister et al., 2012; Werner et al., 2012), MS instrument sensitivity(Yu et al., 2020; Robitaille et al., 2020) and pY-specific enrichment techniques 4-7 have improved pY sequencing depth, these advances alone are not sufficient for deep pY sequencing from scarce samples. Similar to methods in single cell proteomics(Petelski et al., 2021; Vistain and Tay, 2021), the BOOST method takes advantage of TMT multiplexing, state-of-the-art MS instruments and pY specific enrichment techniques to improve pY sequencing in low abundance samples. Proteomics experiments using carrier channels to improve sequencing are frequently used during single-cell TMT proteomics experiments(Specht and Slavov, 2020; Cheung et al., 2021; Petelski et al., 2021; Vistain and Tay, 2021; Dwivedi and Rose, 2022; Tsai et al., 2020; Derks et al., 2023). Artefacts can arise from impurities in TMT reagents(Stopfer et al., 2021; Peterson et al., 2024), which are exacerbated with uneven sample loading(Chua et al., 2021; Stopfer et al., 2021; Peterson et al., 2024), and it is therefore imperative to evaluate the pY carrier channel in advance of a BOOST experiment. Further, fatal differences in quantitation are observed when using either MS3 or MS2 quantitation during carrier channel experiments(Chua et al., 2021; Stopfer et al., 2021), thus highlighting the need to evaluate MS acquisition settings to produce the most quantitatively reliable data. Regardless, it is possible to achieve deep, quality pY sequencing in scarce samples using the BOOST method.

Here, we show the utility of the BOOST method for studying signalling events in primary T cells from mice using 5- to 50-fold less starting material than traditional pY proteomics protocols. We evaluate the accuracy, precision and reproducibility of

the BOOST method using a multiplexed TMT setup with SPS-MS3 quantitation(Chua et al., 2021; McAlister et al., 2014) (Figures 3.1, 3.3, and 3.4). Our experimental design considers reporter ion interference, a phenomenon by which impurities in the synthesis of reporter ions leads to false positive identifications in unexpected channels(Stopfer et al., 2021; Peterson et al., 2024), by discarding TMT127C, where we have previously observed significant reporter ion interference during BOOST experiments(Chua et al., 2020, 2021). Using the BOOST method, we quantify over 2000 pY sites from TCR stimulated CD8+ T cells that are not observed in previously published literature using 100 million CD4+ T cells for pY enrichment followed by label free DDA acquisition(Locard-Paulet et al., 2020). Of these pY sites, many are sites of unknown function on proteins critical to T cell signal transduction, including ZAP70, CD45, ITK, TEC and PLC γ 1, while reliably sequencing pY sites that are commonly observed by traditional methods. We further show that the BOOST method can uncover new TCR responsive pY sites from 1 mg of protein input, including sites of unknown function like SLP76^{Y483}, CSK^{Y184} and Cblb^{Y763}, while reproducibly sequencing known TCR responsive sites like Erk1^{Y205}, Erk2^{Y185}, ζ -chain^{Y83}, ζ -chain^{Y123}, ζ -chain^{Y142}, ζ -chain^{Y153}, CD3 ϵ ^{Y170}, Tec^{Y518} and PLC γ 1^{Y771} (Figure 3.11). Our understanding of the regulation and timing of tyrosine phosphorylation on well-established proteins like LCK and ZAP70 is still evolving, and the role of novel pY sites on TCR signalling proteins are continuously being established(Kästle et al., 2022; Barreira et al., 2018; Courtney et al., 2019; Gallagher et al., 2021; Hallumi et al., 2021; Huszenicza et al., 2024; Kagohashi et al., 2024; Kumar et al., 2018; Kumaradevan et al., 2018; Lazer et al., 2010; Lo et al., 2018, 2019; Robles-Valero et al., 2021; Saitoh et al., 2022; Sridaran et al., 2022; Strazza et al., 2021; Sun and Ayrapetov, 2023; Tawaratsumida et al., 2022; Voisinne et al., 2022; Wada et al., 2022; Wang et al., 2021; Yang et al., 2020; Zhang et al., 2021, 2024). Thus, establishing the TCR responsiveness of novel pY sites, including those on SLP76, Cblb, CSK and others that are observed only using BOOST, provide new hypotheses regarding the regulation of T cell signalling proteins. Although the BOOST method does provide a new set of TCR responsive pY sites, some sites seen in the TMT control samples are not significantly responsive (Figure 3.11C–G), possibly due to the slightly inflated variance associated with using carrier channels in multiplexed proteomics experiments(Cheung et al., 2021). The most comprehensive picture of TCR

responsive pY sites comes from the combination of TMT experiments with either PV and unstimulated carrier channels, each of which reveals a unique set of differential pY sites on T cell signalling proteins.

We also unexpectedly find that instrument parameters can have a profound influence on the efficacy of the BOOST method. We find that enabling TurboTMT during sample acquisition on the Orbitrap Eclipse Tribrid MS can severely reduce the depth of pY sequencing and the quality of data in BOOST experimental samples (Figures 3.6, 3.7, 3.13 and 3.14). The TurboTMT setting uses the Φ SDM to increase the speed of sample acquisition and spectral resolution in multiplexed TMT experiments (Yu et al., 2020; Grinfeld et al., 2017; Kelstrup et al., 2018). Despite the potential benefits from TurboTMT, Grinfeld et al., Kelstrup et al., and Yu et al. have shown that the benefits of the Φ SDM require optimisation of MS settings, including shorter gradients and lower transient times, and that low abundance samples generally tend to suffer from implementing the Φ SDM (Yu et al., 2020; Grinfeld et al., 2017; Kelstrup et al., 2018).. To minimise parameter differences in our experiments, the acquisition settings differ only in whether TurboTMT, and therefore Φ SDM, is implemented. Our acquisition settings may, therefore, not be optimal for the Φ SDM to be maximally effective. This, along with the generally small protein input we use compared with other studies evaluating the Φ SDM, may explain the decrease in sequencing depth we observe in the control samples when TurboTMT is implemented (Figure 3.6). These parameters do not, however, explain the severe degradation of sequencing depth, quality and TCR-responsive pY sites we see in the BOOST experimental samples acquired with TurboTMT implemented (Figures 3.13 and 3.14). Optimisation and evaluation of the Φ SDM for pY proteomics requires more investigation such as titring carrier channel abundance, MS2/MS3 transients, gradient length, and AGC settings, and we caution others attempting similar pY proteomics studies to evaluate the acquisition settings on their MSs before these experimental samples.

With an expanding interest in global PTM evaluation by LC-MS/MS, single cell proteomics, and evaluation of scarce samples, methods to increase sequencing depth and experimental reproducibility with reduced sample input are required. Here, we evaluate

the BOOST method for increasing pY sequencing depth in primary T cells and show that a novel computational method such as Φ SDM may harm pY quantitation. Our results provide confidence that the BOOST method is viable for samples with reduced protein input, particularly scarce samples like primary cells or clinical samples.

3.5 Associated Data

The mass spectrometry proteomic data have been uploaded to the ProteomeXchange Consortium(Jones et al., 2006) via the PRIDE partner repository with the dataset identifier PXD053863.

Acknowledgements

The authors wish to thank Drs. Ricky Edmoson and Samuel G. Mackintosh from the University of Arkansas for Medical Sciences (UAMS) for collecting proteomic data. We acknowledge financial support from NIH grants R01AI083636, P20GM121293, P01AI091580, R24GM137786, HL148120, and HL161127. Aurora Callahan was supported by T32GM136566 and the Sidney E. Frank Fellowship.

Conflict of Interest

The authors have declared no conflict of interest.

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CHAPTER 4

CSF1R -CAR T cells induce CSF1R signalling and can promote target cell proliferation

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Abstract Chimeric antigen receptor (CAR) T cells have revolutionised the landscape of cancer therapy, demonstrating unprecedented success in treating relapsed or refractory blood cancers. Previous studies of the mechanisms underlying the interactions and responses of CAR T cells and their targets have focused on the activation of CAR T cells and attempted to optimise CAR design to increase efficacy, while ignoring tumours and their responses to CAR ligation. We evaluated the signalling of a second-generation, ligand-based CAR built from the colony-stimulating factor 1 (CSF1) ligand to target the CSF1 receptor (CSF1R) on CSF1R-expressing target cells, and compared it to a conventional single-chain variable fragment (scFv)-based CAR against the B-cell antigen CD19 using stable isotope labeling by amino acids in cell culture (SILAC) co-culture with phosphotyrosine (pY) enrichment and liquid chromatography–tandem mass spectrometry (LC–MS/MS).. We showed that ligation of CSF1R-expressing THP-1 cells with CSF1R-CAR T cells stimulated CSF1R-like signalling in the THP-1 cells, whereas no target cell signalling response was observed after the ligation of CD19-CAR T cells with target Raji cells. In experiments with small-molecule inhibitors of the tyrosine kinase Lck, actin polymerisation, and CSF1R, we found that CAR-induced CSF1R signalling in THP-1 cells depended exclusively on the kinase activity of CSF1R with no participation from T cell activation. Consistently, CSF1R-CAR T cells promoted THP-1 cell growth at low effector-to-target (E:T) ratios but prevented THP-1 cell growth at high E:T ratios. Our data provide evidence for an unintended consequence of CARs: CAR-induced signalling

in cancer cells. These data may have broad implications for the choice of CAR antigen for optimal clinical efficacy.

4.1 Introduction

The development of cancer immunotherapies, including cancer-targeted antibodies, bispecific T cell receptors (TCRs), and chimeric antigen receptors (CARs), has substantially improved survival rates among patients with aggressive forms of cancer. Currently, six CARs are US Food and Drug Administration-approved for use in treating relapsed or refractory multiple myeloma, B cell lymphoma, acute lymphoblastic leukaemia, and mantle cell lymphoma (Abbasi et al., 2023). The success and development of CARs were greatly aided by their modular design because each CAR contains one or more single-chain variable fragments (scFvs) from an antibody, providing target specificity toward one or more proteins overexpressed on the tumour, as well as a transmembrane domain and multiple intracellular signalling domains from receptors involved in T cell activation. All currently approved CARs include the intracellular domain of the ζ -chain, a component of the TCR complex that is involved in TCR signal initiation by acting as a primary T cell activation signal (Lindner et al., 2020; Nix and Wiita, 2024). Costimulation, a requirement for full activation and effector function of cytotoxic T cells, is achieved in CAR T cells through the inclusion of intracellular signalling domains from canonical T cell costimulatory receptors, such as CD28 or 4-1BB (Cappell and Kochenderfer, 2021; Eisenstein et al., 2016; Zhang et al., 2007; Zheng et al., 2004). Although only six CARs have been approved, many CARs targeting a diverse array of target-associated antigens (TAAs) are being developed and tested in animal models and patients (Albinger et al., 2021; Luksik et al., 2023; Qu et al., 2021).

Selecting viable target proteins that minimise off-target effects and retain specificity toward the tumours is necessary for the efficacy of CAR T cell therapy. The approved CARs target cluster of differentiation 19 (CD19) or B cell maturation antigen (BCMA), which are expressed widely on cells of the B cell lineage (Dogan et al., 2020; Ginaldi et al., 1998; Wang et al., 2012). Although these CARs have been effective in treating multiple myelomas, non-Hodgkin lymphomas, and acute lymphoblastic leukaemias, CAR T cell

treatment indiscriminately lyses B lineage cells expressing target antigens, leading to B cell aplasia (Gabelli et al., 2024; Wang et al., 2022). Many patients experience antigen escape, whereby CAR T cells eradicate tumours expressing the TAA and inadvertently select for a TAA-negative tumour population or are reduced in number by the tumour microenvironment (Lin et al., 2024). Immune evasion is a hallmark of cancer development, which is a limitation of immunotherapies and an active area of research (Pecorino, 2021; Vinay et al., 2015).

There has been a wave of research evaluating the use of CAR T cells for the clearance of tumour-associated macrophages (TAMs), which reside in and are heavily regulated by tumours to promote a reduction in the number of immune cells that would otherwise respond to the tumour (Li et al., 2022b; Rodriguez-Garcia et al., 2021; Sánchez-Paulete et al., 2022). Therapies targeting TAMs can tremendously improve standard cancer therapeutics (Li et al., 2022a) and are thus an attractive secondary target for CAR T cell therapies. Although few studies have evaluated CAR T cell efficacy against TAMs, two notable studies by Rodriguez-Garcia et al. (2021) and Achkova et al. (2022) provided different targeting strategies to deplete TAMs or lymphoma cell lines expressing TAM markers with some success. Rodriguez-Garcia et al. (2021) showed that a subpopulation of TAMs express folate receptor β (FR β) and have an M2-like macrophage phenotype, and FR β + TAMs decrease mesothelin-targeting CAR T cells during antigen ligation. Furthermore, by targeting FR β + TAMs with a third-generation scFv-based CAR in conjunction with a third-generation CD19 CAR, Rodriguez-Garcia et al. (2021) observed targeting and depletion of FR β + TAMs, which improved CD19-CAR T cell-mediated tumour lysis (Rodriguez-Garcia et al., 2021). Achkova et al. (2022) opted to target colony-stimulating factor 1 receptor (CSF1R), a receptor tyrosine kinase that drives macrophage growth and differentiation, which is highly abundant on TAMs and some cancers, using CSF1 and the cytokine interleukin-34 (IL-34), two native ligands of CSF1R with different binding affinities. The authors showed that using CSF1 as the targeting domain for CD28-TCR ζ or CD28-41BB-TCR ζ CARs (28 ζ and 28bb ζ , respectively, leads to cytokine production and the depletion of CSF1R-expressing lymphoma cell lines in vitro (Achkova et al., 2022). Whereas Rodriguez-Garcia et al. (2021) showed the potential for targeting and depleting TAMs, Achkova et al. (2022) showed that targeting with

native ligands may also be effective and did so while targeting a protein necessary for macrophage development and persistence.

Considering the high abundance of signalling active proteins with known ligands in many cancers and on TAMs, we expect many researchers to evaluate ligand-based CARs for tumour and TAM clearance in the future. Here, we evaluated the signalling capacity of a second-generation CSF1R-targeting CAR constructed with CSF1 as the targeting moiety (CSF1-28tm-28co- ζ ; CSF1R-CAR, hereafter) in comparison to a well-established CAR design targeting CD19 (CD19scFv-8tm-28co- ζ ; CD19-CAR, hereafter). We showed that CAR expression was stable in a Jurkat T cell model system, and we used liquid chromatography–tandem mass spectrometry (LC-MS/MS) to evaluate bi-directional phosphotyrosine (pY) signalling using a stable isotopic labelling of amino acids in cell culture (SILAC) co-culture method, which our laboratory previously established (Griffith et al., 2022). We found that CAR signalling differed between CD19-CAR T cells and CSF1R-CAR T cells, with a higher signalling response observed in the CD19-CAR T cells. In contrast, Raji cells (the targets of the CD19-CAR T cells) showed no trend of activation during co-culture with CD19-CAR T cells, whereas THP-1 cells expressing CSF1R showed robust signalling during co-culture with CSF1R-CAR T cells. Small-molecule inhibition of CSF1R ablated the signalling phenotype in THP-1 cells, whereas inhibition of the tyrosine kinase Lck or of actin polymerisation in CSF1R-CAR T cells had no effect. Finally, we found that CSF1R-CAR T cells induced THP-1 cell proliferation at low effector-to-target (E:T) ratios and can cleared THP-1 cells at high E:T ratios in vitro. Our data show that the method of targeting influences the propensity for target cell clearance, which should be considered during the design of new CARs.

4.2 Results

4.2.1 Stimulation of phosphotyrosine signalling differs between CD19-CAR and CSF1R-CAR

To determine whether a ligand-based CAR was capable of productively engaging its target receptor, we expressed a second-generation CSF1R-targeting CAR that utilises

CSF1, a native ligand of CSF1R, as the targeting moiety in Jurkat cells, a human T cell leukaemia cell line. As a control, we expressed a CD19-targeting CAR modelled after the therapeutic Yescarta (Axicabtagene ciloleucel; Figure 4.1A, 4.2A-B)(Locke et al., 2019). Both the CSF1R-CAR and the CD19-CAR were expressed on the cell surface (Figure 4.1B-C) with high GFP expression in CD19-CAR T cells (Figure 4.2C). To evaluate signal induction during CAR-target ligation, we used CSF1R-expressing THP-1 cells (Figure 4.2D) and CD19-expressing Raji cells in co-culture stimulation experiments with a 2:1 T cell to target cell ratio (effector-to-target, E:T). We found that CD19-CAR T cell and Raji cell co-cultures showed a characteristic, CAR-dependent T cell signalling-like induction pattern as determined by Western blotting analysis with an anti-phosphotyrosine antibody (α -pY, clone 4G10)(Courtney et al., 2017), with statistically significant increases in the intensities of bands between 50 and 100 kDa, and a high molecular weight band corresponding to PLC γ 1 after 2 and 5 min of co-culture (Figure 4.1D, 4.3A). In contrast, co-cultures of CSF1R-CAR T cells and THP-1 cells showed an altered, but not statistically significant, banding pattern after 2 min, with fewer pY-containing bands of between 50-100 kDa increasing in intensity, and a substantial increase in the intensity of a THP-1 cell-specific, high molecular weight pY-containing protein (Figure 4.1E, 4.3C). In contrast, non-CAR-expressing Jurkat cells showed a significant reduction in global tyrosine-phosphorylated proteins after co-culture with either Raji cells or THP-1 cells (Figure 4.3B, 4.3D), indicating that the CAR was necessary for any changes in pY-containing proteins observed by Western blotting. Together, these data suggest that there are differences in pY signalling between CD19-CAR T cell:Raji cell co-cultures and CSF1R-CAR T cell:THP-1 cell co-cultures.

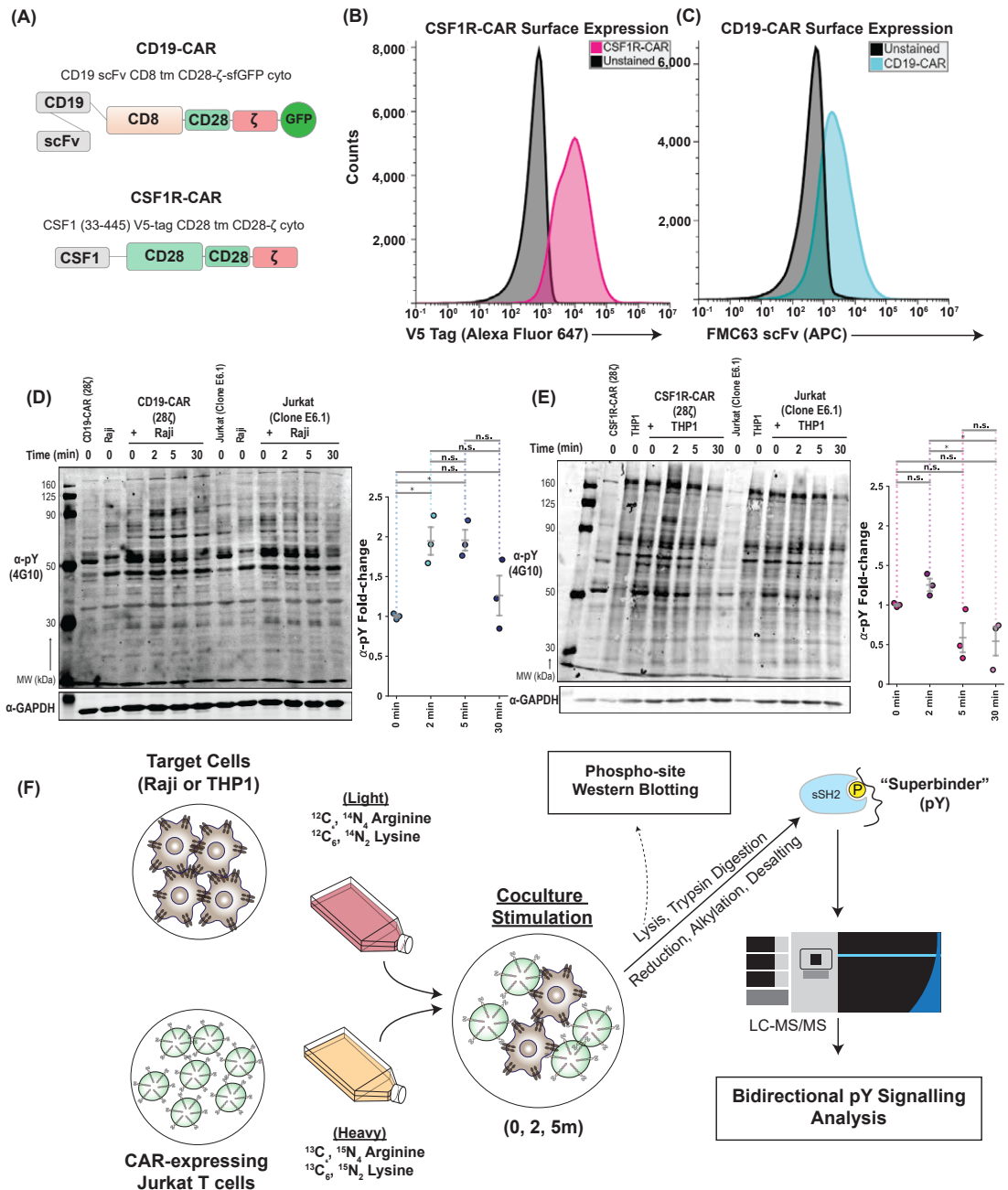


Figure 4.1: (See next page for legend)

Figure 4.1: Stimulation of phosphotyrosine signalling differs between the CD19-CAR and the CSF1R-CAR. (A) Domain construction of the CD19-CAR and the CSF1R-CAR. (B and C) Representative flow cytometry analysis showing the cell surface expression of CSF1R-CAR and (C) CD19-CAR on Jurkat cells. (D) Left: Western blotting analysis of tyrosine phosphorylation (with the 4G10 antibody) for CD19-CAR T cell:Raji cell co-cultures and for parental Jurkat cell:Raji cell cocultures incubated for the indicated times. Blots are representative of three biological replicates. GAPDH was used as a loading control. Right: Fold-change in the relative intensity of 4G10-positive bands as determined by densitometry analysis (blue shades indicating CD19-CAR timepoints). (E) Western blotting analysis (left) and densitometry analysis (right), as described for (D), for CSF1R-CAR T cell:THP-1 cell co-cultures and for parental Jurkat cell:THP-1 cell cocultures incubated for the indicated times (pink shades indicating CSF1R-CAR timepoints). Statistical significance was determined by two-way ANOVA with Fisher's least significant difference and the Holm-Sidak correction for multiple comparisons. * : $p < 0.05$, ** : $p < 0.01$, ***: $p < 0.001$. (F) Workflow for the SILAC co-culture experiments that were analysed by LC-MS/MS.

(A) CD19 scFv CD8 tm CD28-ζ-sfGFP cyto

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MALPVTALLLPLALLLHAARPDIQMTQTSSLSASLGDRV
TISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVP
SRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFG
GGTKLEITGGGGSGGGGGGGSEVKLQESGPGLVAPSQS
LSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETT
YYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAK
HYYYGGSYAMDYWGQGTSTVTVSSGSGSEQKLISEEDLGST
TTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD
FACDIYIWAPLAGTCGVLLLSLVITLYCSRSKRSRLHSD
YMNMTPRRPGPTRKHYPYAPPRDFAAYRSRVKFSRSADA
PAYQQQNQLYNELNLRREEYDVLDKRRGRDPEMGGKPQ
RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGL
YQGLSTATKDTYDALHMQALPPRTSGSGSMSKGEELFTGV
VPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTG
KLPVPWPTLVTTLYGVQCFSRYPDHMKRHDFFKSAMPEG
YVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFK
EDGNILGHKLEYNFNSHNVYITADKQNGIKANFKIRHNV
EDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDP
NEKRDHMLLEFVTAAGITHGMDELYK

```

FMC63 scFv
(PBD 7URV)

CD8α tm (NP_001759.3)

CD28 cyto (NP_006130.1)

ζ-chain cyto (NP_000725.1)

sfGFP (UFQ89826.1)

(B) CSF1 (33-445) CD28 tm CD28-ζ cyto

```

MTMYLWLKLLAFGFAFLDTEVFVTGQSPTGKPIPNLLGL | V5 Tag
DSTGSGSTSEEVSEYCSHMIGSGHLQSLQRLIDSQMETSC
QITFEFVDQEQLKDPVCYLKKAFLLVQDIMEDTMRFRDNT
PNAIAIVQLQELSLRLKSCFTKDYEEHDKACVRTFYETPL
QLLEKVKNVFNETKNLLDKDWNIFSKNCNSFAECSSQDV
VTKPDCNCLYPKAIPSSDPASVSPHQPLAPSMAPVAGLTW
EDSEGTEGSSLLPGEQPLHTVDPGSAKQRPPRSTCQSFEP
PETPVVKDSTIGGSQPPRPSVGAFNPGMEDILDSAMGTNW
VPEEASGEASEIPVPQTELSPSRPGGSMQTEPARPSNF
LSASSPLPASAKGQQPADVTGTALPRVGPVRPTGQDWNHT
PQKTDHPSALLRDPPEPGSPRISSLRPQGLSNPSTLSAQF
QLSRSHSSGSVLPLGELEGRRSSGSGSIEVMYPPPYLDNE
KSNGTIIHVKGKHLCPSPLFPGPSKPFFWVLVVGGVLACY
SLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRPGPTRKH
QPYAPPRDFAAYRSRVKFSRSADAPAYQQQNQLYNELN
LRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKD
KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH
MQALPPR

```

CSF1
(NP_757351.2)

CD28 tm & cyto
(NP_006130.1)

ζ-chain cyto (NP_000725.1)

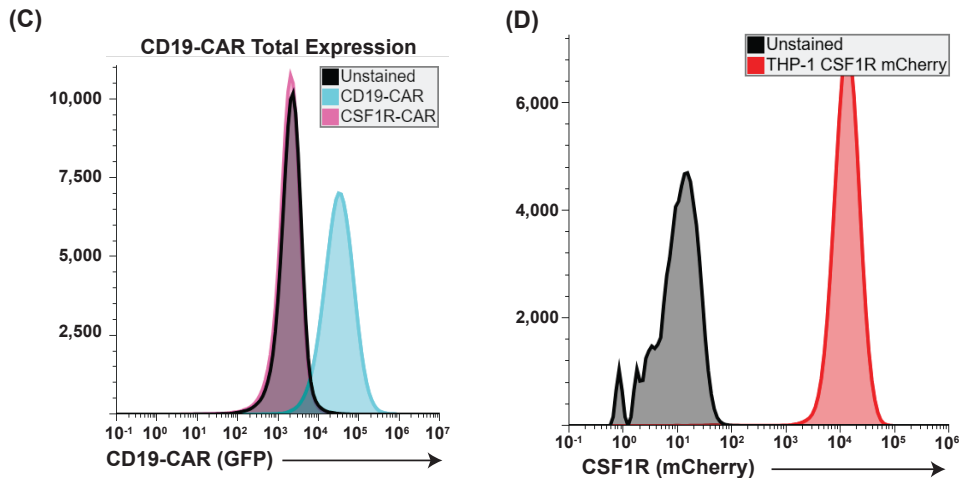


Figure 4.2: (See next page for legend)

Figure 4.2: Protein sequences for (A) CD19-CAR and (B) CSF1R-CAR. Colours represent areas of perfect alignment with the proteins shown to the right. (C) GFP Flow cytometry showing total CD19-CAR expression. (D) Flow cytometry evaluating mCherry abundance in THP1 cells overexpressing CSF1R mCherry.

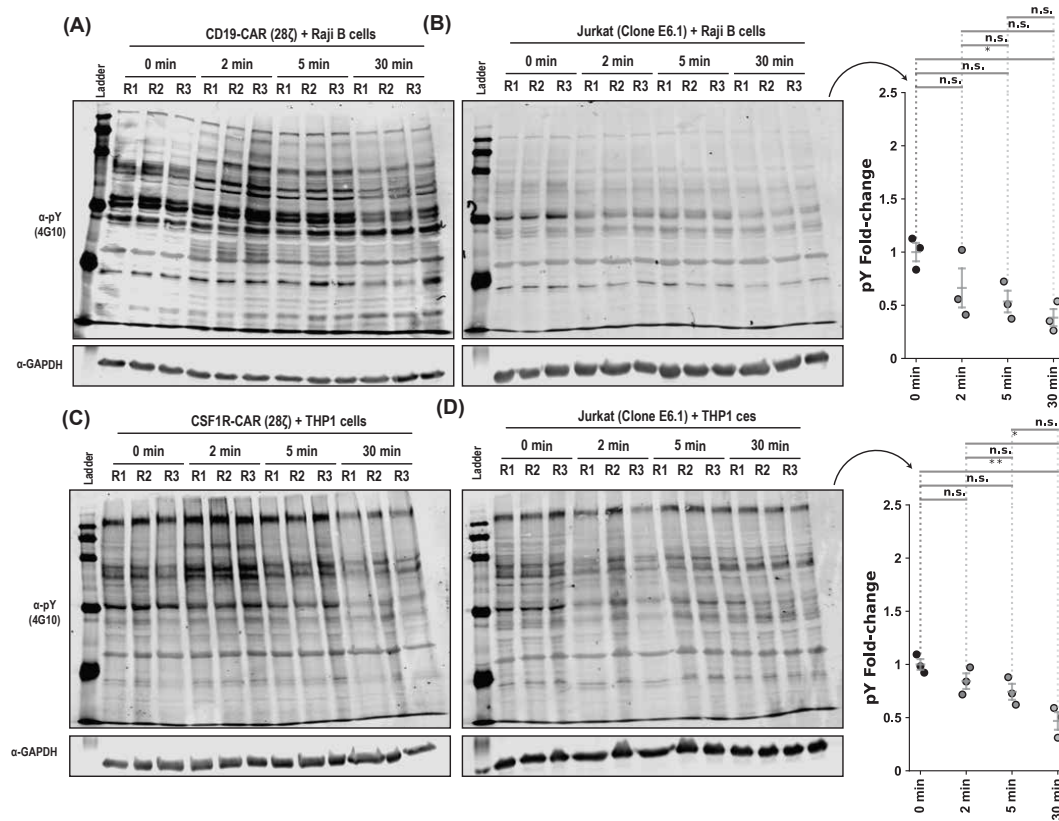


Figure 4.3: CSF1R-CAR/THP1 co-cultures exhibit altered pY induction compared with CD19-CAR/Raji co-cultures, while non-CAR Jurkat T cells elicit no pY responses. Total phosphotyrosine (pY) Western blots on co-cultures of (A) CD19-CAR and Raji B cells, (B) Jurkat (clone E6.1) and Raji B cells, (C) CSF1R-CAR and THP1 monocytes, and (D) Jurkat (clone E6.1) and THP1 monocytes. Total pY quantification, normalised to GAPDH, is displayed to the right of (B) and (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Fisher LSD with the Holm-Sidak FWER adjustment). Quantification for (A) and (C) is shown in Figure 4.1.

4.2.2 The CSF1R-CAR minimally engages TCR signalling pathway

To provide a comprehensive analysis of bi-directional pY signalling during co-culture, we used our previously established SILAC co-culture MS-based methodology (Figure 4.1F), which enables the use of isotopic labelling as a cell identity marker for peptides acquired by MS analysis (Griffith et al., 2022). In our previous study, we found that an FMC63 scFv-based, third-generation CD19-CAR robustly increased the phosphorylation of canonical TCR signalling proteins (Griffith et al., 2022). Here, we report that our CD19-CAR reproducibly engaged the T cell signalling pathway, similarly to that of a parameter-matched third-generation CAR, with large-scale induction of pY signalling after 2 and 5 min of co-culture with Raji cells (Figure 4.4A, 4.5A, 4.6). In contrast, there was very little stimulation of pY signaling in CSF1R-CAR T cells at either 2 or 5 min after co-culture with THP-1 despite reproducible peptide sequencing (Figure 4.4B).

Western blotting analysis of pY proteomics lysates revealed increased global tyrosine phosphorylation in both CD19-CAR T cell:Raji cell and CSF1R-CAR T cell:THP-1 cell co-cultures. However, only CD19-CAR T cell:Raji cell co-cultures showed a significant increase in the amounts of two key TCR signalling pY sites: PLC γ 1^{Y783} and LAT^{Y220} (Figure 4.4C-E, 4.7) (Paz et al., 2001; Weiss et al., 1991). In CD19-CAR T cell:Raji cell co-cultures, our MS data revealed the phosphorylation of TCR ζ immunoreceptor tyrosine-based activation motifs (ITAMs) but not of ITAMs in CD3 ϵ or CD3 γ , which are involved in the forward progression of TCR signalling (James, 2018; Love and Hayes, 2010). Although the MS analysis could not distinguish between TCR ζ sequences originating in the CAR from those in the native TCR ζ protein, previous studies suggest that CAR and TCR signalling are largely independent. The lack of CD3 ϵ or CD3 γ engagement suggests that the phosphorylation of TCR ζ ITAMs originated from the CAR (Barden et al., 2023). We observed an increased abundance of pY sites in proximal T cell signalling proteins, including LAT^{Y220}, PLC γ 1^{Y775}, PLC γ 1^{Y783}, PLC γ 1^{Y775Y783}, Zap70^{Y492}, Zap70^{Y493}, and Zap70^{Y429Y493}, as well as TCR-distal proteins, such as Jnk2^{T183Y185}, Erk1^{T202Y204}, and Erk2^{T185Y187} (Figure 4.4F) (Gaud et al., 2018).

Consistent with our Western blotting analysis, CSF1R-CAR T cells displayed little,

if any, increases in pY-containing TCR signalling proteins, except for Erk1^{T202Y204} and Erk2^{T185Y187}, which are positioned toward the bottom of feedforward CAR signalling (Figure 4.4G). Through post-translational modification signature enrichment analysis (PTM-SEA), an algorithm that reveals the PTM-specific enrichment of signalling pathways, kinases, and drug signatures (Krug et al., 2019), we saw that TCR signalling, and signalling pathways in general, were significantly increased in CD19-CAR and CSF1R-CAR T cells after co-culture, despite there being little significant increase in pY site abundance in the TCR signalling pathway in CSF1R-CAR T cells (Figure 4.4H-I). Substrates for the integral TCR kinases Lck and Zap70 were exclusively enriched in the CD19-CAR T cell:Raji cell co-cultures, whereas CSF1R-CAR T cells showed enrichment of Src substrates (Figure 4.4J-K). CAR-based signalling is critically affected by aspects of the binding domain, including its affinity (Mao et al., 2022), its proximity to the plasma membrane (Hanssens et al., 2022; Haso et al., 2013; Li et al., 2020), receptor clustering as a result of scFv aggregation (Nieba et al., 1997), the abundance of its target antigen (Ramakrishna et al., 2019; Zhang et al., 2023b), and tonic phosphotyrosine signalling (Hartl et al., 2020; Yao et al., 2024), which may explain the relatively low extent of pY signaling. Together, our results demonstrate that ligand-targeted CSF1R-CARs weakly engage the TCR signalling pathway compared to the therapeutically used CD19-CARs under the experimental conditions tested.

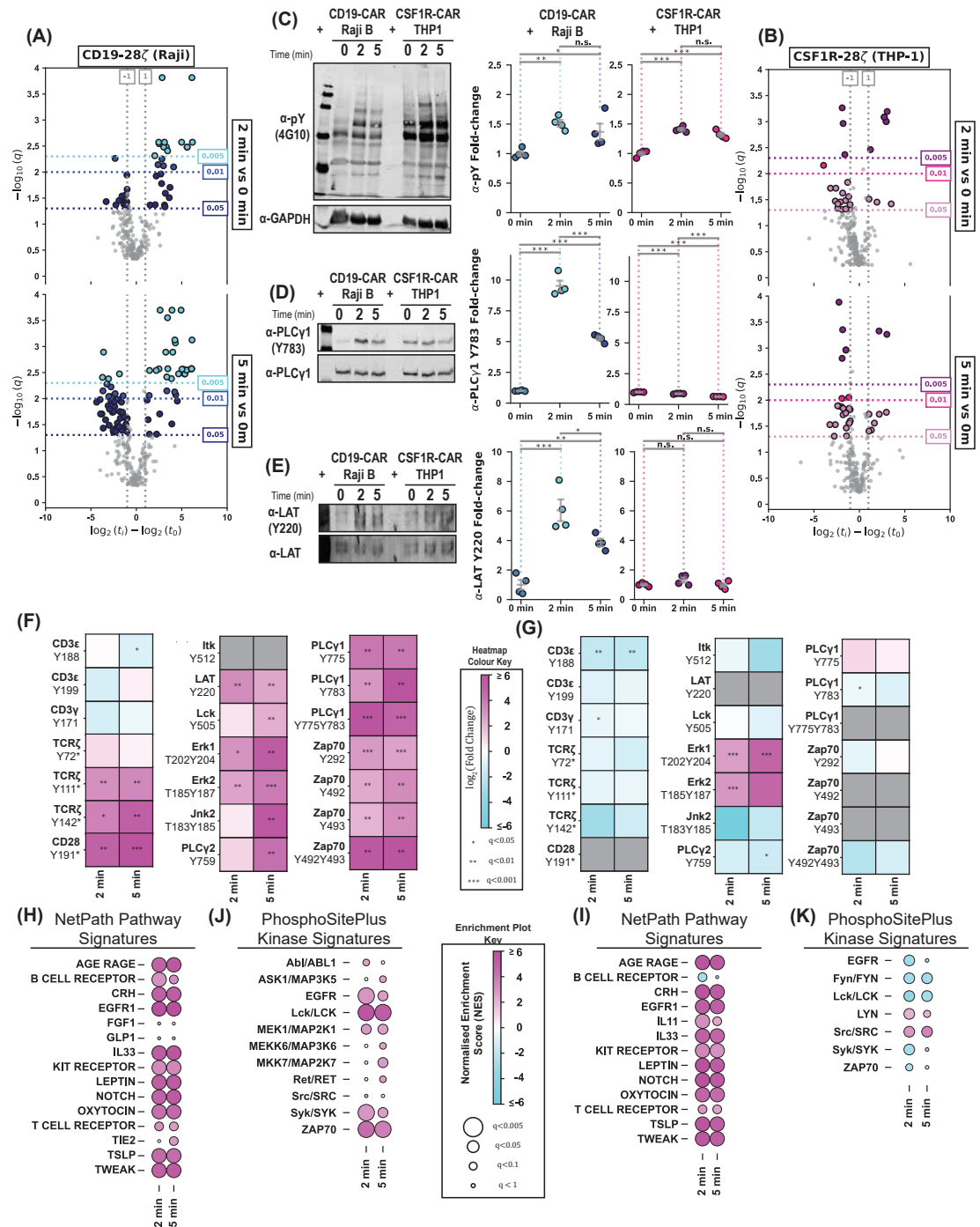


Figure 4.4: (See next page for legend)

Figure 4.4: The ligand-targeted CSF1R-CAR minimally activates the TCR signalling pathway in Jurkat cells. (A) Volcano plot analysis of the phosphotyrosine (pY) sites, as identified by LC-MS/MS, in CD19-CAR T cells after co-culture with Raji cells for the indicated times. Data are from four biological replicates. (B) Volcano plot analysis of the pY sites in CSF1R-CAR T cells after co-culture with THP-1 cells for the indicated times. Data are from four biological replicates. (C) Left: Western blotting analysis of pY-containing proteins (4G10-positive) in proteomics samples, from the indicated cell co-cultures. Right: Quantification of the fold-change in the pY-containing proteins in the indicated cell co-cultures after normalization to GAPDH abundance. Data are from four biological replicates; blue shades indicating CD19-CAR and pink shades indicating CSF1R-CAR timepoints. (D and E) Western blotting analysis (left) and relative quantification (right), as described for (C), of PLC γ 1Y783 (D) and LATY220 (E) in the indicated cell co-cultures. LATY220 abundance was normalised to that of PLC γ 1 because of the low LAT protein signal. Data are from four biological replicates. Statistical significance was determined by two-way ANOVA with Fisher's least significant difference and the Holm-Sidak correction for multiple comparisons. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$. (F and G) Heatmaps showing quantification of the indicated individual phosphorylation sites observed in CD19-CAR T cells after co-culture with Raji cells (F) and in CSF1R-CAR T cells after co-culture with THP-1 cells (G) for the indicated times. Data are from four biological replicates. (H and I) PTM-SEA showing enrichment of CD19-CAR T cell PTM sites in NetPath Pathways (H) and in the PhosphoSitePlus Kinase Signatures database (I). Data are from four biological replicates. (J and K) PTM-SEA showing enrichment of CSF1R-CAR T cell PTM sites in NetPath Pathways (H) and in the PhosphoSitePlus Kinase Signatures database (I). Data are from four biological replicates.

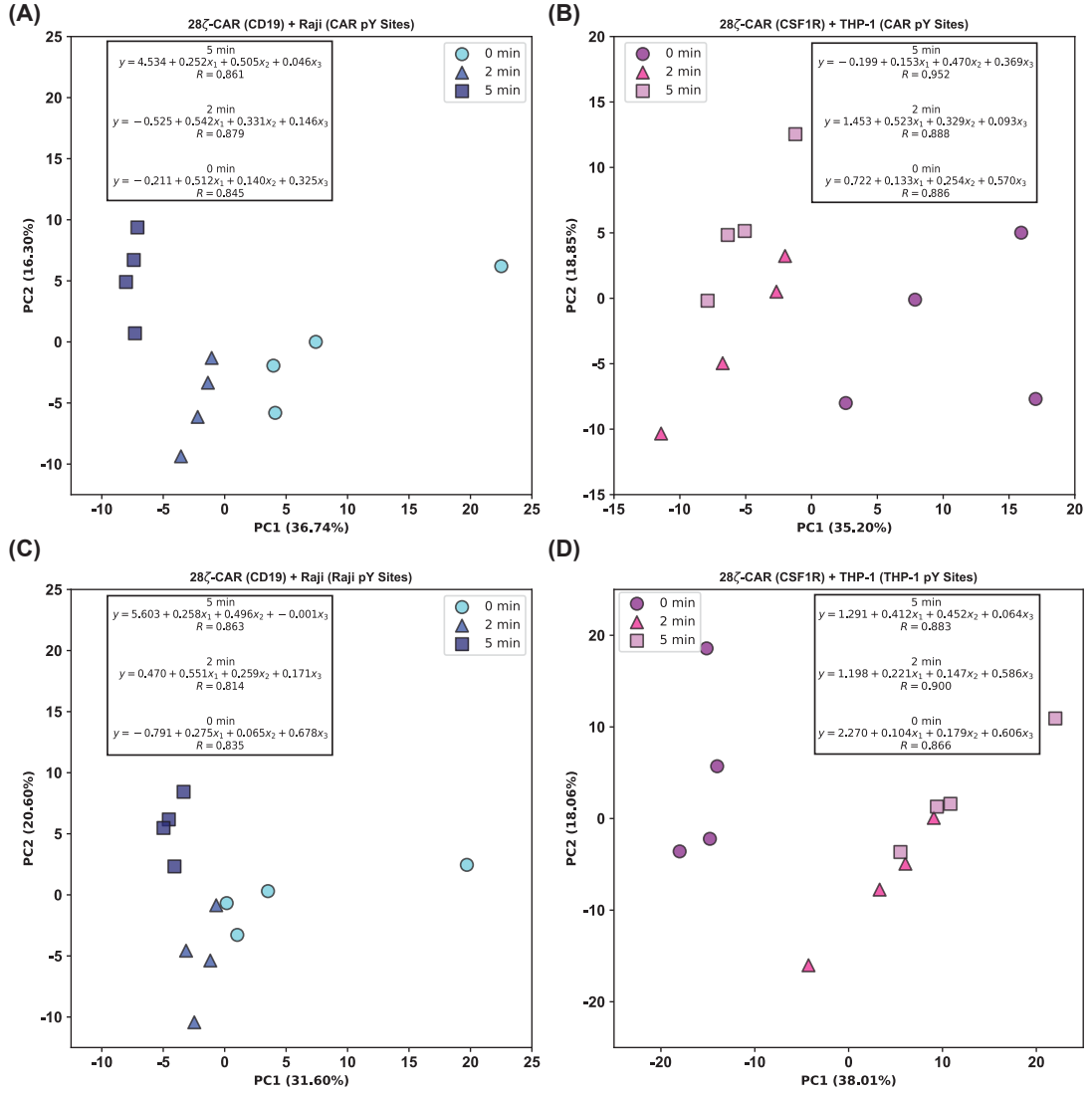


Figure 4.5: Reproducibility and clustering of pY proteomics data. (A) Principal component analysis of CD19-CAR T cell samples. Inset shows the results of multiple regression, including the line of best fit and the coefficient of determination. (B), (C), and (D) as in (A) except for CSF1R-CAR T cell, Raji cell, and THP1 monocytes, respectively.

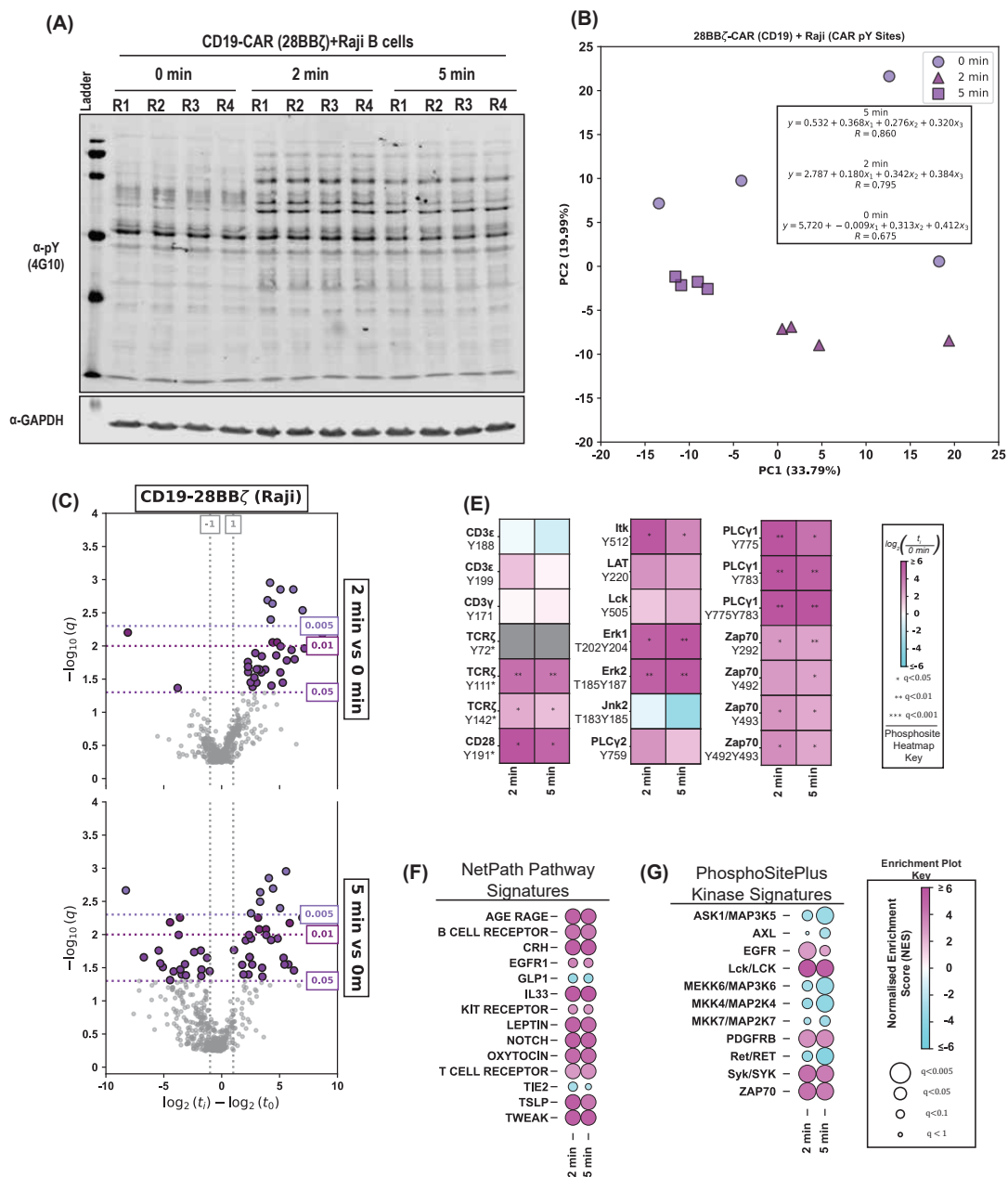


Figure 4.6: A third generation CD19-CAR with CD28 and 4-1BB costimulation shows T cell signalling engagement comparable to the second generation CD19-28 ζ -CAR. (A) Principal component analysis of CD19-28bb ζ -CAR T cell samples. Inset shows the results of multiple regression, including the line of best fit and the coefficient of determination. (B) Volcano plot analysis of pY sites observed in CD19-28bb ζ -CAR T cells during co-culture with Raji cells after 2- and 5-minutes. (C) Heatmaps showing quantification of individual phosphorylation sites observed in CD19-28bb ζ -CAR T cells during co-culture with Raji cells. (D) PTM-SEA showing enrichment of CD19-CAR T cell PTM sites in NetPath Pathways. (E) PTM-SEA showing enrichment of CD19-CAR T cell PTM sites in the PhosphoSitePlus Kinase-signatures database.

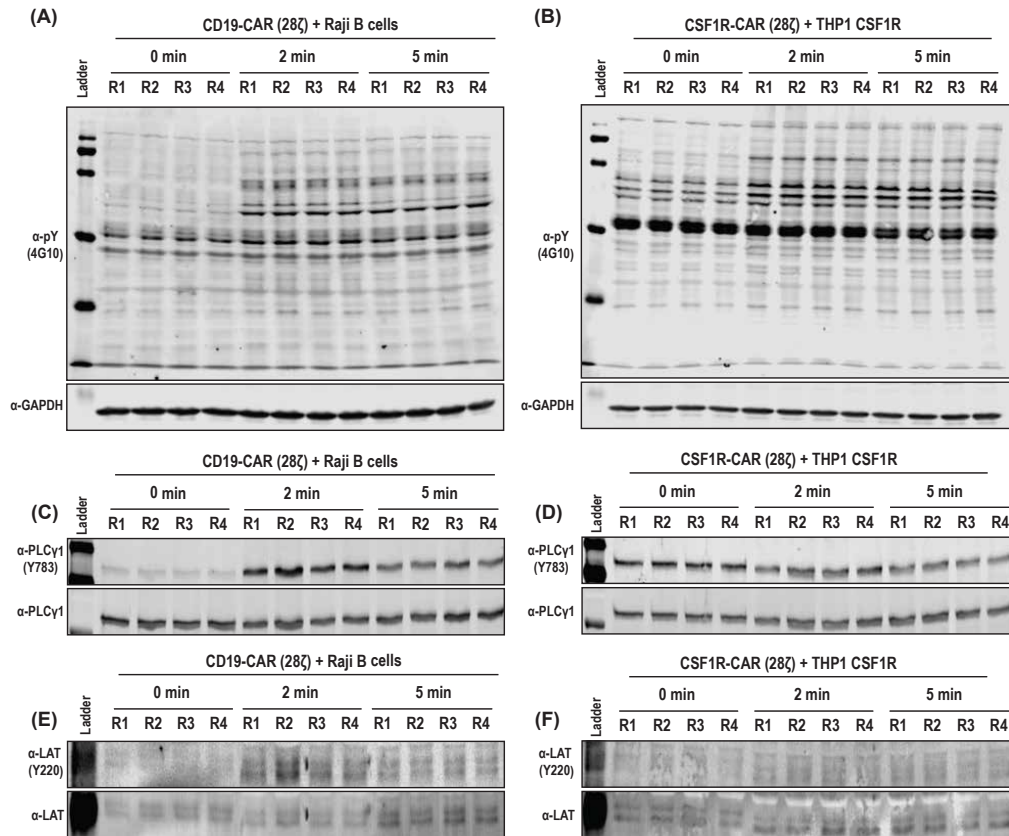


Figure 4.7: Western blot verification of T cell signalling proteins from our co-culture pY proteomics results. α -pY (clone 4G10) Western blots for (A) CD19-CAR/Raji and (B) CSF1R-CAR/THP1 co-culture proteomics samples. α -PLC γ 1^{Y783} Western blots for (C) CD19-CAR/Raji and (D) CSF1R-CAR/THP1 co-culture proteomics samples. α -LAT^{Y220} Western blots for (E) CD19-CAR/Raji and (F) CSF1R-CAR/THP1 co-culture proteomics samples. Quantification for these Western blots are presented in Figure 2.

4.2.3 The CSF1R-CAR activates CSF1R signalling in THP-1 cells

Ligand-based targeting systems benefit from native receptor-ligand interactions, which provide specific and efficacious targeting (Figure 4.1B, 4.2C)(Chinsuwan et al., 2023; Nakazawa et al., 2016; Wong et al., 2022). Using our SILAC co-culture methodology (Figure 4.1G), we gained precise information about pY signaling in the target Raji or THP-1 cells during co-cultures with CAR-expressing cells. We observed no clear trend in pY signaling in Raji cells after co-culture with second-generation CD19-CAR T cells, consistent with our results for third-generation CD19-CAR T cells (Figure 4.8A, 4.5C, 4.9)(Griffith et al., 2022). In contrast, pY sites in THP-1 cells showed patterns consistent with effective CSF1-CSF1R engagement (Figure 4.8B, 4.5D). By Western blotting analysis, we showed increased phosphorylation of Erk1^{T202Y204} and Erk2^{T185Y187} in both CD19-CAR T cell:Raji cell and CSF1R-CAR T cell:THP-1 cell co-cultures, to a much greater extent in the former, which likely originated from CD19-CAR signalling (Figures 4.4F, 4.8C, 4.107A-B). We did not observe phosphorylation of CD19^{Y531} in CD19-CAR T cell:Raji cell co-cultures; however, we observed significant phosphorylation of CSF1R^{Y723}, a phosphorylation site that promotes the binding of phosphoinositide-3-kinase (PI3K) signaling components(van der Geer and Hunter, 1993), in CSF1R-CAR T cell:THP-1 cell co-cultures (Figure 4.8D-E, 4.10C-F).

Site-specific analysis of our CD19-CAR T cell:Raji cell pY proteomics data revealed few, if any, significant changes in B cell signaling-related phosphorylation sites in Raji cells, particularly in kinases involved in forward progression of the signalling cascade (Figure 4.8F). In contrast, CSF1R-CAR T cell:THP-1 cell co-cultures induced the phosphorylation of many sites involved in CSF1R signalling, including CSF1R^{Y561}, CSF1R^{Y699}, CSF1R^{Y809}, and CSF1R^{Y873}, with a significant increase after 2 or 5 min of co-culture (Figure 4.8G). CSF1R signalling is not well characterised and is heavily cell-dependent(Han et al., 2022; McVicar and Trinchieri, 2009; Mun et al., 2020; Otero et al., 2009), however we observed tyrosine phosphorylation in various proteins thought to be involved in CSF1R signalling in macrophage lineage cells, including Pyk2^{Y573Y580}, which regulates its binding capacity(Wu et al., 2006), SHC1^{Y349}, which increases GRB2 re-

cruitment(Patrussi et al., 2005), the activating sites PLC γ 2^{Y753} and PLC γ 2^{Y759}(Özdener et al., 2002), and the activation sites Erk1^{T202Y204} and Erk2^{T185Y187}(Butch and Guan, 1996; Ettehadieh et al., 1992). There was no significant increase in the abundance of the activating pY site SFK^{Y416} (conserved sequence LIEDNEYTAR for Src family kinases)(Amata et al., 2014), but there was a significant reduction in the phosphorylation of the negative regulatory site Syk^{Y323}(Keshvara et al., 1998; Sada et al., 2000). Similarly, specific pY sites in CSF1R did not show significant changes after co-culture, including CSF1R^{Y276}, CSF1R^{Y556}, CSF1R^{Y571}, and CSF1R^{Y923} (Figure 4.8G). PTM-SEA revealed that, despite a few significant changes in pY abundance, many signalling pathway signatures, but only the kinase signatures of MEK1 and Src, were increased in Raji cells after 2 min of co-culture (Figure 4.8H-I). In THP-1 cells, pathway signatures and kinase signatures for Btk, MEK1, and Src, which are integral for the progression of proximal and distal CSF1R signalling, were increased after 2 and 5 min of co-culture (Figure 4.8J-K). Together, our data demonstrate that a ligand-targeted CSF1R-CAR activates pY signaling in CSF1R-expressing THP-1 cells.

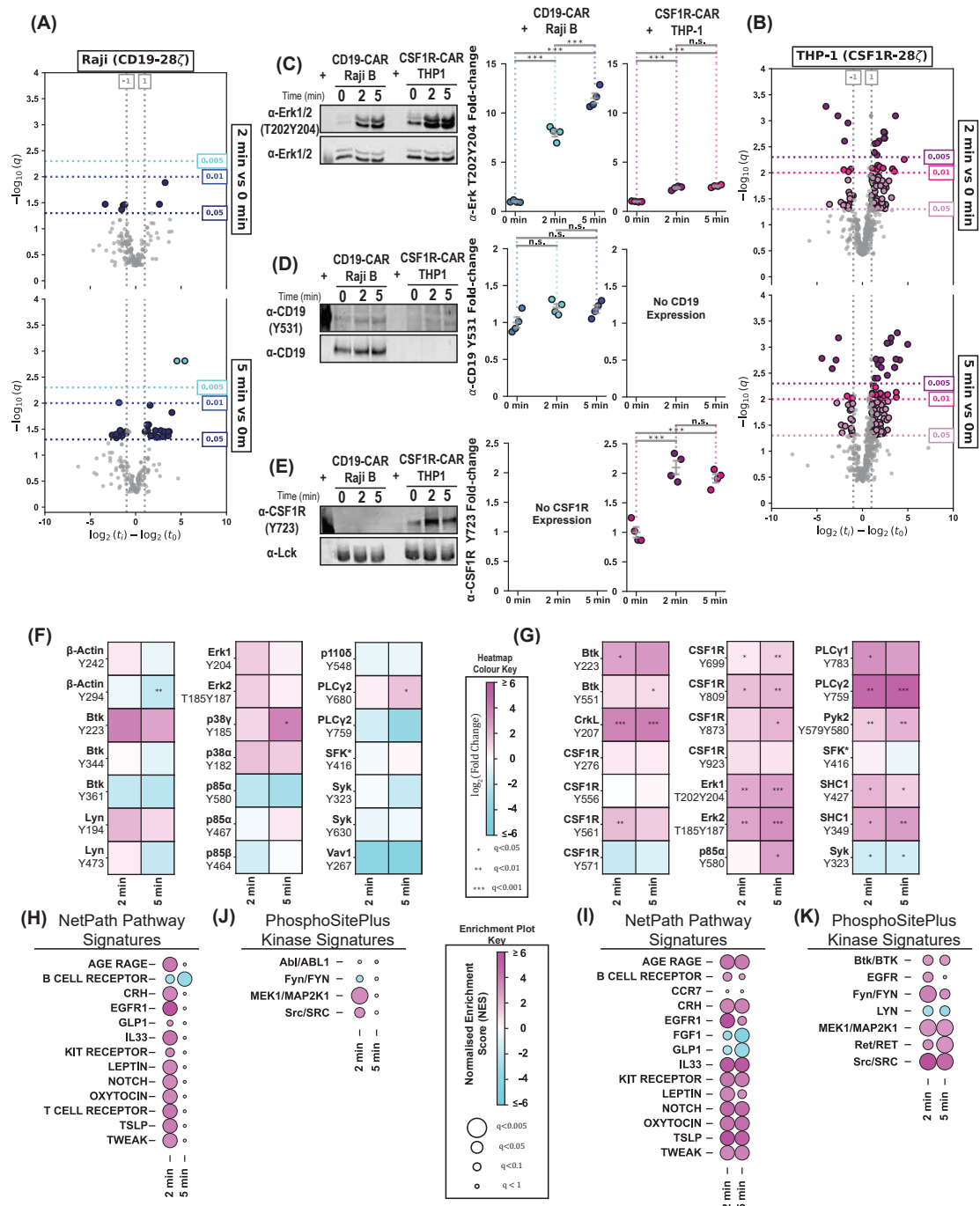


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Figure 4.8: The ligand-targeted CSF1R-CAR activates CSF1R signalling in THP-1 cells. (A) Volcano plot analysis of the phosphotyrosine (pY) sites, as identified by LC-MS/MS, in Raji cells after co-culture with CD19-CAR T cells for the indicated times (Data are from four biological replicates). (B) Volcano plot analysis of the pY sites in THP-1 cells after co-culture with CSF1R-CAR T cells for the indicated times. Data are from four biological replicates. (C) Left: Western blotting analysis of Erk1/2^{T202Y204} phosphorylation in proteomics samples from the indicated cell co-cultures. Right: Quantification of the fold-change in phosphorylated Erk1/2 in the indicated cell co-cultures. Data are from four biological replicates. (D and E) Western blotting analysis (left) and relative quantification (right) as described for (C) of CD19^{Y531} (D) and CSF1R^{Y723} (E) in the indicated cell co-cultures. CSF1R^{Y723} abundance was normalised to that of Lck. Data are from four biological replicates. Statistical significance was determined by a two-way ANOVA with Fisher's least significant difference and the Holm-Sidak correction for multiple comparisons. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$. (F and G) Heatmaps showing quantification of the indicated individual phosphorylation sites observed in Raji cells after co-culture with CD19-CAR T cells (F) and in THP-1 cells after co-culture with CSF1R-CAR T cells (G) for the indicated times. Data are from four biological replicates. (H and I) PTM-SEA showing enrichment of Raji PTM sites in NetPath Pathways (H) and in the PhosphoSitePlus Kinase Signatures database (I). Data are from four biological replicates. (J and K) PTM-SEA showing enrichment of THP-1 PTM sites in NetPath Pathways (H) and in the PhosphoSitePlus Kinase Signatures database (I). Data are from four biological replicates.

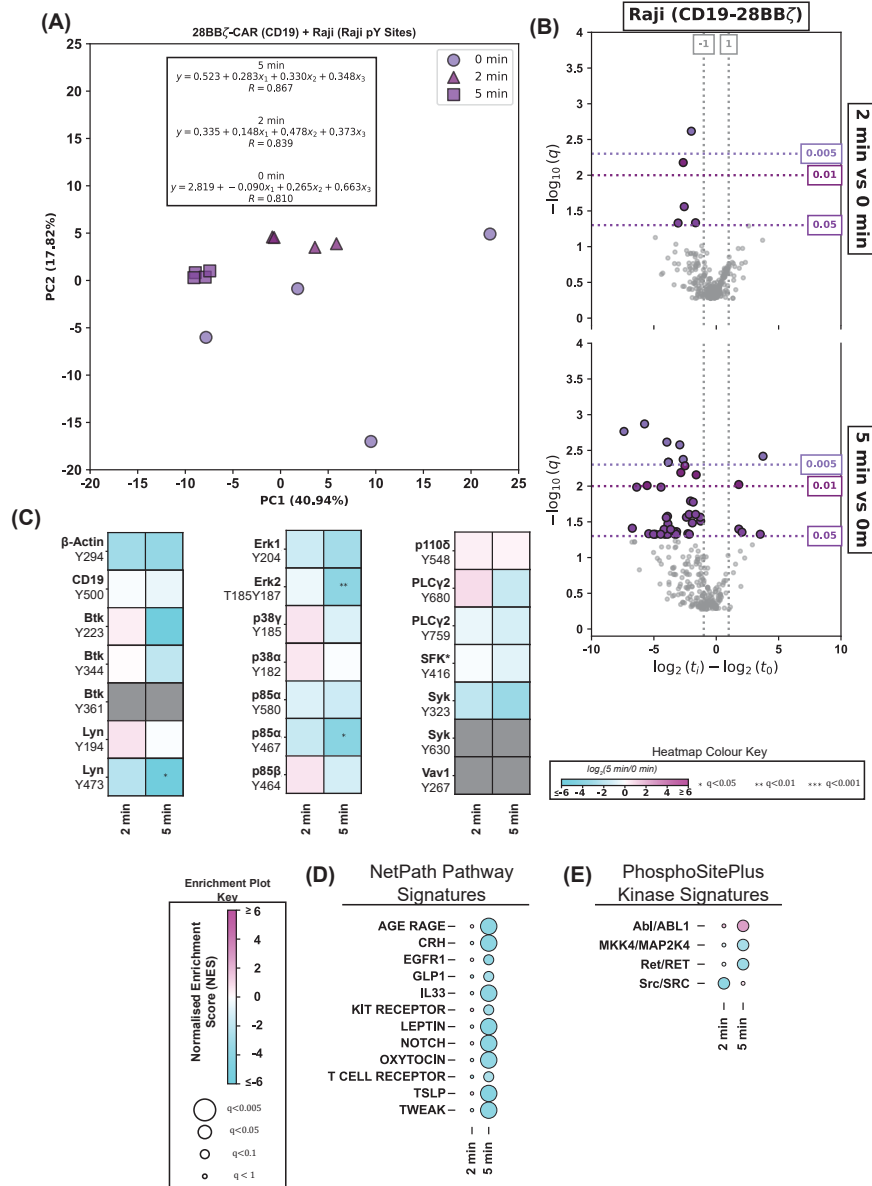


Figure 4.9: Raji cells show minimal signal induction after co-culture with CD19-28bbζ-CAR T cells (A) Principal component analysis of Raji cell (28bbζ) samples. Inset shows the results of multiple regression, including the line of best fit and the coefficient of determination. (B) Volcano plot analysis of pY sites observed in Raji cells during co-culture with CD19-28bbζ-CAR T cells after 2- and 5-minutes. (C) Heatmaps showing quantification of individual phosphorylation sites observed in Raji cells during co-culture with CD19-28bbζ-CAR T cells. (D) PTM-SEA showing enrichment of Raji cells PTM sites in NetPath Pathways. (E) PTM-SEA showing enrichment of Raji cells PTM sites in the PhosphoSitePlus Kinase-signatures database.

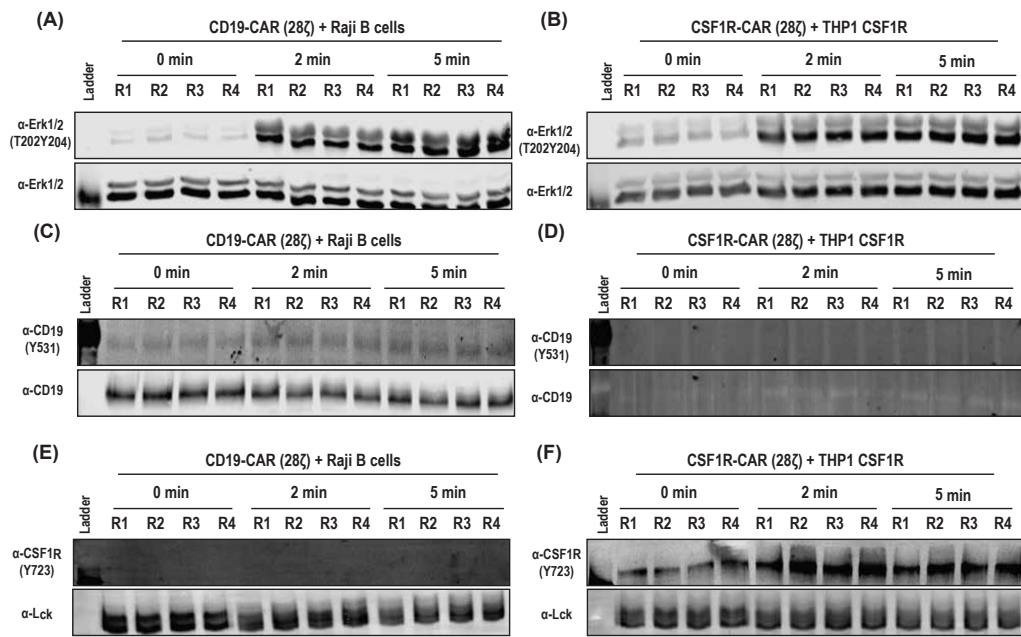


Figure 4.10: Western blot verification of target cell signalling proteins from our co-culture pY proteomics results. α -Erk^{T202Y204} Western blots for (A) CD19-CAR/Raji and (B) CSF1R-CAR/THP1 co-culture proteomics samples. α -CD19^{Y531} Western blots for (C) CD19-CAR/Raji and (D) CSF1R-CAR/THP1 co-culture proteomics samples. α -CSF1R^{Y723} Western blots for (E) CD19-CAR/Raji and (F) CSF1R-CAR/THP1 co-culture proteomics samples. Quantification of these Western blots are presented in Figure 4.8.

4.2.4 CSF1R-CAR-stimulated CSF1R signalling requires CSF1R kinase activity, not T cell activation or immune synapse formation

During TCR- and CAR-based T cell activation, actin polymerisation is induced, which pushes signalling proteins into a small space in the cell-to-cell junction, termed the immune synapse. Although CARs are less effective than TCRs in forming an immune synapse, previous studies suggest that immune synapse formation is still involved in CAR-based T cell activation (Dong et al., 2020; Xiong et al., 2024), which could promote or enhance the activation of CSF1R during CSF1R-CAR T cell:THP-1 cell co-culture. To uncouple T cell activation and CSF1R signaling in THP1 cells, we inhibited Lck, the activating kinase for T cell signalling (Denny et al., 2000; Straus and Weiss, 1992), with PP1 and inhibited actin polymerisation with Cytochalasin D in CSF1R-CAR T cells (Figure 4.11A) (Bijian et al., 2007; Celeste Morley et al., 2003; Hanke et al., 1996; Nolz et al., 2007). PP1 pretreatment of CSF1R-CAR T cells for three hours confirmed that low-micromolar concentrations of PP1 inhibited CAR-proximal T-cell signalling while leaving CSF1R autophosphorylation intact (Figure 4.11B-C, 4.12A-B). At concentrations of 0.2 and 2 μ M, which fall within the 1 to 5 μ M range that reliably abolishes IL-2/IL-2R promoter activity in Jurkat cells (Hanke et al., 1996), PP1 had no effect on the phosphorylation of CSF1R^{Y723} (Figure 4.11B-C). However, Erk1/2^{T202Y204} phosphorylation was markedly reduced by 2 μ M PP1 (Figure 4.12A-B), verifying effective Lck inhibition and thus loss of CAR signalling. Only the non-selective concentration of 20 μ M PP1 reduced the abundance of CSF1R^{Y723} to basal amounts, which is most plausibly an off-target action of PP1, because PP1 ceases to be Src-selective at low-micromolar concentrations and directly inhibits other kinases, such as Kit and Bcr-Abl (Tatton et al., 2003). Cytochalasin D (from 0.2 to 20 μ M), in contrast, left both CSF1R^{Y723} and downstream Erk phosphorylation unchanged (Figure 4.12D-E, 4.12C and D), suggesting that actin polymerisation and immune-synapse architecture were not prerequisites for CSF1R activation in THP-1 target cells. Furthermore, Cytochalasin D disrupts actin polymerisation at concentrations greater than 1 μ M with a 30-min pre-treatment without substantially altering Erk activation (Bijian et al., 2007; Celeste Morley et al., 2003; Nolz

et al., 2007). Together, these data sharpen our conclusion: CSF1R^{Y723} phosphorylation is driven mainly by the intrinsic kinase activity of the receptor and is largely independent of Lck-mediated T-cell activation and immune synapse formation.

To uncouple CSF1R-CAR-CSF1R ligation from CSF1R kinase activity, we used Vimseltinib(Caldwell et al., 2022), Pexidartinib(Lamb, 2019), and PLX5622(Liu et al., 2019), three small-molecule inhibitors of CSF1R (Figure 4.12F). At all tested concentrations of each inhibitor (0.2, 2, and 20 μ M), inhibition of CSF1R significantly reduced the phosphorylation of CSF1R^{Y723} compared with that in untreated control cells (Figure 4.11G-L). In particular, PLX5622 at all tested concentrations reduced CSF1R^{Y723} abundance below that seen under basal conditions (Figure 4.11K-L). We saw that inhibiting CSF1R kinase activity also significantly reduced the abundance of Erk1^{T202Y204}/Erk2^{T185Y187}, usually reducing induction from approximately 4- to 6-fold greater than basal without treatment to 2- to 3-fold with treatment (Figure 4.12E-J), which is consistent with our proteomics data showing a significant increase in Erk1^{T202Y204}/Erk2^{T185Y187} in THP-1 cells and less of an increase in CSF1R-CAR T cells (Figure 4.4G, 4.8C, 4.8G). Together, our results demonstrate that the kinase activity of CSF1R is the primary determinant of signalling in THP-1 cells during CSF1R-CAR T cell:THP-1 cell co-culture, with Lck activity in the CSF1R-CAR T cells playing a minimal role.

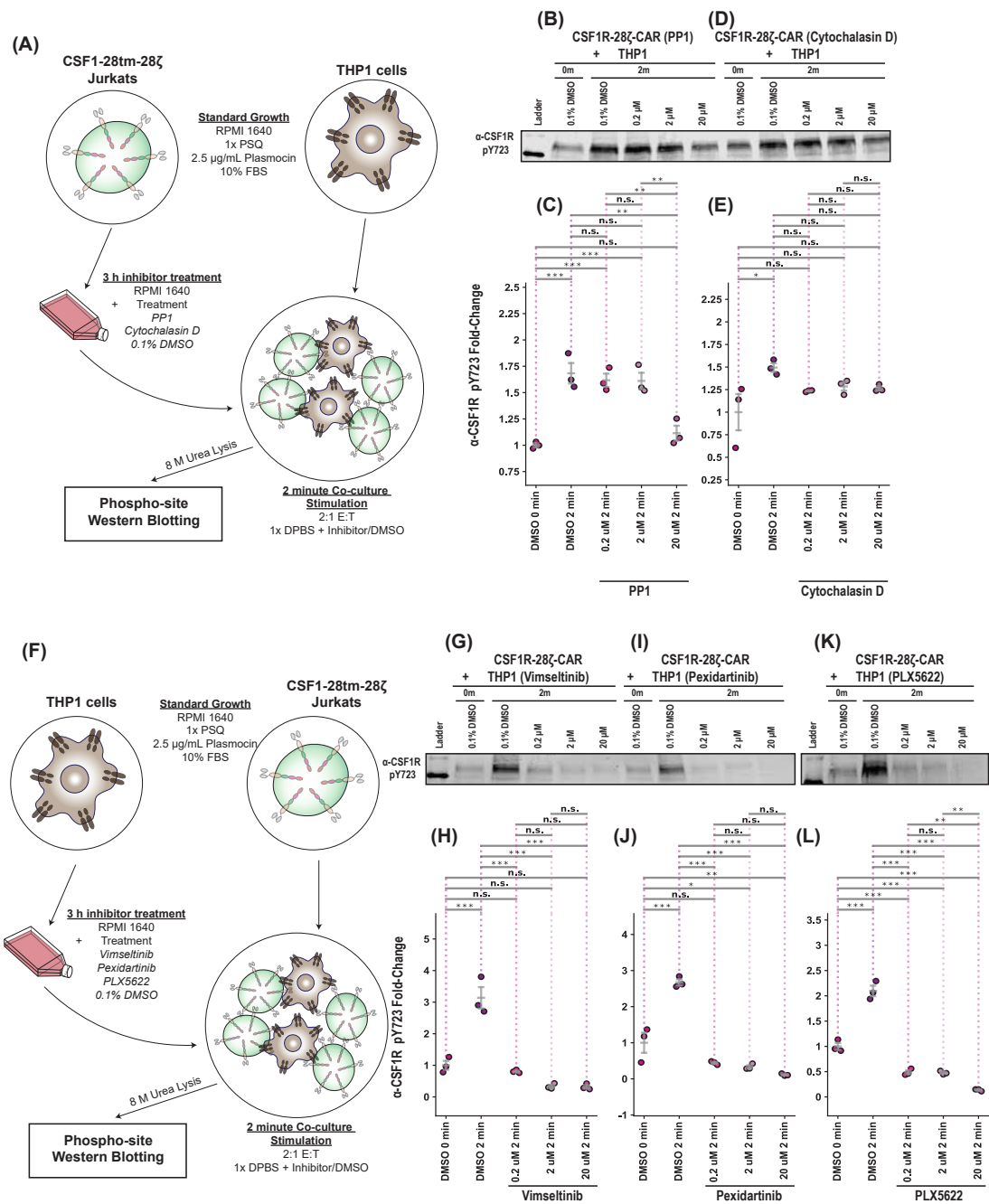


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Figure 4.11: CSF1R signalling requires CSF1R kinase activity but not T cell activation or immune synapse formation. (A) Schematic depicting the pretreatment of CSF1R-CAR T cells with PP1 (Lck inhibitor) or Cytochalasin D (actin polymerisation inhibitor) before co-culture with THP-1 cells. (B and C) Western blotting analysis (B) of CSF1R^{Y723} after pretreatment of CSF1R-CAR T cells with the indicated concentrations of PP1 and co-culture with THP-1 and (C) quantification of the fold-change in band intensity. Data are from three biological replicates. (D and E) Western blotting analysis (D) of CSF1R^{Y723} after pretreatment of CSF1R-CAR T cells with the indicated concentrations of Cytochalasin D and co-culture with THP-1 and (E) quantification of the fold-change in band intensity. Data are from three biological replicates. (F) Schematic depicting the pretreatment of THP-1 cells with Vimseltinib, Pexidartinib, and PLX5622 (CSF1R kinase inhibitors) before co-culture with CSF1R-CAR T cells. (G and H) Western blotting analysis (G) of CSF1R^{Y723} after pretreatment of THP-1 cells with the indicated concentrations of Vimseltinib and co-culture with CSF1R-CAR T cells and (H) quantification of the fold-change in band intensity. Data are from three biological replicates. (I and J) Western blotting analysis (I) of CSF1R^{Y723} after pretreatment of THP-1 cells with the indicated concentrations of Pexidartinib and co-culture with CSF1R-CAR T cells and (J) quantification of the fold-change in band intensity. Data are from three biological replicates. (K and L) Western blotting analysis (K) of CSF1R^{Y723} after pretreatment of THP-1 cells with the indicated concentrations of PLX5622 and co-culture with CSF1R-CAR T cells and (L) quantification of the fold-change in band intensity. Data are from three biological replicates. CSF1R^{Y723} band abundance was normalised to a 100 kDa off-target band due to the low CSF1R protein signal (Figure 4.12). Statistical significance was determined by two-way ANOVA with Fisher's least significant difference and the Holm-Sidak correction for multiple comparisons. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

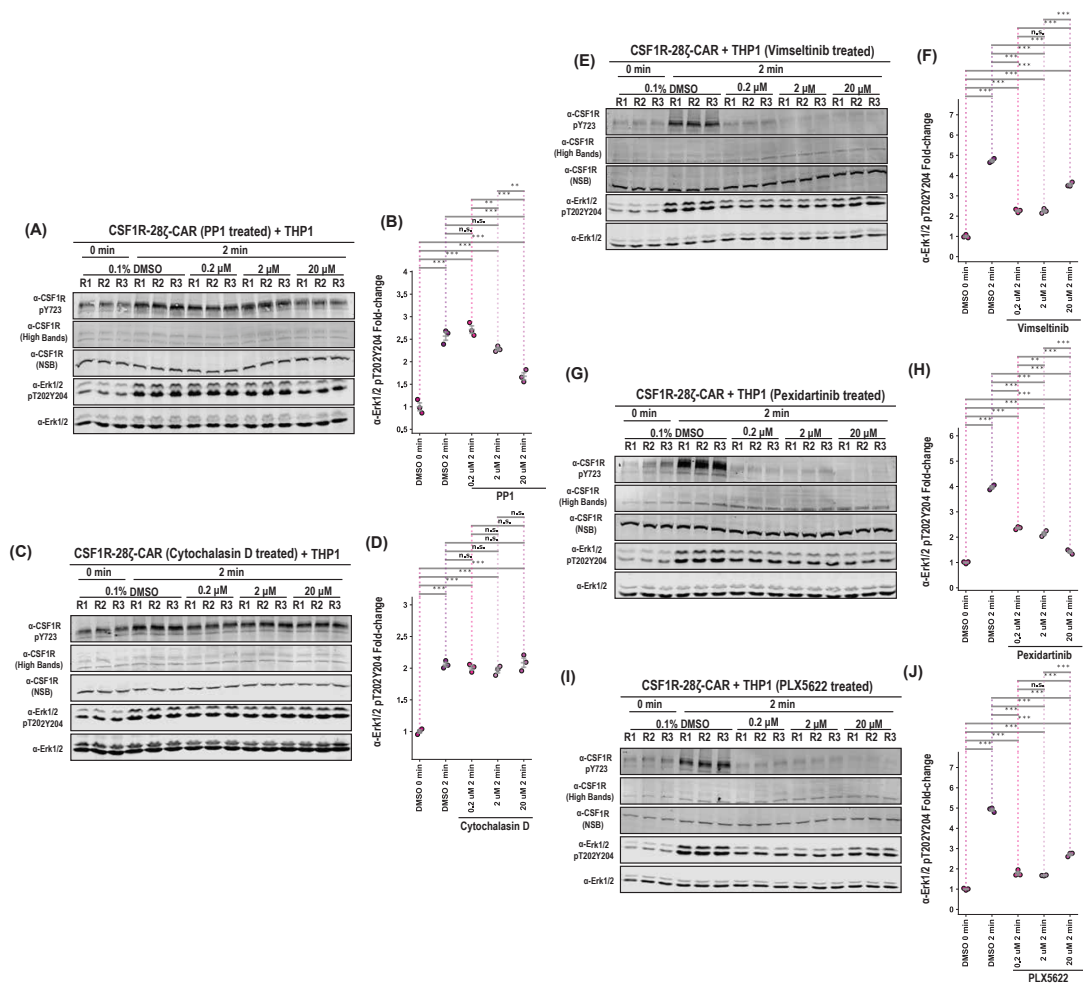


Figure 4.12: CSF1R kinase inhibitors ablate CSF1R^{Y723} induction and significantly reduce phosphorylation of Erk1^{T202Y204} and Erk2^{T185Y187}. (A) Western blot analysis of α-CSF1R^{Y723} and α-Erk^{T202Y204} in PP1-treated CSF1R-CAR T cells after co-culture with THP1 monocytes, with (B) α-Erk^{T202Y204} quantification. (C-D) As in (A-B), except for Cytochalasin D-treated CSF1R-CAR T cells. (E) Western blot analysis of α-CSF1R^{Y723} and α-Erk^{T202Y204} in Vimseltinib treated THP1 monocytes after co-culture with CSF1R-CAR T cells, with (F) α-Erk^{T202Y204} quantification. (G-H) As in (E-F), except for Pexidartinib-treated THP1 monocytes. (I-J) As in (E-F), except for PLX 5622-treated THP1 monocytes. Statistical significance is determined by a two-way ANOVA with Fisher's least significant difference and the Holm-Sidak correction for multiple comparisons. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

4.2.5 E:T-dependent biphasic effects of CSF1R-CAR T cells on the proliferation of THP-1 cells

Considering that CSF1R promotes macrophage proliferation (Keshvari et al., 2021), we sought to determine whether CSF1R-targeted CARs could induce proliferation of targeted THP-1 cells in a primary CAR T cell model. To this end, we first expressed CD19-CAR, CSF1R-CAR, and a CSF1R-CAR with a shortened CSF1 ligand (sCSF1R-CAR; Figure 4.14C) in primary T cells and characterised their effects on T cell activation. Compared to untransduced T cells (UTD), sCSF1R-CAR, CSF1R-CAR, and CD19-CAR T cells showed an increase in the percentage of CD69-positive cells in both unstimulated and co-cultured states. Note that sCSF1R-CAR T cells showed significantly reduced basal (tonic) CD69 expression compared to that of CSF1R-CAR T cells and significantly greater increases induced by co-culture (Figure 4.13A-B). These results were supported by evaluating extracellular $\text{TNF}\alpha$ abundance, a functional marker of T cell activation that is typically induced downstream of early activation events such as CD69 upregulation. Consistent with the CD69 data, $\text{TNF}\alpha$ secretion was elevated in CSF1R-CAR T cells under basal conditions and showed greater increases upon co-culture (Figure 4.13C-D). Furthermore, CD19-CAR T cells exhibited the lowest basal percentage of CD69-positive cells, maintained similar CD69 expression to that of CSF1R-CAR T cells after co-culture, and showed similar, albeit variable, basal $\text{TNF}\alpha$ secretion to that of sCSF1R-CAR T cells. However, the CD19-CAR T cells secreted significantly lower amounts of $\text{TNF}\alpha$ after co-culture (Figure 4.13A-D). Together, these results demonstrate that although CSF1R-CAR T cells exhibit enhanced tonic signaling compared to that of sCSF1R-CAR and CD19-CAR T cells, they could still be activated nonetheless.

To determine the numbers of THP-1 cells and Raji cells during co-culture with CSF1R-CAR or CD19-CAR T cells, respectively, we used THP-1 and Raji cells expressing a luciferase reporter and calculated the percentage of target cell lysis compared with a control without T cells (Figure 4.13E) (Nakajima and Ohmiya, 2010). Co-culture of Raji cells with CD19-CAR T cells showed a significant concentration-dependent increase in cell lysis ($p < 0.001$ comparing 1:3 to all other E:T ratios) compared to co-culture with UTD, and at a high E:T ratio (10:1), UTD showed a significant, albeit modest, increase

in cell lysis (Figure 4.13F). Because CSF1R signaling promotes myeloid proliferation, we hypothesized that ligand-based CSF1R engagement might produce bi-modal outcomes in THP-1 cells, supporting growth at low E:T while driving cytolysis at high E:T. Consistent with this, CSF1R-CAR T cells from healthy donors (Figure 4.149D, middle) promoted THP-1 proliferation at 1:10 ($p = 0.00687$) and 1:3 ($p = 0.00334$) but induced significant lysis at 10:1 ($p < 0.00001$) relative to UTD (Figure 4.13G). In contrast, co-culture of primary CSF1R-CAR T cells with native THP-1 cells only induced lysis at an E:T ratio of 10:1 ($p = 0.00012$) although less than that for CSF1R-overexpressing THP-1 cells ($p = 0.03193$), and similar results were found at higher E:T ratios for CSF1R-CAR-expressing Jurkat cells (Figure 4.14E). Lastly, co-culture of CSF1R-expressing THP-1 cells with primary sCSF1R-CAR T cells showed a similar proliferation phenotype as was observed with CSF1R-CAR T cell co-cultures at low E:T ratios ($p < 0.01$ at 1:30); however, sCSF1R-CAR T cells showed more lytic activity than that of CSF1R-CAR T cells at higher E:T ratios ($p = 0.000172$ at 1:3), and both types of CAR T cell showed almost 100% cell lysis at E:T ratios greater than 1:1 (Figure 4.13H). Note that analyses with CSF1R-CAR T cells from two independent donors (Figure 4.13G-H) confirmed that the proliferation-promoting effect on THP-1 cells was reproducible, even though the E:T ratio at which THP-1 cell proliferation was apparent was shifted by approximately three-fold between donors, an outcome most likely driven by donor-specific differences in CAR T cell potency, basal activation, and greater CAR expression in the new batch (Figure 4.14B, 4.14D). These data indicate an E:T-dependent, biphasic effect of CSF1R-CAR T cells on THP-1 targets: they promote proliferation at low E:T ratios but inhibit growth at high ratios.

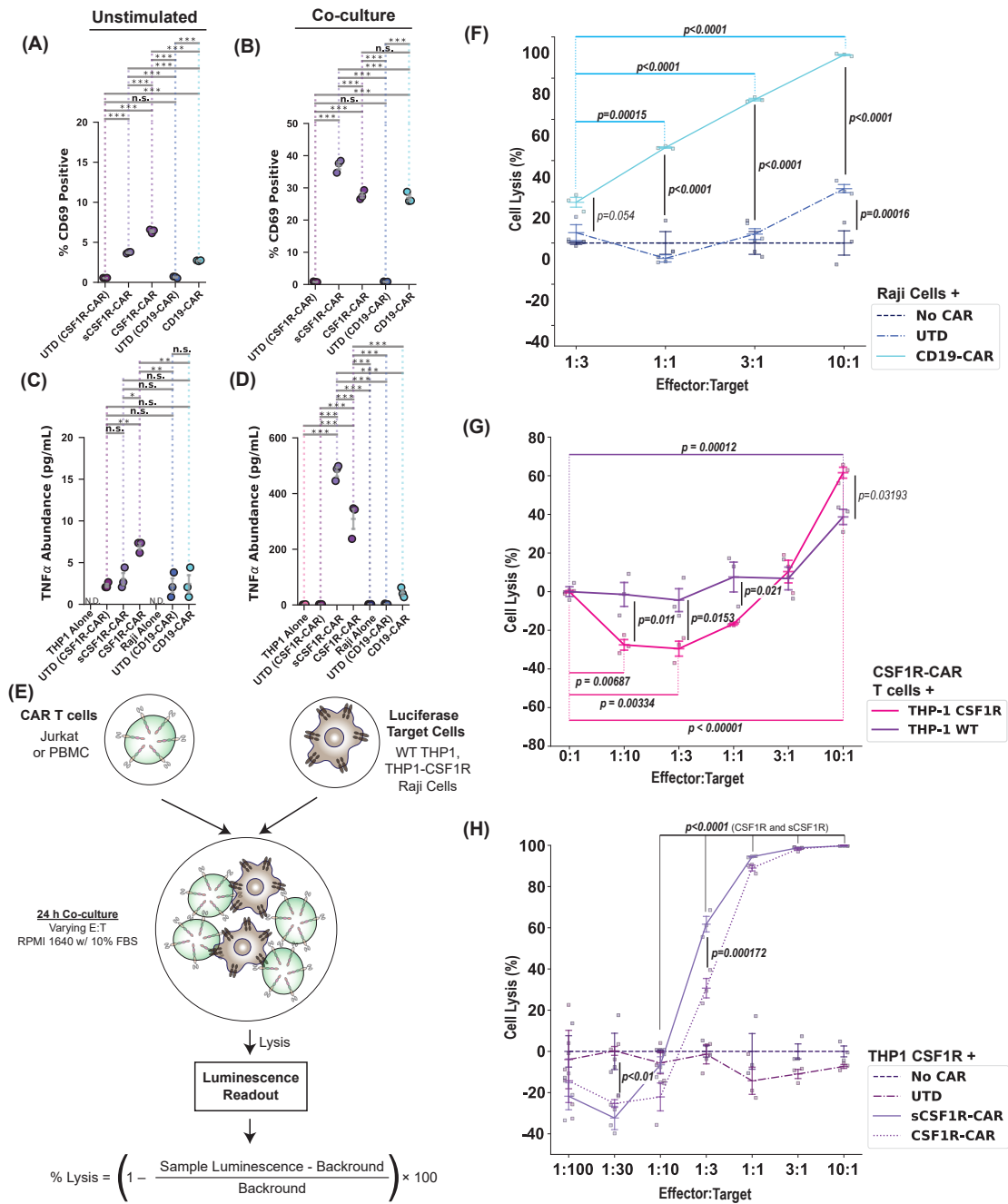


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Figure 4.13: E:T-dependent biphasic effects of primary CSF1R-CAR T cells on the proliferation of target THP-1 cells. (A and B) Flow cytometry analysis of CD69 expression in primary CAR T cells (A) before and (B) after co-culture with THP-1 target cells. Data are from three biological replicates. (C and D) ELISA analysis of extracellular TNF α (C) before and (D) after co-culture of primary CAR T cells with THP-1 target cells. Data are from three biological replicates. (E) Schematic depicting luciferase co-culture growth assays. (F) Luciferase cell lysis assay of primary CD19-CAR T cells co-cultured with Raji cells. Data are from three biological replicates. (G) Luciferase cell lysis assay of primary CSF1R-CAR T cells co-cultured with THP-1 cells with WT levels or overexpression of CSF1R. Data are from three biological replicates. (H) Luciferase cell lysis assay of THP-1-CSF1R cells co-cultured with untransduced (UTD), CSF1R-CAR with a shortened CSF1 ligand (sCSF1R-CAR) and CSF1R-CAR primary T cells. Data are from three biological replicates. Statistical significance was determined by one-way ANOVA with Fisher's LSD and the Holm-Sidak correction for mean percentage lysis comparisons. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

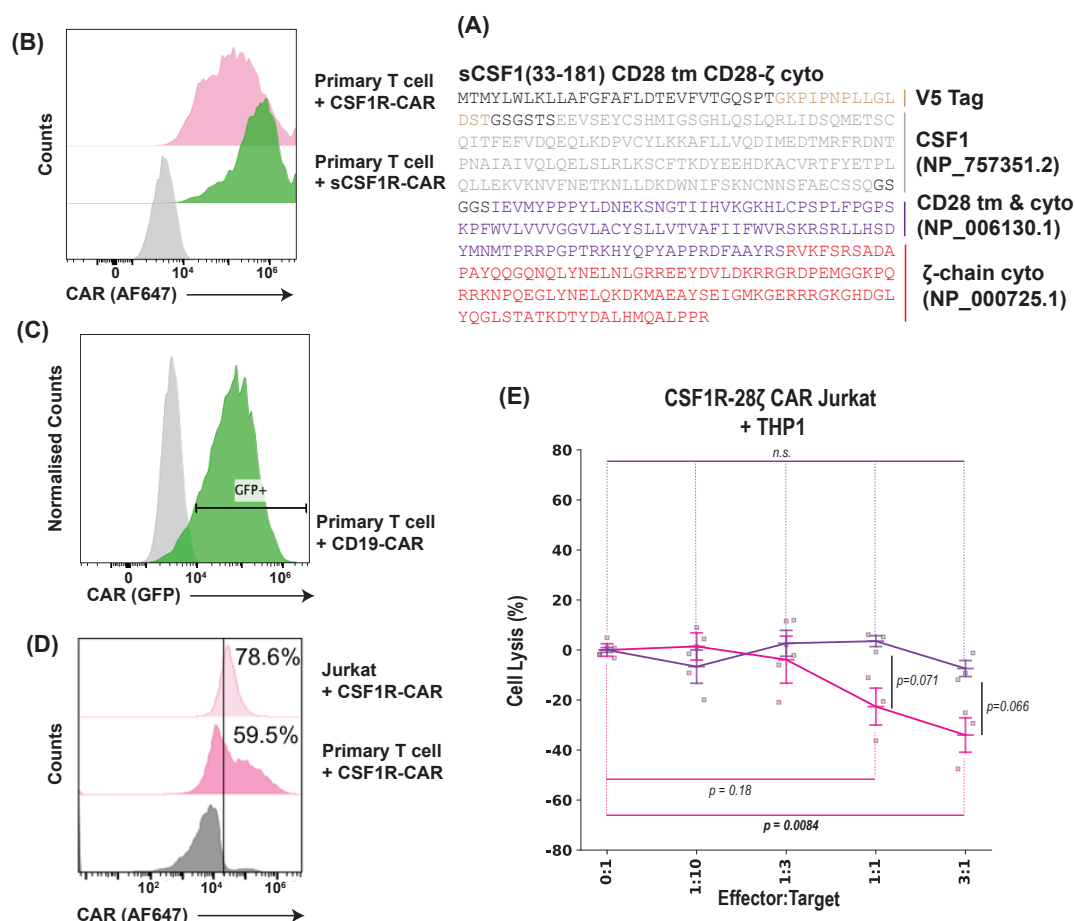


Figure 4.14: CSF1R-CAR, sCSF1R-CAR, and CD19-CAR express well in primary human T cells. (A) Sequence of sCSF1R-CAR. (B) Flow cytometry analysis of surface expression of CSF1R-CAR and sCSF1R-CAR. (C) Total expression of CD19-CAR. (D) Expression of CSF1R-CAR in Jurkat T cells and primary T cells. (E) Luciferase cell lysis assay showing cell lysis percentage as a function of E:T ratio in CSF1R-CAR Jurkat T cells. p-values determined by a one-way ANOVA with Fisher's LSD and the Holm-Sidak correction for mean percent lysis comparisons.

4.3 Discussion

The boom in CAR T cell therapy has revolutionised targeted cancer immunotherapies and is currently under investigation for use in the treatment of autoimmune disorders and fungal infections and in various non-T immune cells(da Silva, 2021; Qin et al., 2021; Schett et al., 2023; Seif et al., 2022). Despite the clinical success of second-generation CARs in treating relapsed or refractory B cell acute lymphoblastic leukaemia, B cell lymphoma, and multiple myelomas, many CAR T cells fail in clinical trials(Baguet et al., 2024; White et al., 2022), and patients suffer from a number of side effects, including off-target activation, cytokine release syndrome, and neurotoxicity, which can vary severely between patients(Namuduri and Brentjens, 2016; Schubert et al., 2021). Side effects of CAR T cell therapy can be due to CAR design, which have been ameliorated in newer generations and designs, or off-target CAR-target ligation(Hammill et al., 2020; Wei et al., 2019). The current age of CAR design focuses heavily on rational design of new CARs, leading to the generation of new targeting moieties(Nix and Wiita, 2024) and receptor designs(Giordano-Attianese et al., 2020; Labanieh and Mackall, 2023; Tousley et al., 2023), scFv screening(Duan et al., 2021; Kang et al., 2020; Ochi et al., 2021; Qiu et al., 2024; Zhang et al., 2023a), costimulatory domains, primary stimulation domains(Castellanos-Rueda et al., 2022; Goodman et al., 2022), and cytotoxicity(Jahan et al., 2023; Subham et al., 2023), which has improved the discovery of new, effective CARs. In particular, the therapeutically approved designs are well-studied, with mechanistic studies characterising CAR signalling and using these insights to improve CAR design(Boucher et al., 2021; Guedan et al., 2020; Hsieh et al., 2020; Muller et al., 2021; Philipson et al., 2020; Salter et al., 2018, 2021; Tserunyan and Finley, 2022). However, most studies focus on modulating the activation timing and strength of CARs, and little is known regarding how and if CAR engagement promotes responses in the targeted cells.

In their study, Zhong et al. (2023) evaluated the role of tumour-derived small extra-cellular vesicles (sEVs) in the resistance to CAR T cells, showing that sEVs released by tumours carry large amounts of CAR-targeted antigens and PD-L1(Zhong et al., 2023). These sEVs act as both decoys, preventing binding of the CAR T cells to the targeted

tumour, and negative regulators of CAR T cell function, because of the high abundance of the target antigen mesothelin and PD-L1(Zhong et al., 2023). Zhong et al. (2023) use an scFv-based CAR targeting mesothelin, a transmembrane protein overexpressed in many cancers that can stimulate RAS/MAPK, JAK/STAT, and AKT/mTOR signalling pathways(Faust et al., 2022). Interestingly, sEV production by tumours correlates with tumour–CAR T cell interactions, suggesting that CAR T cells can induce sEV release from the pancreatic ductal adenocarcinomas (PDAC) studied by Zhong et al. (2023). Although the authors did not provide a precise mechanism for sEV production by PDAC in response to CAR T cell treatment, they provided evidence of CAR-specific tumour responses. Whereas our CAR constructs and targets are different, our study provides a similar conclusion, that CARs can induce specific, unexpected responses from tumours. We showed that using CSF1, a native ligand of CSF1R, as the targeting moiety for second generation CAR T cells resulted in a signalling response in THP-1 cells expressing CSF1R (Figure 4.1, 4.3, 4.8, 4.10). This is in stark contrast to CD19-targeted CAR T cells, which consistently produced no response in Raji cells expressing CD19 irrespective of the CAR generation (Figure 4.1D, 4.3, 4.7 4.8)(Griffith et al., 2022). The observed signalling response depended on the kinase activity of CSF1R (Figure 4.11F-L), which is required for the many functions of CSF1R(Roussel, 1994), with minimal input from the activity of the CAR T cells (Figure 4.11A-E). Furthermore, we found that CAR T cells stimulated proliferation of THP-1 cells in vitro when the E:T stoichiometry favoured the target cell (Figure 4.13G-H). Together, our findings and those of Zhong et al. (2023) highlight a potentially important consequence of CAR T cell therapy, the activity of target cells, which should be screened during the development of new CAR-target pairs to avoid unintended outcomes.

Because of the current limited clinical usage of CAR T cells(Sterner and Sterner, 2021), expanding the specificity and retention of CAR T cells is a current priority in the field(Flugel et al., 2023). Many researchers have proposed and researched the use of ligand-based CARs, those containing a native ligand for a TAA highly expressed on the targeted tumour, with results that appear favourable overall. A study by Chinsuwan et al. (2023) showed that EGF- and TGF α -CARs targeting epidermal growth factor receptor (EGFR) can be expressed stably and can kill a number of non-small cell lung cancer

(NSCLC) cell lines in vitro, although the efficiency of cell killing in vitro is reduced in some cases when the target cells are more abundant than the effector T cells (Chinsuwan et al., 2023). However, Chinsuwan et al. (2023) also showed that EGF-CAR T cells induce clearance in lympho-depleted mouse models at clinically relevant doses, suggesting that some ligand-targeted CAR T cells can overcome the low effector to target cell ratios in vivo (Chinsuwan et al., 2023). In another study, Achkova et al. (2022) proposed two CSF1R-directed CARs using CSF1 and IL-34 as targeting moieties and showed that CSF1-targeted CARs have cytolytic activity and can clear lymphoma cell lines expressing CSF1R in vitro to a greater degree than can IL-34-targeted CARs (Achkova et al., 2022). As with many other CAR T cell studies, those of Chinsuwan et al. (2023) and Achkova et al. (2022) assumed that the target cells are static during treatment and did not investigate any possible mechanisms by which ligand-targeted CAR T cells differentially activate (or target cells participate in) phenotypes such as cytokine production.

Here, we found that CAR activation at the level of phosphotyrosine signaling varied markedly with both the target and the binding modality (Figure 4.4). Using THP-1 macrophage-lineage cells as a model, we evaluated a CSF1-ligand-based CSF1R CAR and showed efficient receptor engagement with downstream CSF1R signaling, accompanied by THP-1 proliferation in vitro that depended on both the effector-to-target (E:T) ratio and CSF1R abundance (Figure 4.8, 4.11, 4.13). As the clinical use of CAR T cells rapidly expands, including applications in B-cell-mediated autoimmunity, alternative effector lineages such as CAR-NK T cells and CAR macrophages, and fungal infections (da Silva, 2021; Schett et al., 2023; Seif et al., 2022; Labanieh and Mackall, 2023), rigorous assessment of both efficacy and off-target consequences becomes increasingly important to ensure patient safety. Together, these data argue that the prevailing focus on T-cell-intrinsic optimization should be broadened to incorporate target-cell signaling and fate, and that CAR-dependent effects on targeted cells should be evaluated at organismal and clinical scales to protect patient safety and maximize therapeutic benefit.

4.4 Methods

4.4.1 Cell culture, CAR expression, and inhibitor treatment

Jurkat cells (clone E6.1, ATCC #TIB-152), Raji cells (ATCC #CCL-86), and THP-1 cells (ATCC #TIB-202) were cultured in RPMI 1640 supplemented with 10% FBS (PeakSerum #PS-FB3), 1× penicillin-streptomycin-glutamine (PSQ; HyClone #SV30082.01), 2.5 µg/mL Plasmocin (InvivoGen #ant-mpp) at 37 C, 5% CO₂ in a humidified incubator. For co-culture mass spectrometry experiments, Jurkat cells were grown to confluence, washed twice in 1× DPBS, and then expanded in SILAC RPMI (Thermo #A33823) supplemented with 0.38 mM ¹³C₆ ¹⁵N₄ Arginine (Millipore #608033), 0.22 mM ¹³C₆ ¹⁵N₂ Lysine (Cambridge Isotopes #CNLM-291-H-1), 10% dialysed FBS, 1× PSQ, and 2.5 µg/mL Plasmocin for 7 days. All cell lines were tested for mycoplasma before each experiment (ABM #G238). Primary human T cells were isolated from the peripheral blood mononuclear cells (PBMCs) of healthy donors (Zenbio #SER-PBMC-200) using the EasySep Human T cell Isolation Kit (StemCell #17951), cultured in RPMI 1640 supplemented with 10% FBS, 50 nM β-mercaptoethanol, 300 U/mL IL-2 (PEPROTECH #200-02), and treated with α-CD3/α-CD28 Dynabeads (Thermo #11161D) for expansion.

For CAR T cell production, HEK293T cells were cultured in DMEM supplemented with 10% FBS and 1× PSQ. One million HEK 293T cells were plated in one well of a six-well plate before transfection with pMD2.G, poPAX, and CAR plasmids (0.5 µg each) with PEI MAX. After 10 hours, the culture medium was replaced with primary T cell or Jurkat cell culture medium, and allowed to incubate for 24 hours, twice, to collect viral particles. Jurkat cells were cultured with 1.5 mL of viral medium per 0.5 million Jurkat cells, with half of the medium replaced with fresh cell culture medium after 24 hours. Jurkat cells were allowed to expand before use. Primary human T cells were infected with 1.5 mL of fresh lentivirus per 0.5 million cells by spinoculation at 800 ×g for 90 min at 32 C, and half of the culture medium was replaced with fresh culture medium after 24 hours. Five days after infection, Dynabeads were removed, and the

CAR T cells were grown in fresh medium for two more days before use.

For T cell inhibitor treatment experiments, Jurkat CSF1R-CAR T cells were collected by centrifugation, washed once with unsupplemented RPMI 1640, and then suspended in RPMI 1640 supplemented with PP1 (Cayman Chemical Company #14244), Cytochalasin D (Cayman Chemical Company #11330), or DMSO (Fisher Scientific #BP231-100) at a concentration of 1×10^7 cells/ml for 3 hours at 37 C, 5% CO₂ in a humidified incubator. For THP-1 cell inhibitor experiments, THP-1 cells were processed similarly to Jurkat CSF1R-CAR T cells and resuspended in RPMI 1640 supplemented with Pexidartinib (Cayman Chemical Company #18271), Vimseltinib (Cayman Chemical Company #38783), PLX 5622 (Cayman Chemical Company #28927), or DMSO. After treatment, the cells were washed once in warm DPBS before undergoing co-culture stimulation.

4.4.2 Co-culture stimulation and mass spectrometry sample preparation

Co-culture stimulations were performed as previously described (Griffith et al., 2022). Briefly, CAR T cells and target cells (Raji cells or THP-1 cells) were grown to confluence and collected by centrifugation. For Western blotting analysis, 1×10^7 CAR T cells (at 2×10^8 cells/mL) and 5×10^6 target cells (at 1×10^8 cells/mL) per replicate were used, whereas for MS experiments, 1×10^8 T cells (at 2×10^8 cells/mL) and 5×10^7 target cells (at 1×10^8 cells/mL) were used. To promote cell-to-cell contact, the mixed cells were immediately centrifuged at $600 \times g$ for 30 s. Stimulation was stopped by the addition of 9 M Urea Lysis Buffer (9 M urea, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM β -glycerophosphate in 20 mM HEPES) to a final concentration of 4 M urea at the times indicated in the figure legends. Cell lysates were chilled on ice for about 15 min, sonicated at 70% amplitude for 30 s, and centrifuged at $1800 \times g$ for 15 min to collect the insoluble fraction. Samples for Western blotting analysis were diluted 1:1 in 2X Lamelli sample buffer [4% SDS, 125 mM TRIS-HCl (pH 6.8), 20% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue), boiled at 95 C for 5 min, and then frozen until used. For MS analysis, protein concentrations were evaluated by BCA (Thermo

#23225) and each sample was normalised to 9 mg of total protein. Samples were then treated with DTT at a final concentration of 10 mM for 30 min at 30 C and then with iodoacetamide at a final concentration of 10 mM for 15 min at room temperature in the dark before undergoing overnight trypsin digestion (1:100 trypsin:protein, Promega #V5113). The following day, samples were acidified to 1% trifluoroacetic acid (TFA, v/v) and centrifuged at 1800 $\times$ g for 15 min to remove the insoluble fraction. Peptides were desalted with Sep-Pak C18 plus cartridges (Waters #WAT020515) and lyophilised as previously described(Griffith et al., 2022; Nguyen et al., 2009).

4.4.3 Phosphotyrosine enrichment with the Src SH2 superbinder

Enrichment of phosphotyrosine-containing peptides was performed with the Src SH2 superbinder (sSH2) domain as previously described(Griffith et al., 2022; Callahan et al., 2024; Chua et al., 2020; Chua and Salomon, 2021) . Briefly, in-house purified recombinant sSH2 protein was conjugated to CNBr-activated sepharose beads at a concentration of 3.2 mg sSH2/mL. Nine milligrams of total desalted peptide was resuspended in 1.4 mL of IAP buffer (10 mM sodium phosphate monobasic monohydrate, 50 mM sodium chloride, 500 mM MOPS, pH 7.2) and incubated in 100 μ g of sSH2 beads for 2 hours at 4 C on a 4-rpm end-over-end rotator. The pY peptide-bound beads were washed three times in ice-cold HPLC water and twice in ice- cold IAP buffer before being eluted from the beads twice with 0.15% TFA on a 1150-rpm mixer at room temperature. The eluted pY peptides were desalted with Pierce C18 tips (Thermo #87784) according to the manufacturer’s guidelines and dried by speed-vac before undergoing LC-MS/MS analysis.

4.4.4 LC-MS/MS, database searching, and analysis

After enrichment, pY peptides were separated on a lab-packed C18 column (250 mm $\times$ 75 μ m) with XSelect CSH C18 2- μ m resin with an Agilent 1200 HPLC. Peptides were eluted using a 120-min method starting with a ratio of Buffer A (0.1 M acetic acid in HPLC water) to Buffer B (0.1 M acetic acid in acetonitrile) of 95:5 and progressing to

a final ratio of 70:30 in 95 min, with a 100% buffer B spike at the end of the gradient. Peptides were analysed on a QExactive mass spectrometer in positive ion mode with a spray voltage of 2.0kV, funnel RF level of 40, and heated capillary temperature of 250 C. Precursor mass range was set at 400 to 1800 m/z with a resolution of 70,000, AGC target of 3×10^6 and an IT of 200 ms in centroid mode. Fragmentation spectra were collected with a Top9 DDA method with a dynamic exclusion time of 30 s and an intensity threshold of 1000. Monoisotopic precursor ions with charge states between +2 and +5 were fragmented by higher-energy collision-induced dissociation (HCD) using a normalised collision energy of 28%. Fragmentation spectra were acquired with a 2.5 m/z isolation window at a resolution of 17,500 and an AGC target of 2×10^4 in centroid mode with a maximum injection time of 200 ms.

Thermo .RAW files were analysed with the high-throughput autonomous proteomics pipeline (HTAPP) and PeptideDepot, custom in-house software for the automation of PTM proteomics analysis, as previously described(Griffith et al., 2022; Yu and Salomon, 2009, 2010). Briefly, MS2 spectra were searched with Mascot against the UniProt complete proteome dataset (98,300 non-redundant forward sequences, downloaded in August 2019) to achieve a 0.1% false discovery rate (FDR) using a reverse decoy database. The following parameters were used for the search: trypsin enzyme cleavage (up to two missed cleavages), 7 ppm precursor mass tolerance, 20 mmu fragment ion mass tolerance, variable modifications for phosphorylation (S/T/Y; +79.9963 Da), oxidation (M; +15.9949 Da), and static modification of either carbamamidomethylation (C; +57.0215 Da) for the analysis of THP-1 cells or carbamamidomethylation (C; +57.0215 Da) and SILAC labelling (K: +8.0142 Da, R: +10.0083 Da) for the analysis of CAR -T cells. Retention time alignment and integration of selected ion chromatograms for each peptide spectrum match were used to determine relative peptide abundance as previously described(Helou et al., 2015). Initial mass spectrometry data analysis was performed with R in PeptideDepot, with follow-up analysis using Python 3.12.5. Briefly, comparisons were built using PeptideDepot in which all peptides were filtered to include only unique pY peptides, student's T tests were performed comparing the mean abundance of log₂- transformed 0-, 2-, and 5-min stimulation conditions, and the global rate of false discovery was adjusted using the method of Storey(Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003;

Welch, 1947). All processed quantitative proteomic data can be found in Data file S1. Replicate reproducibility was evaluated using principal component analysis (PCA) and multiple regression. PTM-SEA was performed as previously described (Krug et al., 2019) using the R-script ssGSEA2.0.R and the PTM-signature database (version 2.0.0).

4.4.5 Western blotting analysis

Western blotting samples were resolved on 8 or 10% polyacrylamide gels poured in house at 100 V for 130 min before being transferred to a PVDF membrane (EMD Millipore #IPFL00010) at 100 V for 100 min. Membranes were blocked with Intercept TBS blocking buffer (LI-COR #927-60001) diluted 1:1 in 1× TBS (137 mM NaCl, 20 mM TRIS) for 1 hour at room temperature, before overnight incubation with primary antibodies in 1× TBS with Tween 20 (TBST), 5% BSA. After primary antibody incubation, membranes were washed three times with 1× TBST for 5 min, incubated in secondary antibodies in 1× TBST, 5% BSA, washed three times in 1× TBST for 5 min, and twice in 1× TBS. Membranes were imaged on LI-COR Odyssey CLx or LI-COR Odyssey M imaging systems. Quantification of Western blots was performed as previously described (Griffith et al., 2022; Pillai-Kastoori et al., 2020). Statistical significance between loading control-corrected, zero minute-normalised band intensities was determined using FWER-corrected p-values generated with Fisher’s least significant difference test (Fisher, 1935; Hayter, 1986), followed by the Holm-Sidak FWER step-up correction (Holm, 1979; Šidák, 1967).

The following primary antibodies were used: pY clone 4G10 (1:2000, Cell Signalling Technologies #96215), GAPDH (1:10000, Millipore Sigma #G9545), PLC γ 1 (1:10000, Millipore Sigma #05-163), PLC γ 1 Y783 (1:2000, Cell Signalling Technologies #2821), LAT ([only used for identifying LAT bands] 1:2000, Thermo #MA1-19307; PLC γ 1 protein was used as the loading control), LAT Y220 (1:2000, Cell Signalling Technologies #3584), Erk1/2 (Cell Signalling Technologies #9107), Erk T202Y204 (1:2000, Cell Signalling Technologies #9101), CD19 (1:2000, Thermo #14-0190-82), CD19 Y531 (1:2000, Cell Signalling Technologies #3571), CSF1R (1:2000, Thermo #MA5-37676; an off-target band or Lck protein was used for normalisation in quantification), and CSF1R Y723 (1:2000, Cell Signalling Technologies #3155).

The following secondary antibodies were used: IRDye 680RD goat anti-mouse IgG (LI-COR #926-68070), IRDye 800CW goat anti-mouse IgG (LI-COR #926-33210), IRDye 680RD donkey anti-Rabbit IgG (LI-COR #926-68073), and IRDye 800CW donkey anti-rabbit IgG (LI-COR #926-32213).

4.4.6 Flow cytometry

For flow cytometry analysis, 1×10^6 cells were collected by centrifugation at $400 \times g$ for 5 min and resuspended in FACS buffer ($1 \times$ DPBS, 2% FBS). Cells were incubated with the appropriate fluorescent antibodies for 30 min at 4 C in the dark, then washed three times in 1 mL of FACS buffer before being fixed with paraformaldehyde (2% final concentration) for 15 min at room temperature in the dark. After fixing, the cells were washed three times in 1 mL of FACS buffer before analysis on a Cytex Aurora flow cytometer. Flow cytometry files were analysed with Floreada, an online flow cytometry analysis tool.

The following antibodies were used: FMC63 scFv APC (AcroBiosystems #FM3AY54P1), V5-Tag Alexa Fluor 647 (Santa Cruz Biotechnology #sc-271944), and CD69 Brilliant Violet 421 (BioLegend #310929).

4.4.7 TNF α ELISA

Assessment of extracellular TNF α abundance was performed with a TNF α ELISA kit (BioLegend #430201) according to the manufacturer's instructions. Briefly, the plate was washed four times per well with 300 μ L of Wash Buffer ($1 \times$ PBS, 0.05% Tween 20) by pipetting and blotting residual buffer on absorbent paper. The plate was then incubated with 200 μ L of Assay Diluent ($1 \times$ PBS, 10% FBS) for 1 hour on a shaker while sealed, and then washed four times with Wash Buffer before the addition of 100 μ L of sample, being sealed and then incubated on a shaker for 2 hours. The plate was then washed four times with Wash Buffer before the addition of 100 μ L of Detection Antibody and incubated for 1 hour with shaking, washed four times, and then incubated with 100 μ L of HRP-Avidin solution for 30 min with shaking. The plate was then washed five times with Wash Buffer, and 100 μ L of TMB Substrate Solution was added and

incubated for 30 min in the dark, before the addition of 100 μL of Stop Solution. The plate was then read on a spectrophotometer at 450 nm. Absolute quantification was determined using a standard curve of known $\text{TNF}\alpha$ solutions.

4.4.8 Luciferase co-culture lysis assay

To quantify live THP-1 cells during CAR T cell co-cultures, THP-1 cells expressing a luciferase reporter were washed and resuspended in RPMI 1640 supplemented with 10% FBS and then mixed with CAR T cells (Jurkat or primary T cells) at E:T ratios ranging from 1:10 to 10:1, with a THP-1 only control (0:1). Cells were co-cultured for 24 hours at 37 C, 5% CO_2 in a humidified incubator, and then were collected, washed in $1\times$ PBS, lysed, and measured for luminescence with a Luciferase Assay Kit (Promega #E1500) on a spectrophotometer. Cell lysis (%) was defined as:

$$\text{Cell Lysis (\%)} = 1 - \left(\frac{\text{Sample Luminescence} - \text{Background}}{\text{Background}} \right) \times 100$$

Comparisons between mean percentage lysis values were determined with FWER-corrected p-values generated using Fisher’s least significant difference test followed by the Holm-Sidak FWER step-up correction.

4.4.9 Code and data analysis

All plotting was performed with Python 3.12.5 with dependencies matplotlib (version 3.9.1), scipy (version 1.14.0), numpy (version 2.0.1), scikit-learn (version 1.5.1), pandas (version 2.2.2), and a custom “helpers” module used for beautifying matplotlib graphs. Coding and plotting were performed in Jupyter interactive python notebooks. Statistical analysis and plotting of Western blotting data were performed in Python with a self-coded version of the Holm-Sidak FWER correction algorithm.

RAW files of the MS data are available from the ProteomeXchange Consortium via the PRIDE partner repository (Dataset ID: PXD058576). All code and original plotting outputs are available in the CAR-Target-Signalling-code-master.zip and on GitHub (<https://github.com/Aurdeegz/CAR-Target-Signalling-code>) and at Zenodo (10.5281/zenodo.15546537). All other data needed to evaluate the conclusions in the

paper are present in the paper or the Supplementary Materials.

4.5 Acknowledgements

We thank the Brown University Proteomics Core Facility for managing and maintaining the mass spectrometer used for this work and for allowing the Salomon laboratory to operate the instrument. We thank the Brown University Genomics Facility for the use of the LI-COR Odyssey CLx and Odyssey M scanners, as well as the Flow Cytometry and Cell Sorter Facility for acquiring our flow cytometry samples.

Funding

This work was supported by National Institutes of Health grant P01AI091580 (to A.R.S.), American Cancer Society Research Scholar Grant 135926 (to X.S.), NIGMS MIRA program R35 GM138299 (to X.S.), Gabrielle’s Angel Foundation Medical Research Award (to X.S.), NCI Exploratory/Developmental Research Grant R21 CA286364 (to X.S.), and the NIH Director’s Transformative Research Award EB037112 (to X.S.).

Competing Interests

The authors declare that they have no competing interests.

Data and materials availability

RAW files of the MS data are available from the ProteomeXchange Consortium through the PRIDE partner repository (dataset ID: PXD058576). All code and original plotting outputs are provided in data file S2, on GitHub (<https://github.com/Aurdeegz/CAR-Target-Signalling-code>), and at Zenodo (10.5281/zenodo.15546537). All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

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CHAPTER 5

Phosphotyrosine proteomics reveals novel Zap70 and Itk pathway targets downstream of TCR and CAR in Jurkat T cells

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Abstract ζ -associated protein of 70 kDa (Zap70) and interleukin-2-inducible T cell kinase (Itk) propagate the primary and CD28-integrated phosphotyrosine (pY) signalling, respectively, to achieve full T cell activation. Despite their canonical roles in T cell activation, our understanding of how each kinase controls canonical and noncanonical pY signalling is incomplete. Here, using three T cell activation methods (soluble antibodies, APC-pMHC/TCR, and CD19-CAR/Raji), we evaluated the effects of two novel inhibitors, RDN2150 (RDN, Zap70) and Soquelitinib (Soq, Itk), on T cell activation. We validated

the published working concentrations of RDN and Soq on phosphorylation of key T cell signalling proteins and on T cell activation markers, finding that RDN provides more complete inhibition of T cell signalling and activation. We used LC-MS/MS to evaluate how RDN and Soq treatment affected the phosphotyrosine (pY) signalling and proteome of T cells, finding that RDN, as opposed to Soq, completely downregulated the TCR signalling pathway. Finally, we identified new, noncanonical pY sites responsive to RDN and Soq, providing new insights into the pathways regulated by Zap70 and Itk. Together, our work provides a basis for further study on RDN and Soq, as well as a molecular roadmap for the effects of these inhibitors.

5.1 Introduction

T cell activation through the T cell receptor (TCR) is initiated by binding of a peptide-presenting major histocompatibility complex (pMHC) on an antigen-presenting cell. It requires the coordinated activity of tyrosine kinases, including the Src family kinase Leukocyte C-terminal Src kinase (Lck), the Syk family kinase ζ -associated protein of 70 kDa (Zap70), and the Tec family kinase interleukin 2 inducible T cell kinase (Itk)(Gaud et al., 2018). TCR-pMHC ligation promotes Lck^{Y505} dephosphorylation by CD45 and subsequent Lck^{Y394} autophosphorylation and phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on TCR subunits CD3 ϵ , CD3 γ , CD3 δ , and TCR ζ , which Zap70 is then able to bind through its dual SH2 domains. Lck activates Zap70 and Itk through phosphorylation at Zap70^{Y493} and Itk^{Y512}, respectively, and linker for activation of T cells (LAT), a scaffolding protein necessary for TCR signalling, is activated by Lck-mediated Zap70 phosphorylation at multiple tyrosine residues. Multiple scaffolding proteins, including SLP76-bound GADS and phospholipase C γ 1 (PLC γ 1), dock on LAT, allowing for the phase separation of the TCR signalosome and bringing substrates into proximity with Lck, Zap70, and Itk for phosphorylation and activation. Components of the TCR signalosome then propagate signal to reorganise the actin cytoskeleton and activate transcription factors, promoting T cell activation and feedback regulation of the signalling input (reviewed extensively in Gaud et al. 2018). Notably, mutations to Zap70 are associated with autoimmune disorders and loss of Zap70 ablates

T cell activation, while inhibition of Itk alters the fate of T cells and is associated with better prognosis in immunotherapeutic intervention in various diseases(Weeks et al., 2021; Au-Yeung et al., 2018).

Chimeric antigen receptors (CARs), a novel immunotherapy for the treatment of autoimmune disorders and cancers, are composed of domains from T cell signalling proteins, with the goal of engaging a disease-associated antigen and initiating an immune response(Lindner et al., 2020). Clinically available CAR designs include the intracellular domain of TCR ζ and costimulation via the intracellular domain of either CD28 or 4-1BB, two T cell costimulatory receptors that enhance T cell activation and memory T cell formation, respectively(Sengsayadeth et al., 2022; Srivastava and Riddell, 2015). Despite hundreds of clinical trials evaluating the safety and efficacy of CARs in humans, only six are currently approved for use as a last resort for relapsed or refractory cancers of blood cell lineage(Sengsayadeth et al., 2022). CAR T cell therapy is often plagued by non-specific T cell activation, low antigen sensitivity, and T cell exhaustion, leading to variable patient responses, at worst resulting in no tumour clearance, cytokine release syndrome, and neurotoxicity resulting from enhanced immune activity in the brain(Zhang et al., 2023b). Recent literature suggests that altered T cell activation is the result of aberrant engagement of the TCR signalling pathway, including constitutive docking of active Lck on CD28 costimulated CARs, a lack of recruitment of key TCR signalling proteins after antigen ligation on 4-1BB costimulated CARs, and differential engagement with the TCR signalosome(Salter et al., 2018, 2021; Philipson et al., 2020). Researchers have demonstrated that disrupting the Itk binding site in the CD28 costimulatory domain (PRRP to ARRA) or the GADS binding site (YMNM to FMNM) can significantly enhance the function of second-generation CARs with a CD28 domain, thereby reducing constitutive, continuous CAR activation(Boucher et al., 2021; Guedan et al., 2020). Currently, efforts to restrain CAR-induced toxicities have come from high-throughput Jurkat screens evaluating combinatorial CAR libraries for sustained T cell activation with low exhaustion markers, and from the rational design of new CARs, including dual-antigen split CARs and “fourth-generation CARs,” known as T cells redirected for universal cytokine-mediated killing (TRUCKs)(Chmielewski and Abken, 2020; Tousley et al., 2023; He et al., 2020; Goodman et al., 2022; Gordon et al., 2025, 2022; Kang et al.,

2020; Rydzek et al., 2019). Even with advances in the functional understanding of CAR T cells, basic research evaluating how CARs influence T cell biology, particularly T cell activation, is lagging.

Two small-molecule inhibitors with distinct effects on T-cell signalling have recently been described: RDN2150 (RDN) targeting Zap70 and Soquelitinib (Soq) targeting Itk(Rao et al., 2023; Hsu et al., 2024). RDN covalently inhibits Zap70 and prevents phosphorylation of LAT^{Y220}, thereby destabilising the LAT/SLP76 complex. At concentrations less than 400 nM, RDN reduces interferon- γ and interleukin-17A, and is effective in treating psoriasis in murine models by topical application(Rao et al., 2023). Soq is a highly selective covalent inhibitor of Itk, thought to reduce phosphorylation of PLC γ 1^{Y783} and thus reduce T cell activation strength(Hsu et al., 2024). In this paper, we provide a thorough evaluation of RDN and Soq in the context of Jurkat signalling and activation. By performing inhibitor treatments across three distinct models of T cell activation (soluble antibody crosslinking, APC-pMHC/TCR co-culture, and CD19-CAR/Raji co-culture), we find that RDN’s efficacy in abating T or CAR T cell signalling is higher than its efficacy in disrupting T cell activation markers. Further, Soq appears to thoroughly disrupt PLC γ 1^{Y783} phosphorylation and IL-2 production even at low concentrations, while leaving CD69 expression intact. Finally, we find that while soluble antibody stimulation induces the most pY induction of any stimulation method, it is unable to activate T cells to the same degree as APC-pMHC/TCR or CD19-CAR/Raji stimulation. Thus, we provide the first phosphoproteomic analysis of RDN and Soq in the context of T and CAR T cell signalling and activation, thereby providing a molecular basis for their in vivo effects.

5.2 Results

5.2.1 Soq inhibits PLC γ 1 phosphorylation without large-scale disturbance of the TCR signalling pathway

Given the novelty and potential therapeutic applications of RDN and Soq, we aimed to thoroughly characterise their effects on Jurkat T- and CAR T cell signalling and

activation (Figure 5.1A). Despite genomic instability among Jurkat lines across research groups (Wilson et al., 2025), these lines enable large-scale, deep phosphoproteomic analysis that has shaped the field of T cell signalling (Abraham and Weiss, 2004). We performed a titration of RDN and Soq on Jurkat T cells and assessed phosphorylation of key TCR signalling proteins after T cell activation by soluble antibody stimulation of the TCR and CD28, APC-pMHC/TCR co-culture, and CD19-CD28- ζ CAR/Raji (CD19-CAR/Raji) co-culture. We showed that our Jurkat T cell sublines (J^{OT1} and CD19-CAR Jurkats) are distinguishable by GFP expression and retain high surface expression of CD45, CD28, CD3 ϵ , and TCR $\alpha\beta$ (Figure 5.2A-E). Further, T2-K^b cells (used as APCs for J^{OT1} cells) and Raji cells highly express CD45, CD80, and CD86 (Figure 5.2F-H). We demonstrated that low concentrations (100 nM) of RDN or Soq were sufficient to reduce basal Erk1/2 phosphorylation (Figure 5.3). We found that only 10 μ M RDN was sufficient to impair phosphorylation of LAT^{Y220}, a direct substrate of Zap70 (Paz et al., 2001) (Figure 5.1B-D). Phosphorylation of Lck^{Y394} was unaffected by RDN (Figures 5.1B, 5.4), but the downstream pathway targets Erk1^{T202Y204}/Erk2^{T185Y187} were significantly reduced at 1 and 10 μ M RDN after 2.5 minutes of stimulation. During soluble antibody stimulation, Soq treatment did not significantly disrupt PLC γ 1^{Y783} phosphorylation, a direct substrate of Itk, after 2.5 minutes. It minimally affected Erk1^{T202Y204}/Erk2^{T185Y187} until a concentration of 10 μ M Soq and did not affect LAT^{Y220} or Lck^{Y394} (Figures 5.1B-D, 5.4). These trends remained true irrespective of the method of stimulation (Figures 5.1E-J, 5.5, 5.6), except for PLC γ 1^{Y783}, which was significantly impaired after Soq treatment in APC-pMHC/TCR and CD19-CAR/Raji conditions. Lck^{Y394} was not induced during APC-pMHC/TCR or CD19-CAR/Raji co-cultures (Figures 5.5, 5.6). We also observed that phosphorylation of early TCR signalling proteins peaked approximately 2.5 minutes after stimulation, with a subsequent decline by 10 minutes (Figures 5.1, 5.4-5.6). Importantly, CD19-CAR Jurkats maintain high induction of phosphorylation in response to soluble antibody stimulation (Figure 5.7), thus low induction during CAR/Raji co-culture is not due to general signalling incompetence in CD19-CAR Jurkats. These data suggest that 10 μ M RDN is required to disrupt phosphorylation-based TCR/CAR signalling, whereas 10 μ M Soq or higher is required to achieve maximal Itk inhibition.

Figure 5.1: Soq inhibits PLC γ 1^{Y783} while RDN inhibits both PLC γ 1^{Y783} and LAT^{Y220} phosphorylation. (A) Schematic representation of T cell signalling, indicating the expected effects of inhibiting Zap70 (RDN) or Itk (Soq). Phosphorylation sites depicted in green are probed in subsequent Western blots. (B) Representative Western blots targeting PLC γ 1^{Y783}/PLC γ 1, SFK^{Y416}/Lck, Erk1^{T202Y204}/Erk1 and Erk2^{T185Y187}/Erk2, and LAT^{Y220}/LAT in soluble antibody stimulation of the TCR (C) Quantification of PLC γ 1^{Y783} during a time course soluble antibody stimulation of the TCR. (D) Quantification of LAT^{Y220} during a time course soluble antibody stimulation of the TCR. (E-G) As in (B-D), except for APC-pMHC stimulation of the Jurkat OT-I TCR. (H-J) As in (B-D), except for Raji co-culture stimulation of a second-generation CD19-CAR. Statistical analysis for Western blots was performed using the Holm-Sidak correction of Fisher's LSD pairwise comparisons. $n \geq 3$ independent experiments per treatment group/time point.

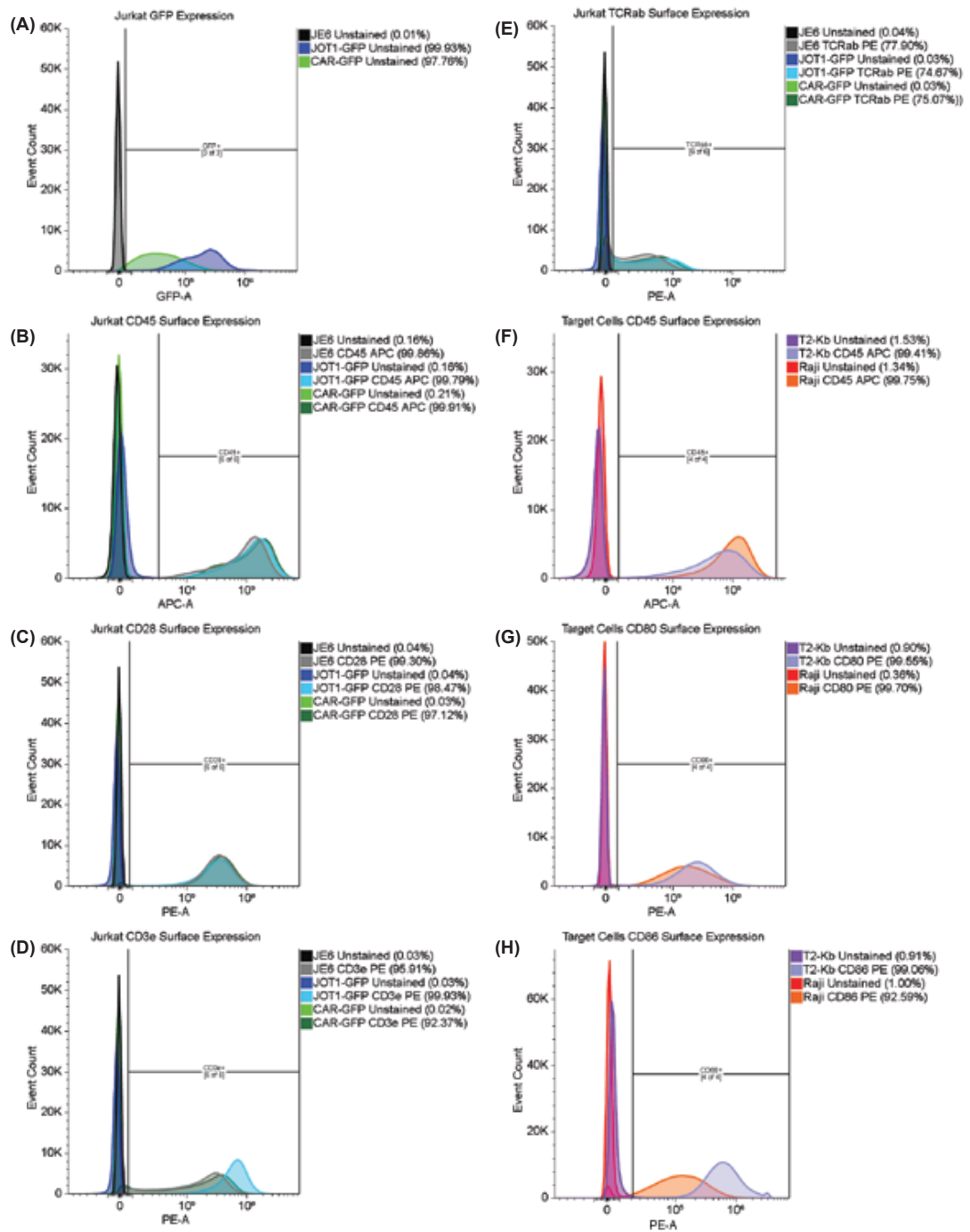


Figure 5.2: Analysis of surface expression on Jurkat T cell sublines and target cells used in activation assays. (A) Flow cytometry analysis of total GFP in Jurkat T cell sublines. (B) Flow cytometry analysis of surface expression of CD45 in Jurkat T cell sublines. (C-E) As in (B), except for CD28, CD3 ϵ , and TCR $\alpha\beta$ surface expression, respectively. (F) Flow cytometry analysis of CD45 surface expression in T2-K^b and Raji cells. (G-H) As in (F), except for CD80 and CD86 surface expression, respectively.

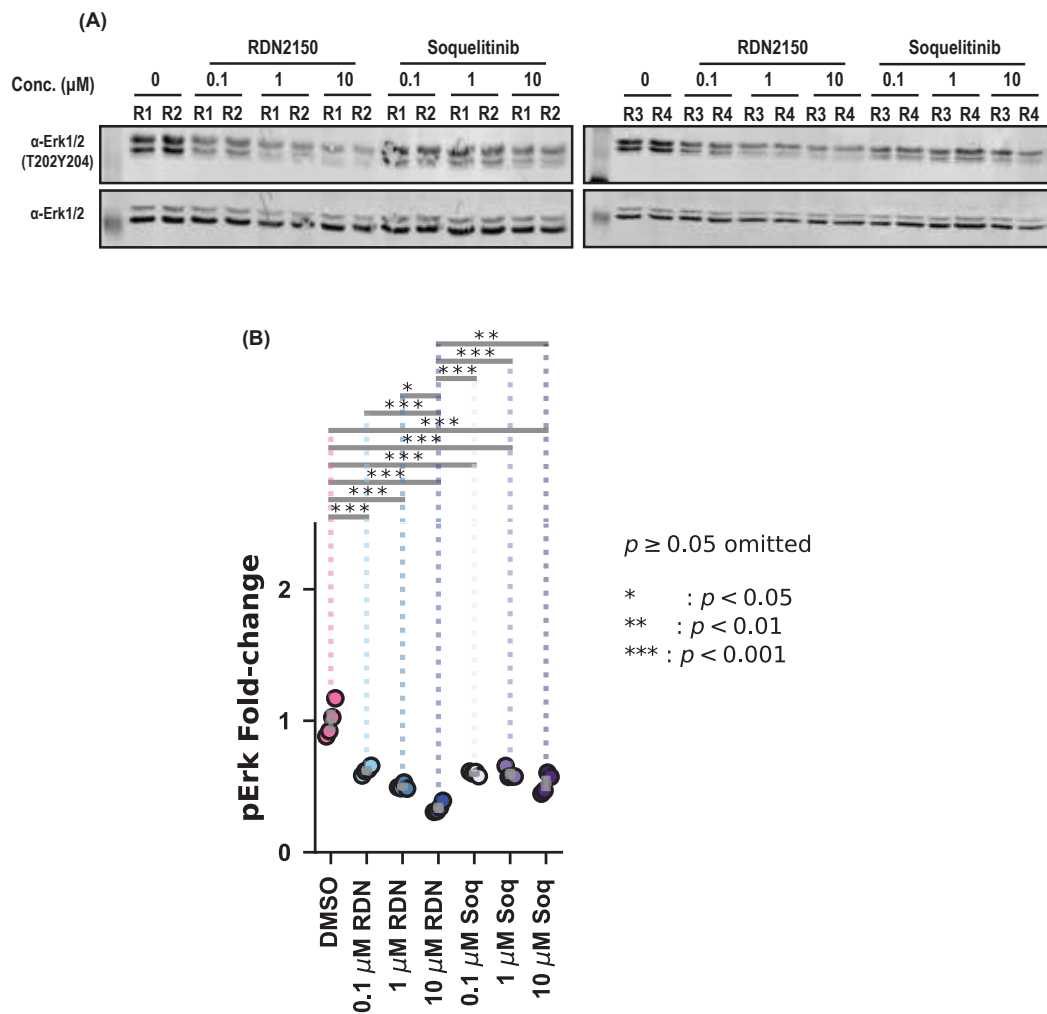
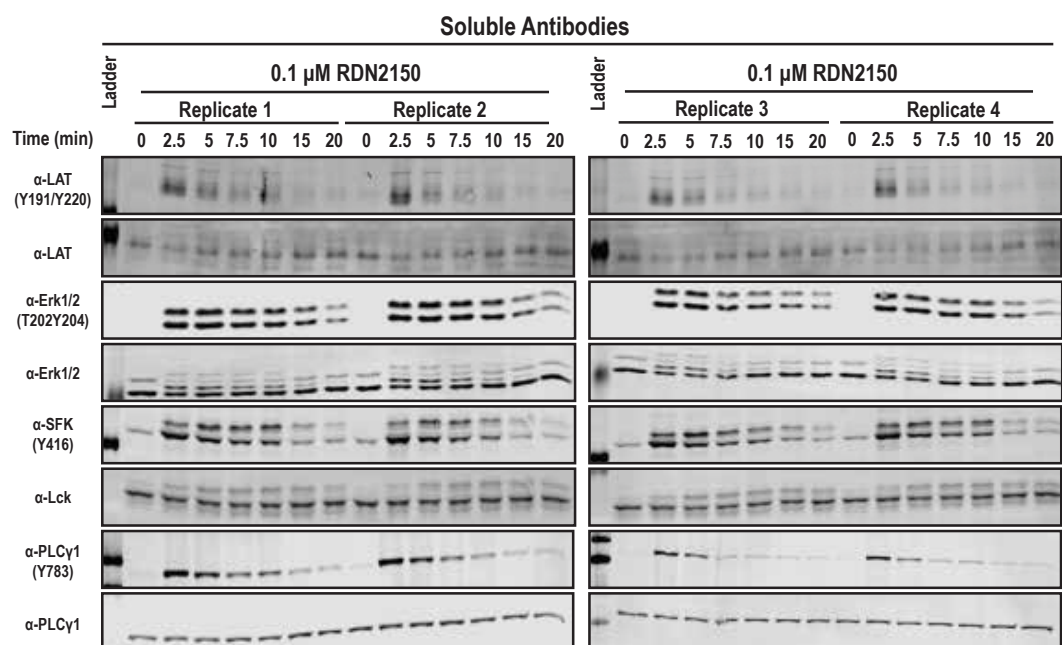
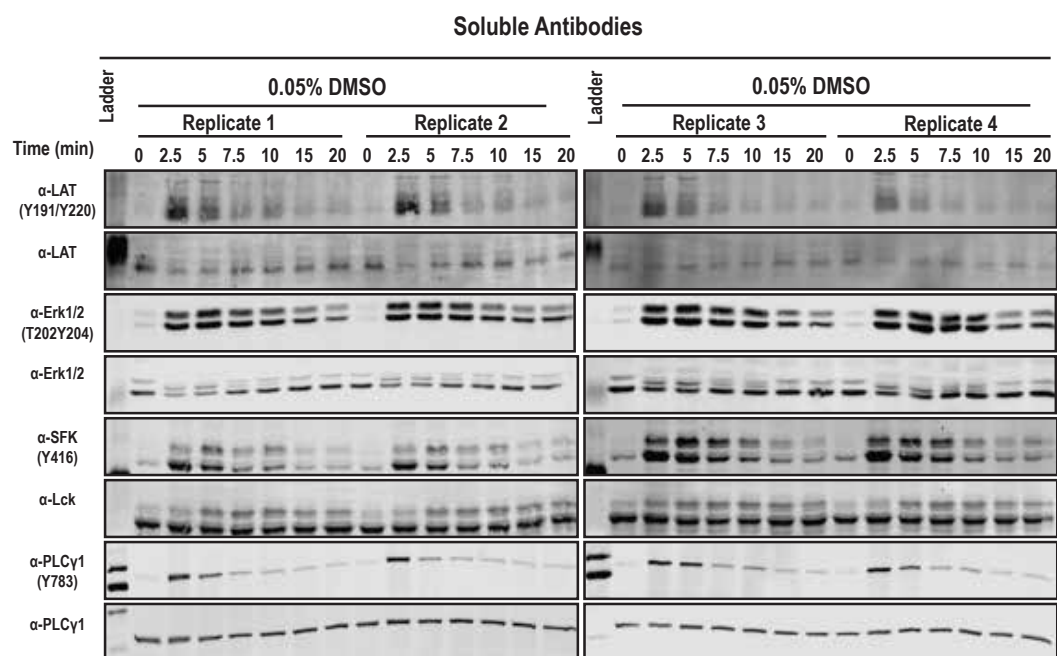
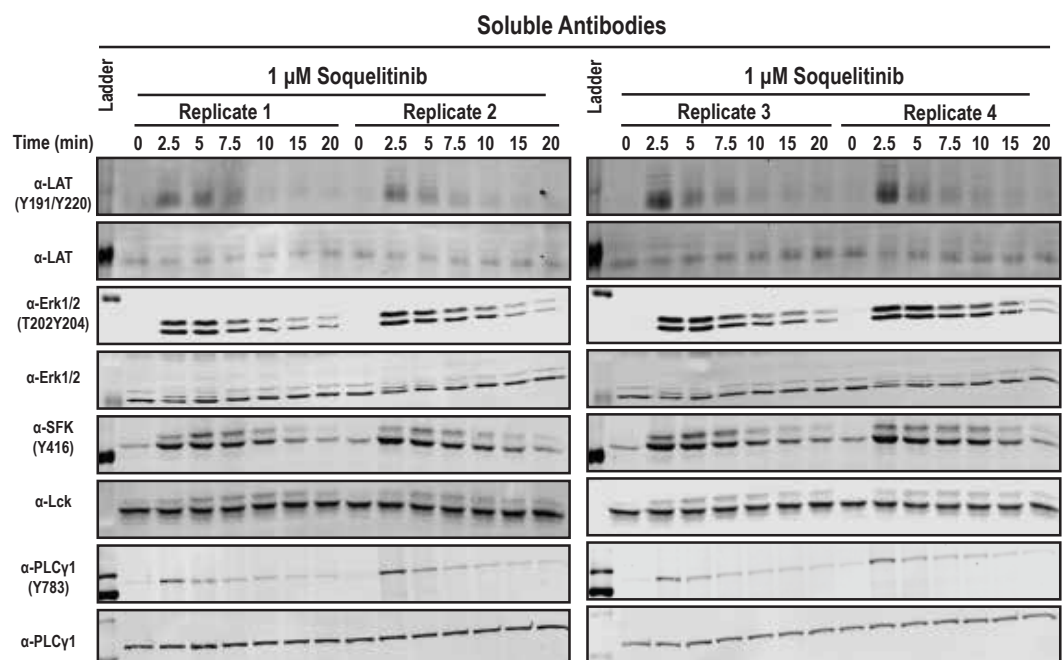
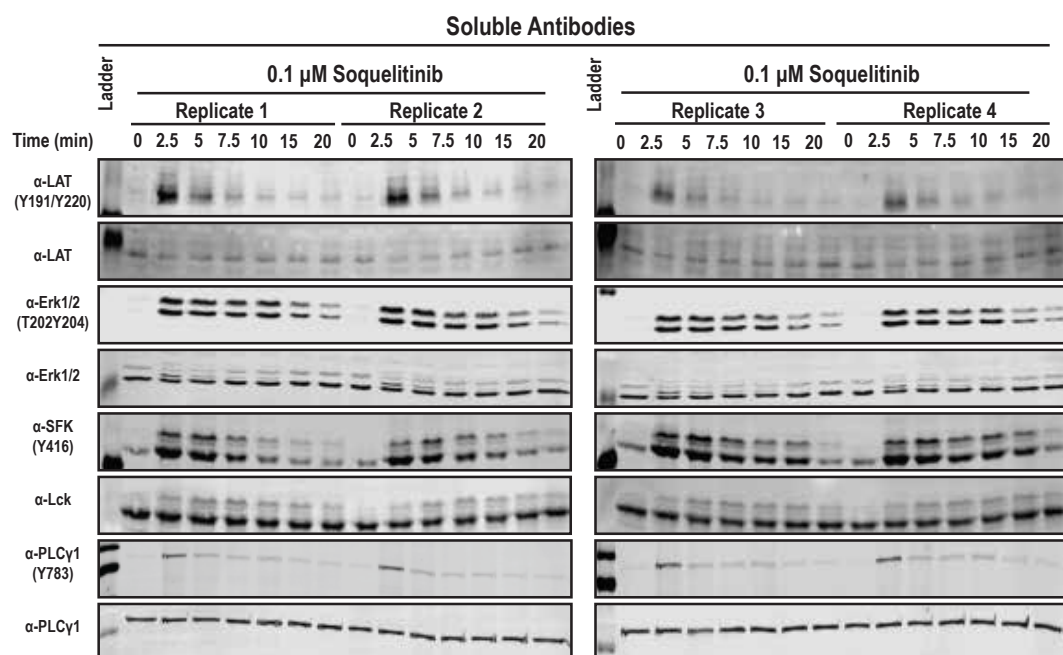


Figure 5.3: Treatment with low-levels of RDN2150 or Soquelitinib reduces Erk1/2 phosphorylation in unstimulated Jurkat T cells. (A) Western blot analysis of Erk1^{T202Y204}/Erk2^{T185Y187} in unstimulated Jurkat T cells with (B) accompanying quantification. Statistical analysis for Western blots was performed using the Holm-Sidak correction of Fisher's LSD pairwise comparisons.



Time (min)	1 μ M RDN2150															
	Replicate 1								Replicate 2							
	0	2.5	5	7.5	10	15	20	0	2.5	5	7.5	10	15	20		
α -LAT (Y191/Y220)																
α -LAT																
α -Erk1/2 (T202/Y204)																
α -Erk1/2																
α -SFK (Y416)																
α -Lck																
α -PLC γ 1 (Y783)																
α -PLC γ 1																

Time (min)	10 μ M RDN2150														Ladder	10 μ M RDN2150														Ladder
	Replicate 1							Replicate 2								Replicate 3							Replicate 4							
	0	2.5	5	7.5	10	15	20	0	2.5	5	7.5	10	15	20		0	2.5	5	7.5	10	15	20	0	2.5	5	7.5	10	15	20	
α -LAT (Y191/Y220)																														
α -LAT																														
α -Erk1/2 (T202/Y204)																														
α -Erk1/2																														
α -SFK (Y416)																														
α -Lck																														
α -PLCy1 (Y783)																														
α -PLCy1																														



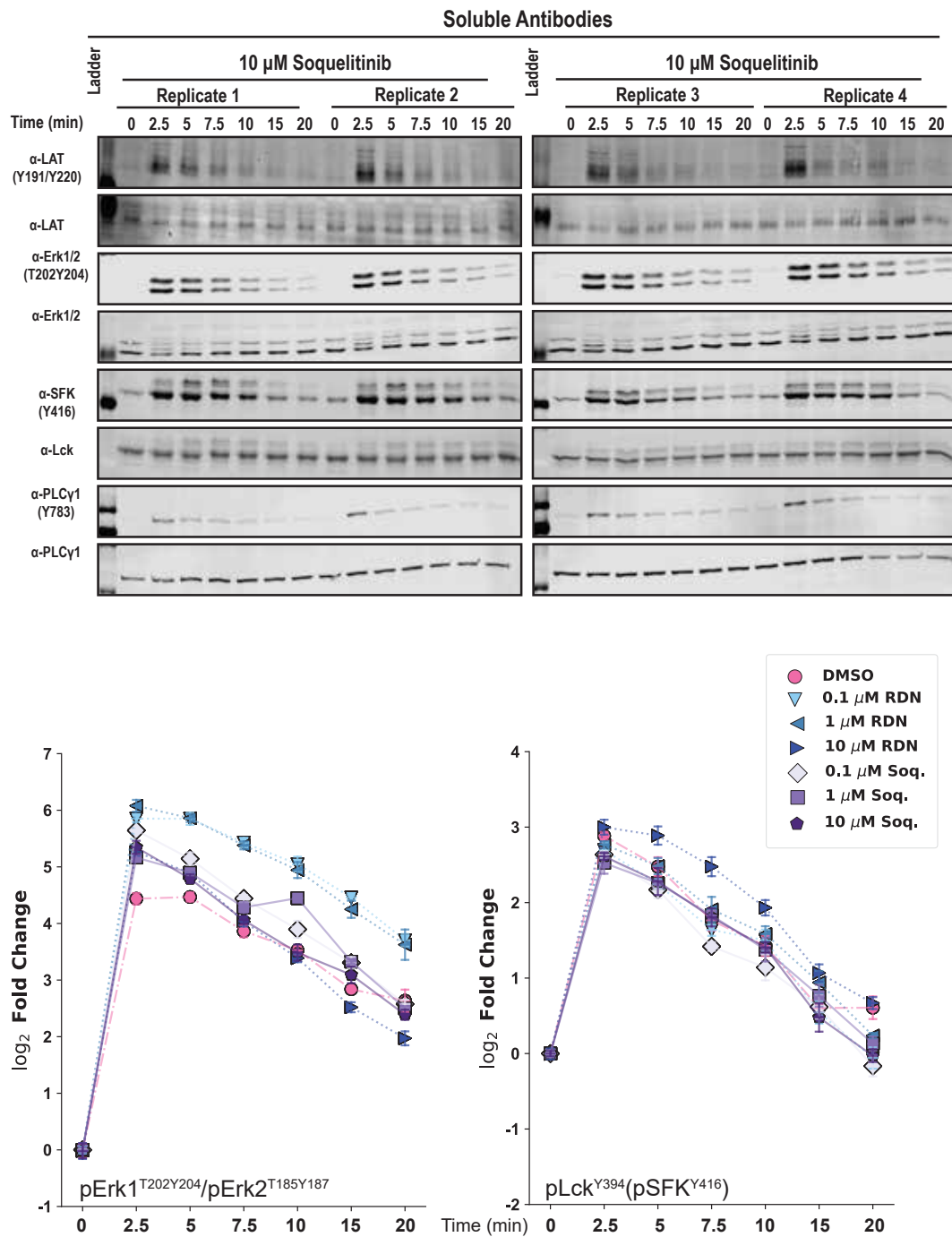
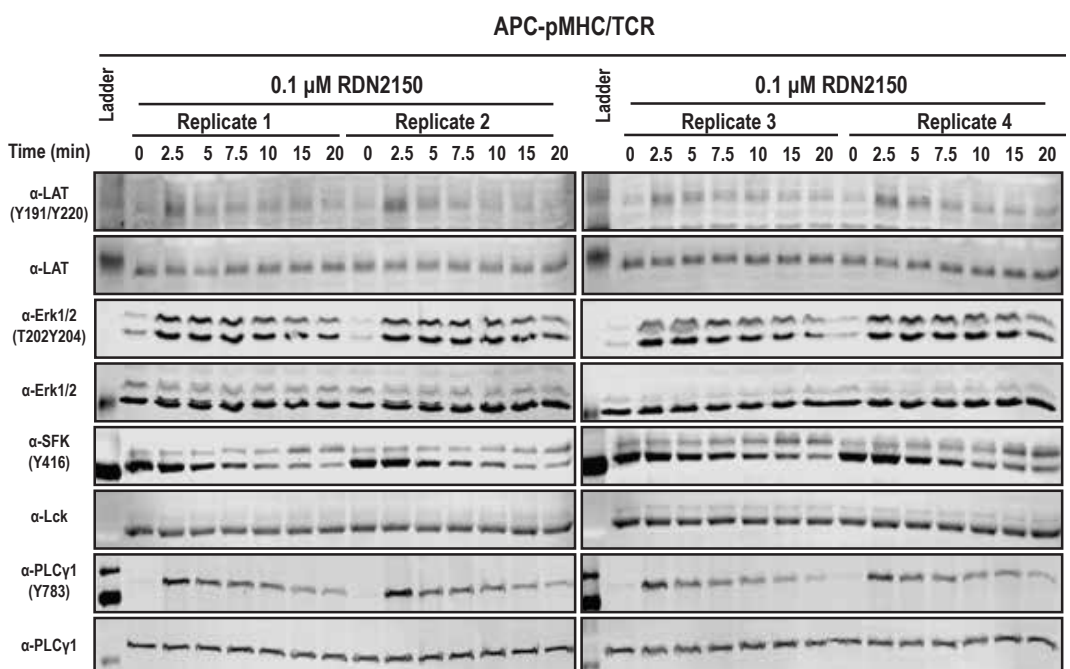
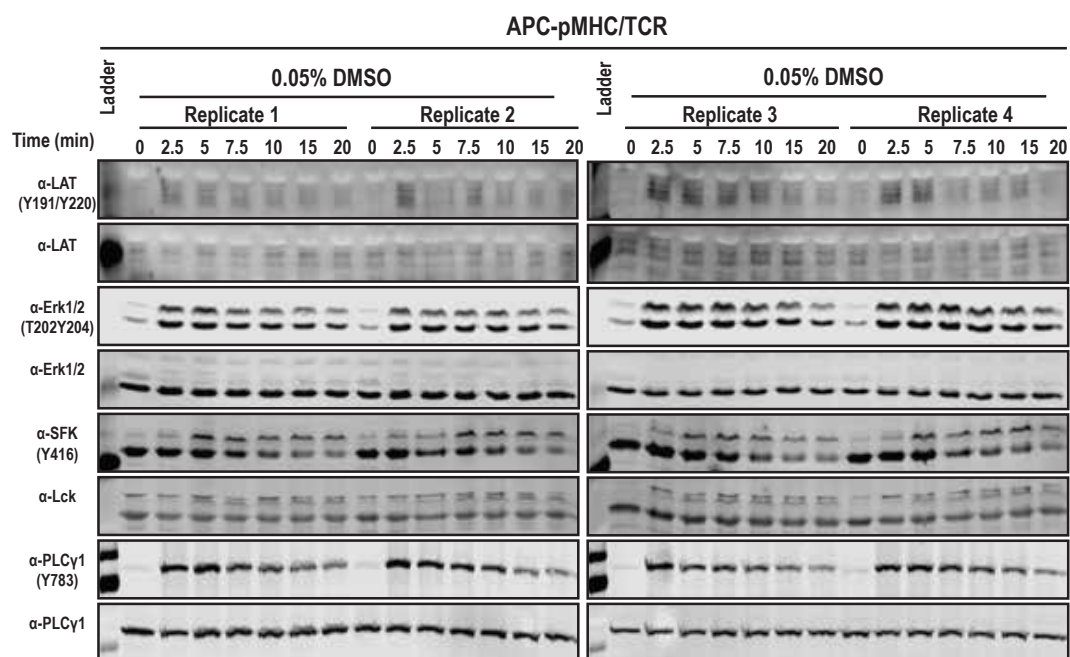
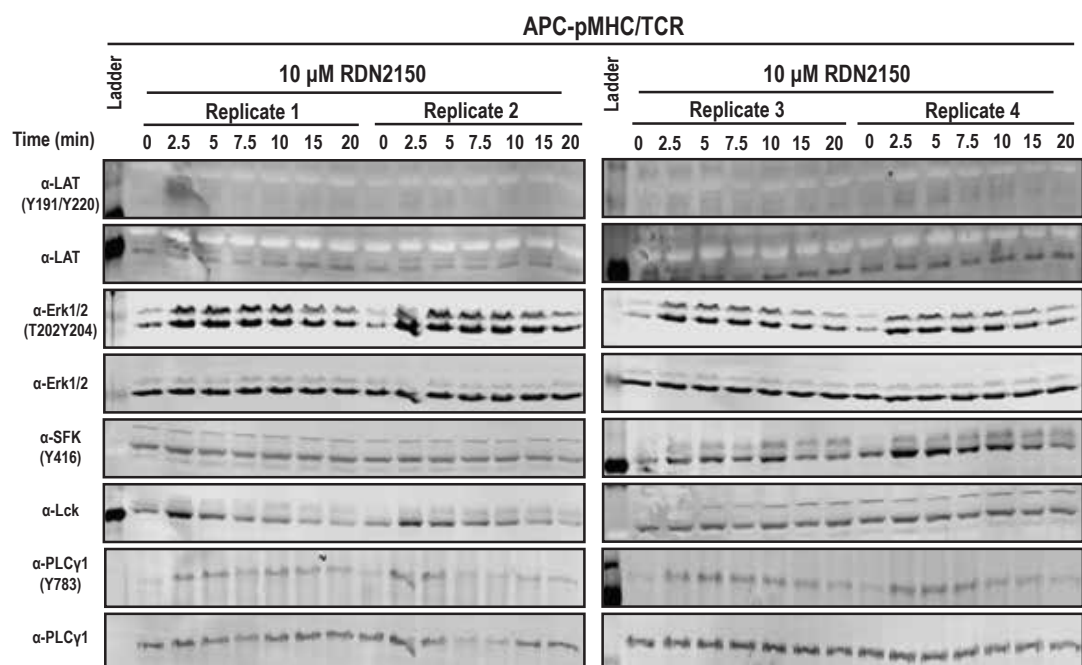
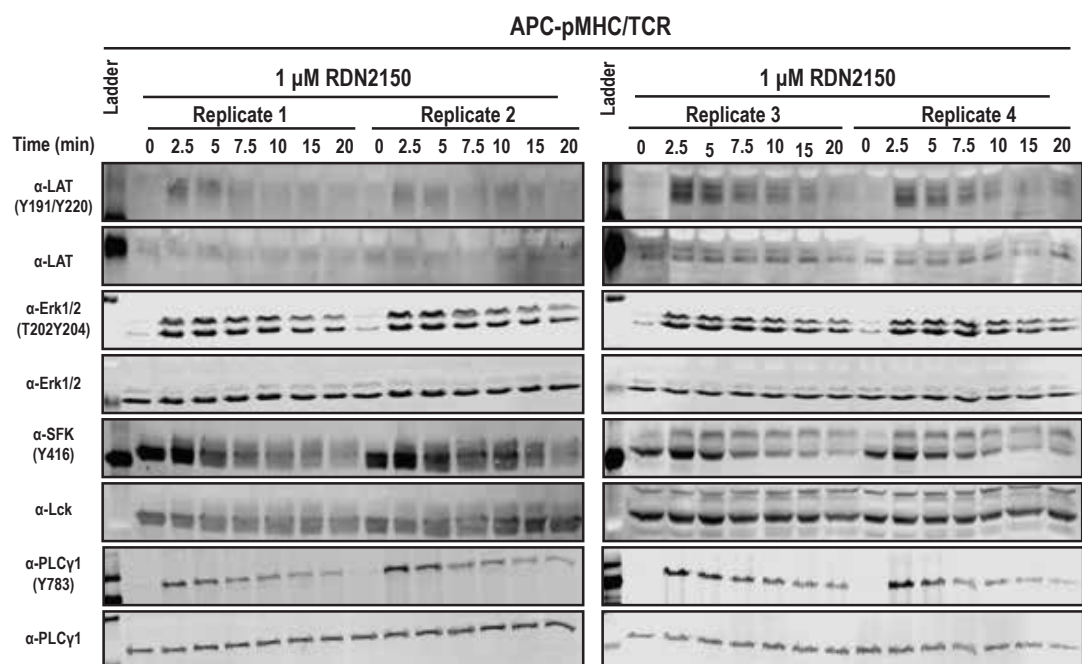
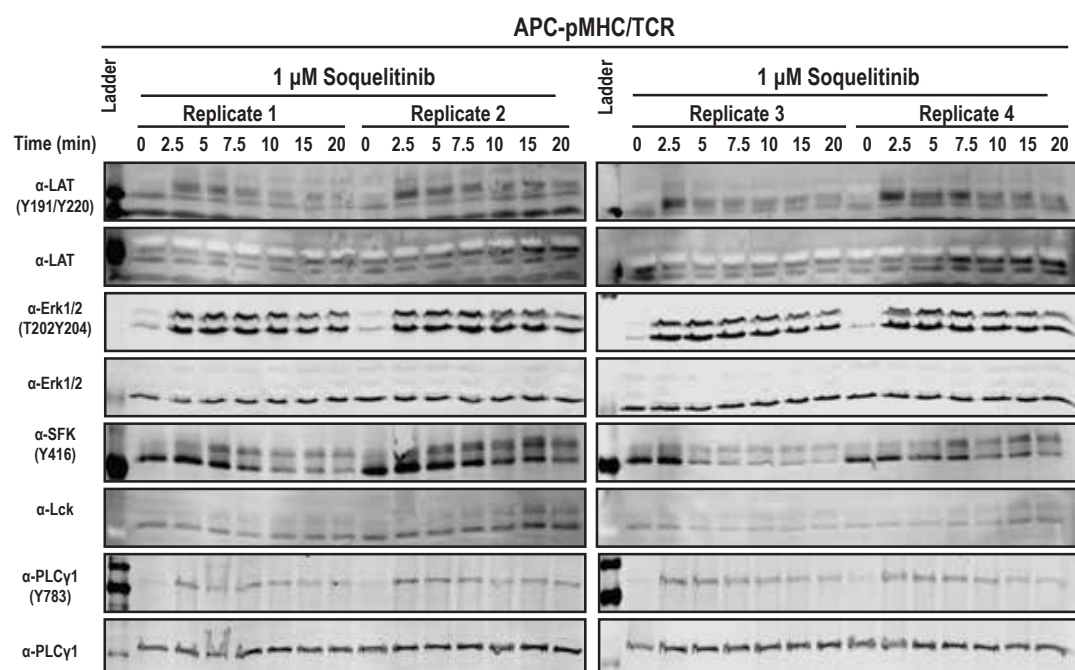
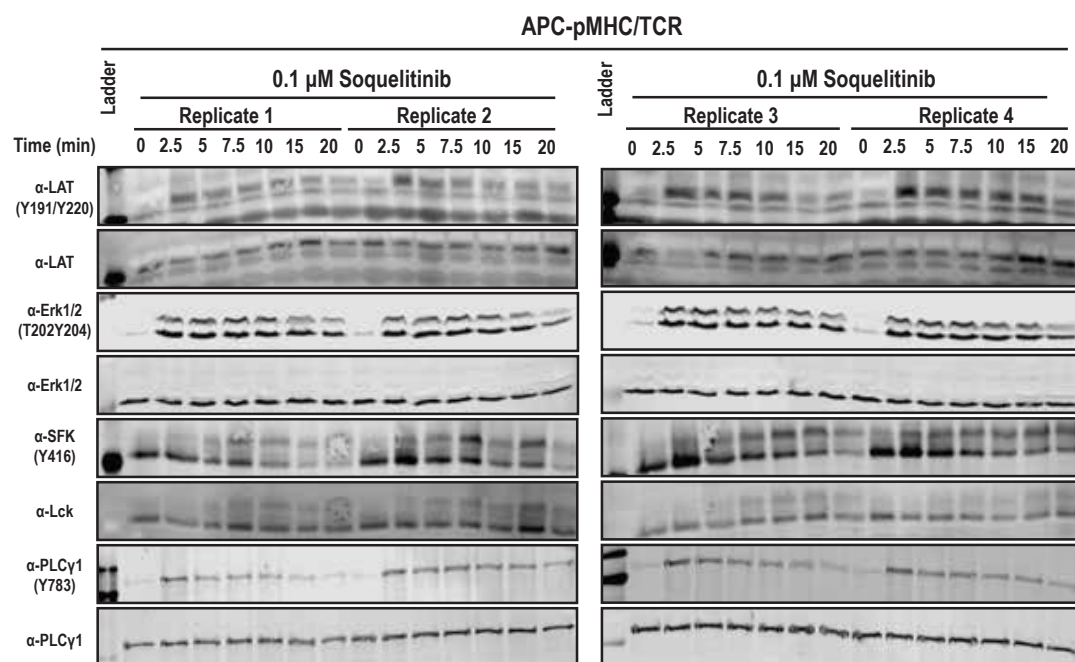


Figure 5.4: (Caption on next page)

Figure 5.4: Quantitative Western blots after time course soluble antibody crosslinking of CD3 ϵ /CD28 and a titration of RDN2150/Soquelitinib reveals a dramatic increase in pY abundance of the key TCR phosphorylation sites LAT^{Y220}, Erk1^{T202Y204}/Erk2^{T185Y187}, Lck^{Y394}/SFK^{Y416}, and PLC γ 1^{Y783}. Quantification for PLC γ 1^{Y783} and LAT^{Y220} are shown in Figure 5.1. Statistical analysis for Western blots was performed using the Holm-Sidak correction of Fisher's LSD pairwise comparisons.







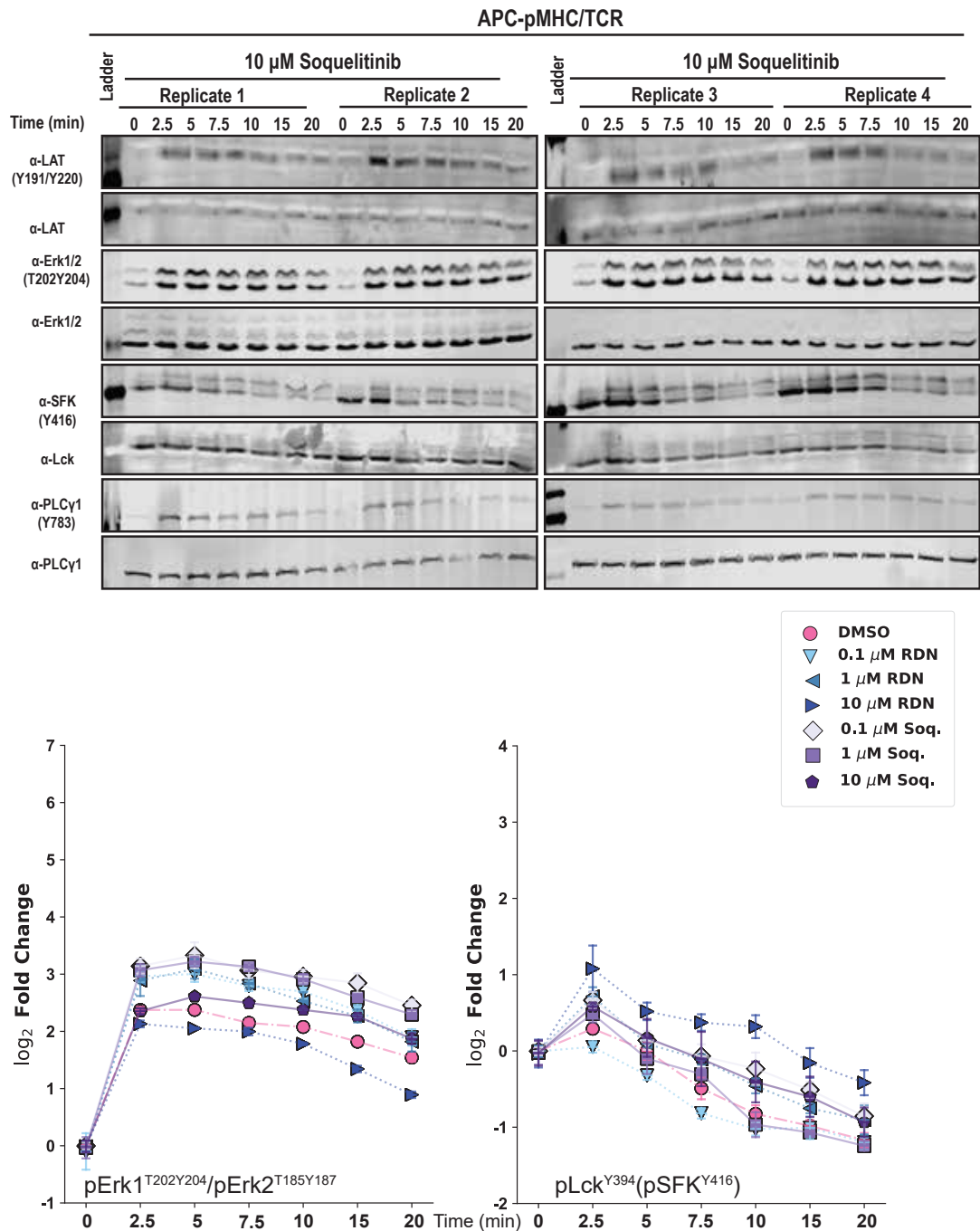
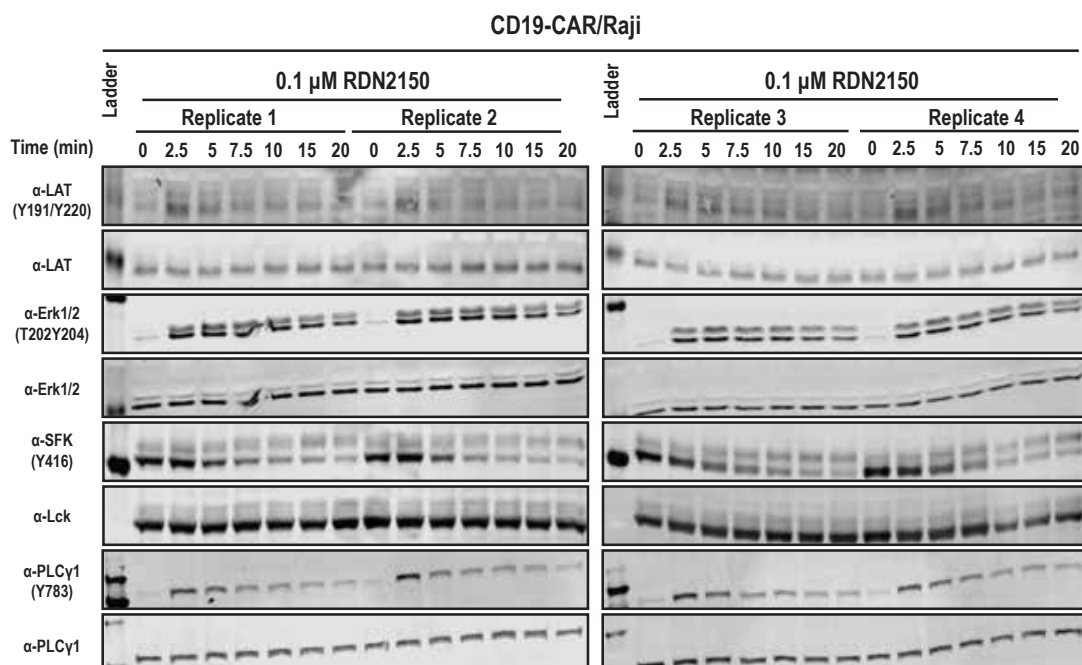
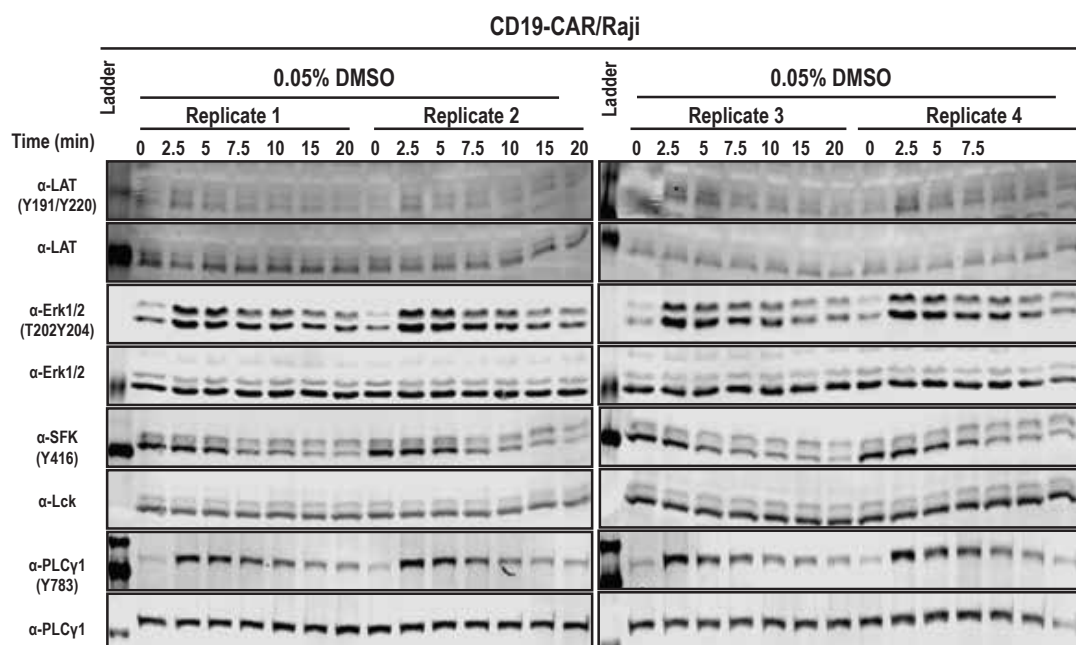
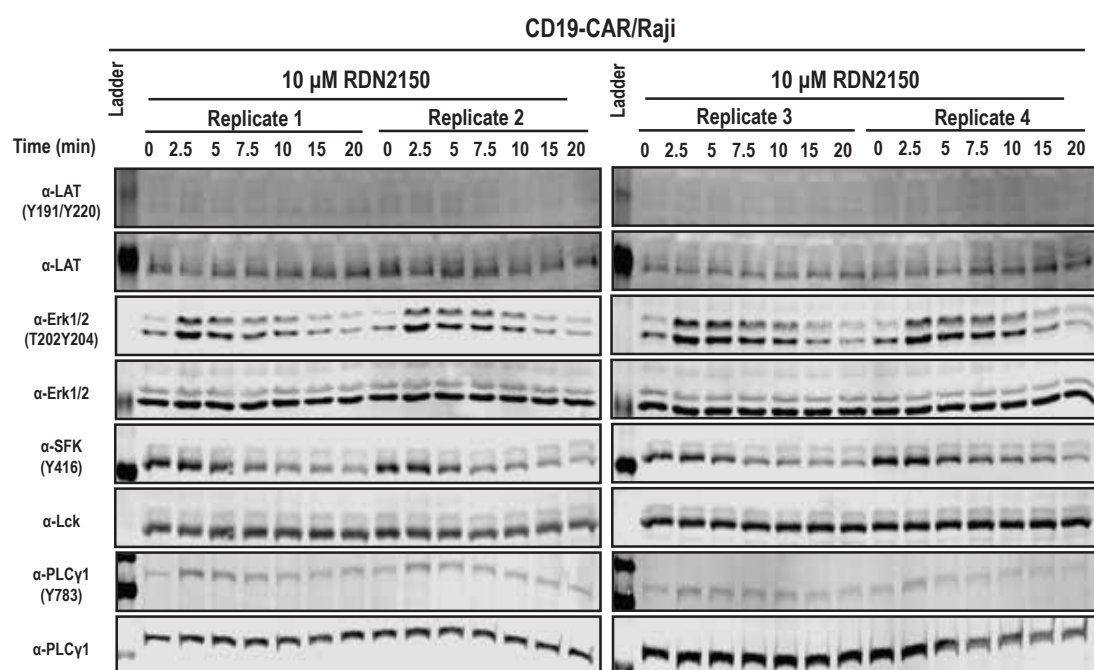
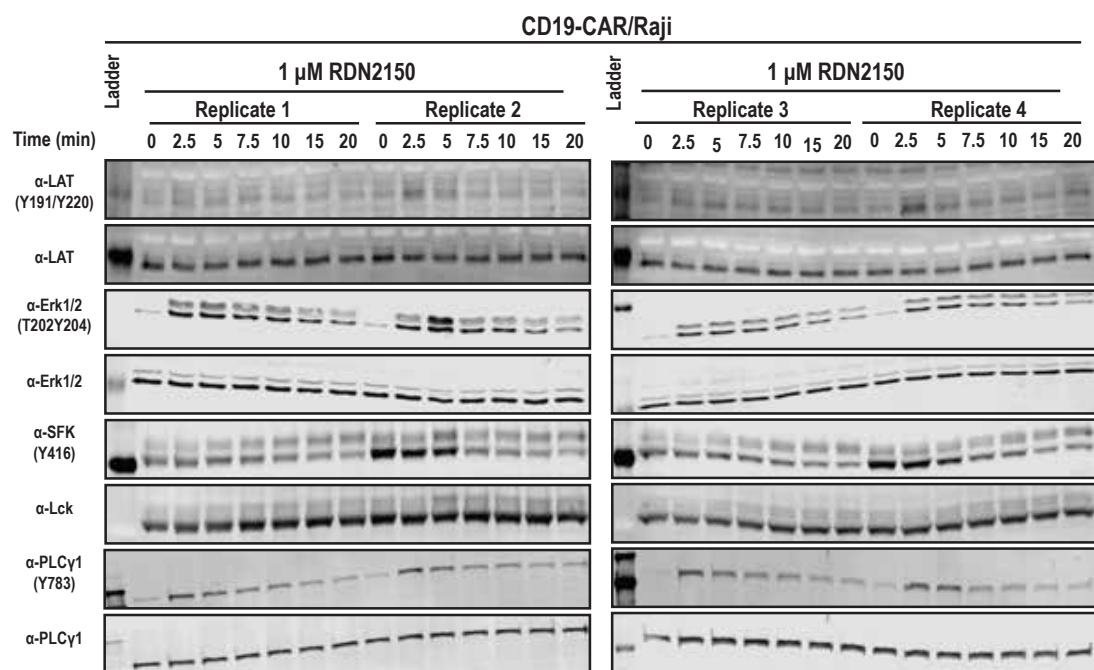
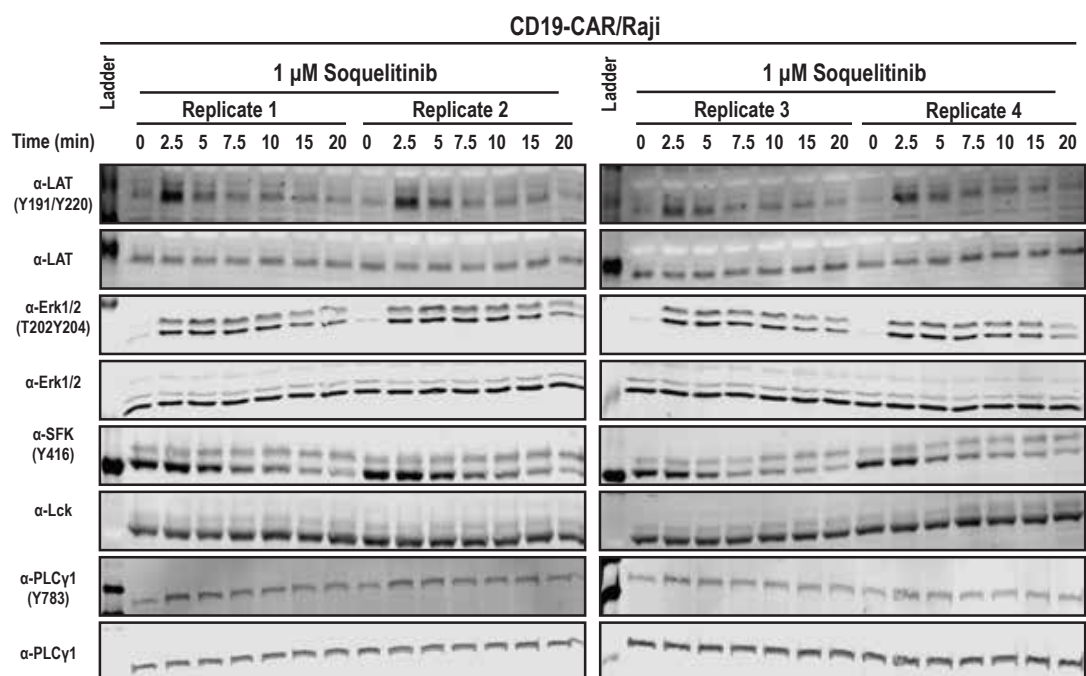
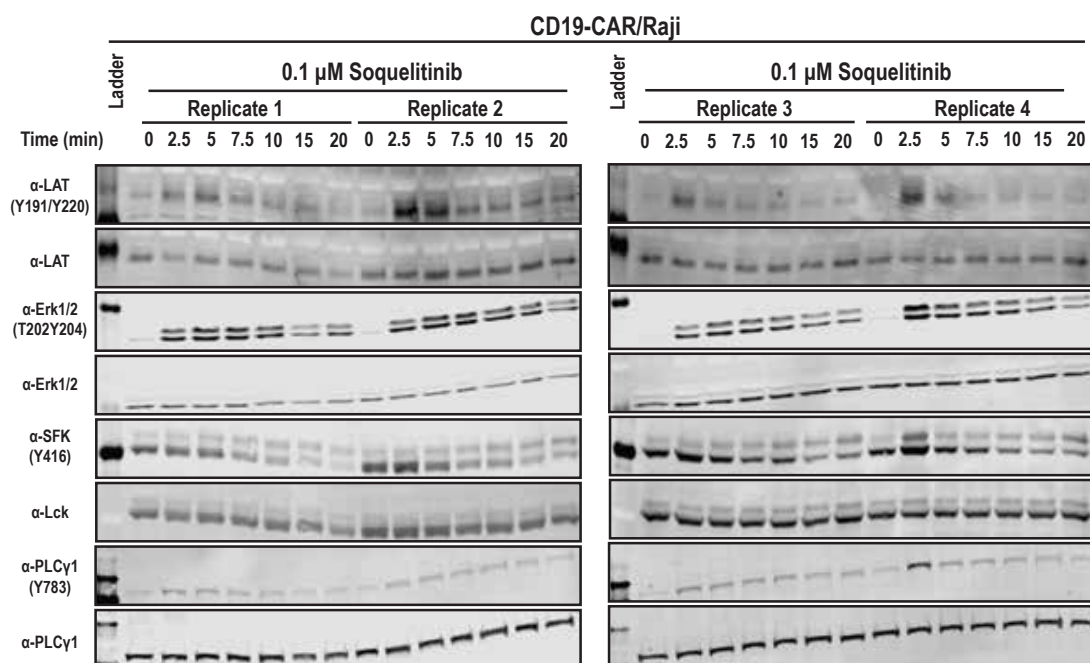


Figure 5.5: (Caption on next page)

Figure 5.5: Quantitative Western blots after time course co-culture of peptide-loaded T2-K^b cells with J^{OT1} cells and a titration of RDN2150/Soquelitinib reveals a modest increase in pY abundance of the key TCR phosphorylation sites LAT^{Y220}, Erk1^{T202Y204}/Erk2^{T185Y187}, Lck^{Y394}/SFK^{Y416}, and PLCγ1^{Y783}. Quantification for PLCγ1^{Y783} and LAT^{Y220} are shown in Figure 5.1. Statistical analysis for Western blots was performed using the Holm-Sidak correction of Fisher's LSD pairwise comparisons.







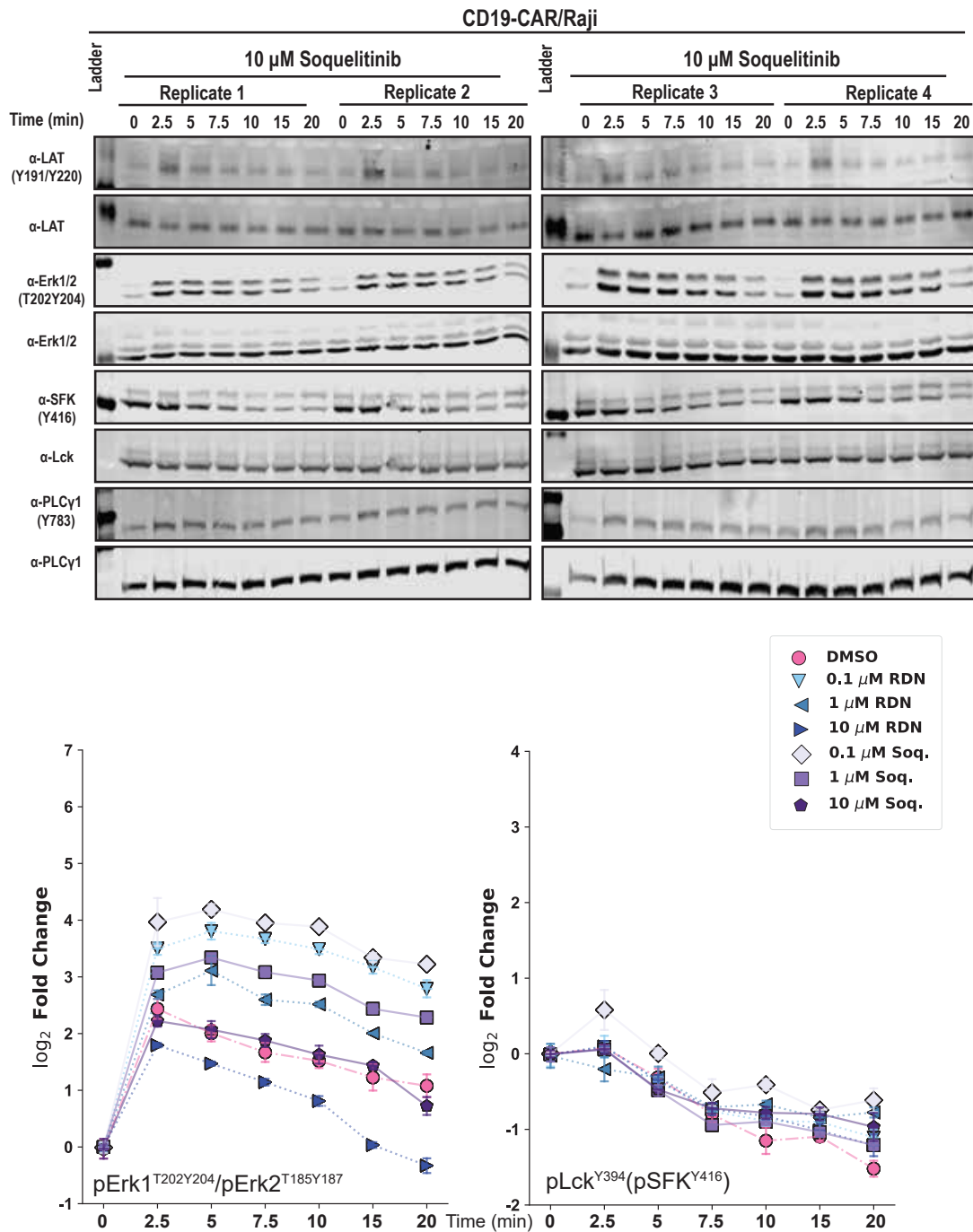


Figure 5.6: (Caption on next page)

Figure 5.6: Quantitative Western blots after time course co-culture of CD19-expressing Raji B cells with CD19-28 ζ -CAR T cells and a titration of RDN2150/Soquelitinib reveals generally weak changes in pY abundance of the key TCR phosphorylation sites LAT^{Y220}, Erk1^{T202Y204}/Erk2^{T185Y187}, Lck^{Y394}/SFK^{Y416}, and PLC γ 1^{Y783}. Quantification for PLC γ 1^{Y783} and LAT^{Y220} are shown in Figure 5.1. Statistical analysis for Western blots was performed using the Holm-Sidak correction of Fisher's LSD pairwise comparisons.

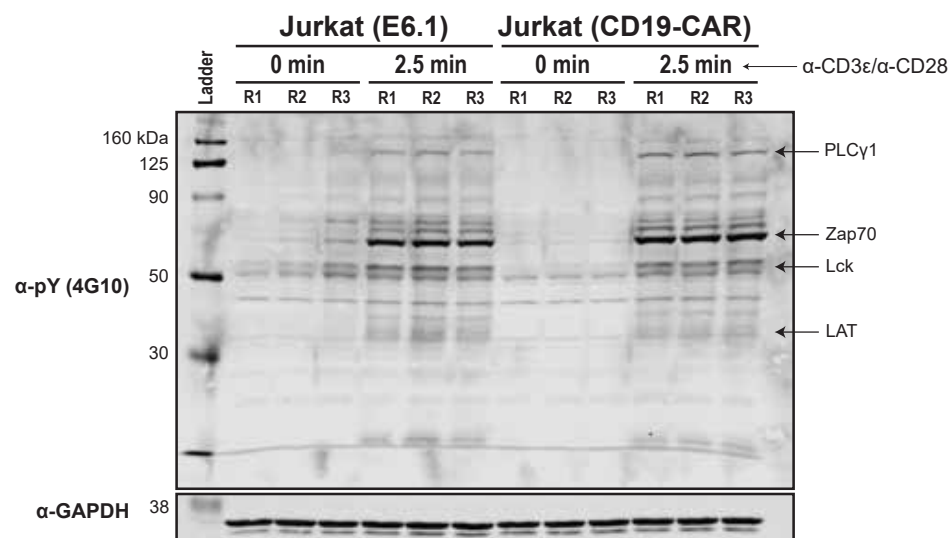


Figure 5.7: CD19-CAR Jurkat T cells retain responsiveness to soluble antibody stimulation of the TCR.

5.2.2 Both RDN and Soq inhibit IL-2 expression, but only RDN blocks CD69 upregulation

Signalling from the TCR/CAR is a rapid process, leading to the expression of new genes and the presentation/release of new proteins that elicit intercellular effects, such as cell death or the activation of neighbouring immune cells. T cell activation is a finely tuned process that requires precise, time-dependent activation of each component of the signalling pathway, and small perturbations can lead to over- or under-activation of T cells, ultimately resulting in autoimmunity (Moulton and Tsokos, 2015). To determine whether RDN or Soq impacted overall T cell activation, we evaluated expression of CD69 and IL-2, two activation markers of T and CAR T cells, after 24 hours of antibody-, pMHC-, and CD19-CAR/Raji-induced T cell activation. We found that soluble antibody stimulation mildly induced CD69 expression but failed to induce IL-2 expression (Figure 5.8A-B) (Geppert et al., 1988). These data are consistent with the literature showing that complete T cell activation requires mechanical forces and that clustering of TCR/CD28-bound antibodies using beads or tethering TCR/CD28 antibodies to a surface is necessary for antibody-based T cell activation (Hu and Butte, 2016; Fritzsche and Kruse, 2024; Trickett and Kwan, 2003; Manger et al., 1987). Treatment with either RDN or Soq significantly reduced soluble antibody stimulation-induced CD69 expression at all tested concentrations, but only 10 μ M RDN completely ablated CD69 induction (Figure 5.8A). After APC-pMHC/TCR stimulation, CD69 and IL-2 were highly upregulated and significantly reduced after treatment with RDN (Figures 5.8C-D). CD69 expression was not affected by any tested concentration of Soq, but Soq treatment significantly reduced IL-2 production after APC-pMHC/TCR stimulation (Figures 5.8C-D). These trends were most evident in CD19-CAR/Raji stimulation conditions, as CD69 was widely expressed after co-culture and was reduced only by RDN treatment. IL-2 production was highest in CAR T cells ($\sim$ 1000 pg/mL in DMSO control samples) and was significantly reduced with both RDN and Soq treatment (Figures 5.8E-F). These data in the context of the known TCR pathway ordering confirm that soluble antibody stimulation is insufficient to induce full T cell activation and support that the Itk-mediated branch of TCR signalling is responsible for IL-2 production.

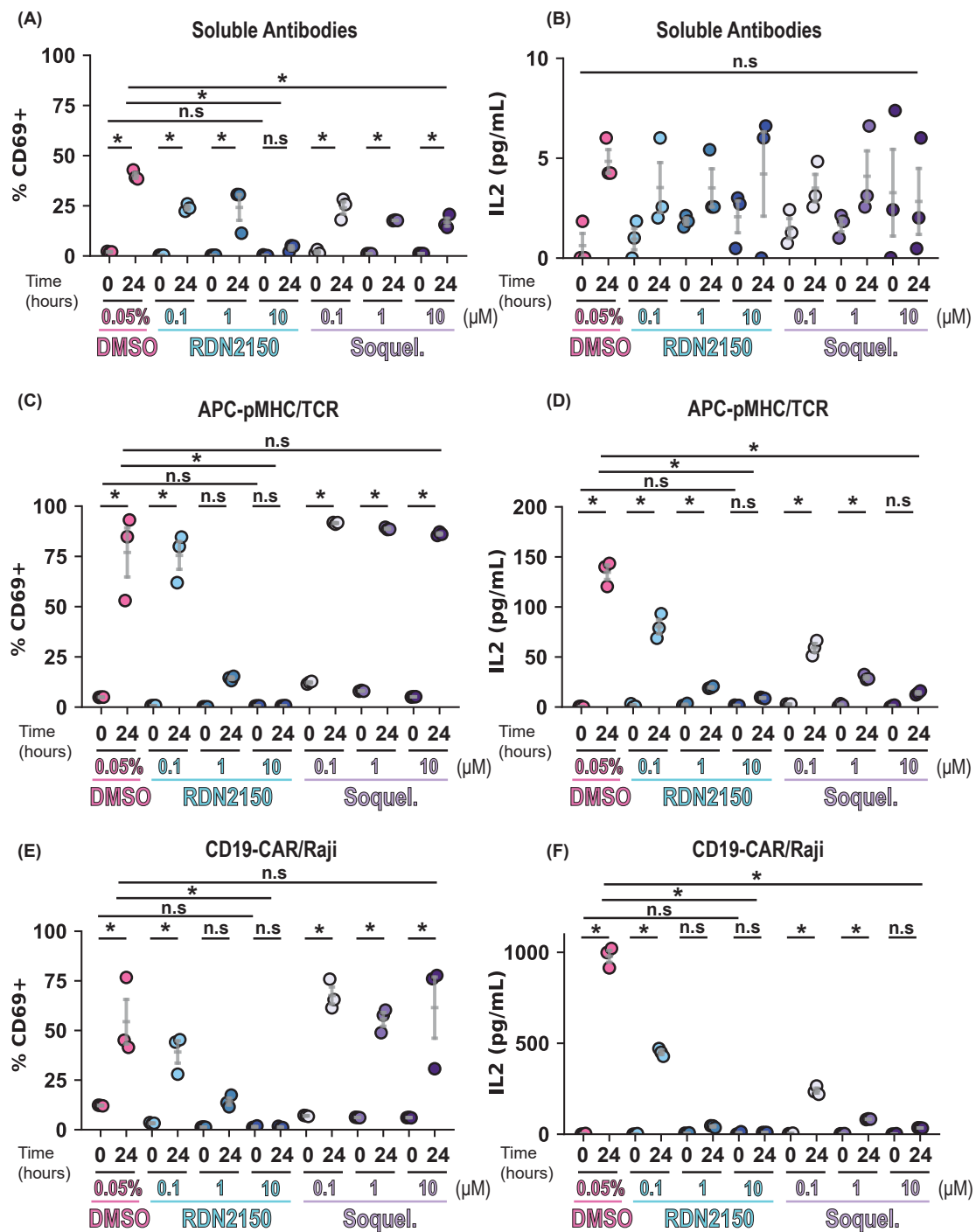


Figure 5.8: (Caption on next page)

Figure 5.8: Interleukin-2 secretion, but not CD69 expression, is significantly reduced by Soq treatment. (A) Dotplot showing the percentage of CD69-positive cells measured by flow cytometry after 24 hours of soluble antibody stimulation. (B) Dotplot showing the concentration of IL-2 in the cell culture supernatant measured by ELISA after 24 hours of soluble antibody stimulation. (C-D) As in (A-B), except for APC-pMHC stimulation. (E-F) As in (A-B), except for Raji-co-culture stimulation of a second-generation CD19-CAR. Statistical analysis for flow cytometry and ELISA data was performed using a one-way ANOVA followed by the Holm-Sidak correction of Fisher's LSD pairwise comparisons, if applicable. n=3 independent experiments per treatment group.

5.2.3 RDN treatment reduces ligand-induced pY signalling more than Soq

To gain precise information regarding the roles of Zap70 and Itk in TCR- and CAR-mediated T cell activation, we leveraged the sensitivity of LC-MS/MS coupled with pY enrichment using the Src SH2 superbinder (sSH2) as previously described (Callahan et al., 2024, 2025b; Griffith et al., 2022; Callahan et al., 2025c). We identified a total of 773 T cell-specific pY sites in antibody-stimulated samples with high reproducibility ($R > 0.8$ by multiple regression; see <https://github.com/Aurdeegz/Zap70-Itk-Inhibitor-Profiling>). Phosphotyrosine signalling from the TCR/CAR is well characterised, and Zap70/Itk have canonical roles in the progression of T cell activation (Figure 5.1A) (Gaud et al., 2018; Salter et al., 2018, 2021; Griffith et al., 2022; Belmont et al., 2017; Helou et al., 2013; Sjölin-Goodfellow et al., 2015). Using our pY proteomics data, we observed that 2.5 minutes of soluble antibody stimulation of the TCR induced large-scale pY induction irrespective of RDN or Soq treatment (Figures 5.9A, top row, 5.10). Known components of the TCR signalling cascade, including ITAMs on CD3 ϵ and TCR ζ , LAT^{Y220}, Erk2^{T185Y187}, Nck^{Y105}, PLC γ 1^{Y775}, PLC γ 1^{Y783}, SFK^{Y416}, and Zap70^{Y492} were increased after 2.5 minutes with or without RDN and Soq. After 10 minutes, some of these pY sites returned to basal, although some pY sites, including ITAMs, Erk1^{T202Y204}/Erk2^{T185Y187}, PLC γ 1^{Y775}, and PLC γ 1^{Y783}, remained elevated in DMSO and/or RDN treatment conditions, in line with our titration data (Figures 5.1|, 5.9B). Analysis of time course data using post-translational modification signature enrichment analysis (PTM-SEA), a GSEA-like algorithm for PTM proteomics data (Krug et al., 2019), showed that soluble antibody stimulation strongly engaged TCR signalling kinases (Figure 5.11A), components of various signalling pathways (Figure 5.11B), and Perturbation Signatures associated with cell activation (Figure 5.11C). In contrast, APC-pMHC/TCR stimulation modestly induced pY signalling by Western blot and proteomics for pY, pLAT, and pPLC γ 1 (Figures 5.9A, middle row, 5.12). The effects of RDN treatment were more evident with fewer significantly induced pY sites after 2.5 minutes (365 total pY sites, Figure 5.9A, middle row). APC-pMHC/TCR stimulation did not uniformly induce phosphorylation along CD3/TCR ζ ITAMs, and Soq treatment did very little to

reduce pY induction along the T cell signalling pathway (Figure 5.9C). In agreement with the antibody stimulation data, APC-pMHC/TCR stimulation significantly upregulated Kinase Signatures (Figure 5.13A), Pathway Signatures with the notable exception of TCR signalling (Figure 5.13B), and perturbation signatures associated with cell activation, including ‘ANTI CD3’ and ‘IL 2’ (Figure 5.13C). Interestingly, CD19-CAR/Raji co-culture significantly increased few pY sites (299 total pY sites, Figures 5.9A, 5.14) after 2.5 minutes with TCR signalling pY sites like Erk2^{T185Y187}, Nck1^{Y105}, PLC γ 1^{Y775}, PLC γ 1^{Y783}, and Zap70^{Y492} being among them in the DMSO control (Figure 5.9D). Treatment with RDN ablated the induction of all TCR signalling pY sites at 2.5 minutes after co-culture. In contrast, Soq treatment had little effect on pY induction along the TCR signalling pathway (Figure 5.9D). Finally, although many Kinase Signatures (Figure 5.15A), Pathway Signatures (Figure 5.15B), and Perturbation Signatures (Figure 5.15C) were significantly upregulated in CD19-CAR/Raji stimulations with DMSO or Soq treatment, almost no signatures were significantly induced during RDN treatment, supporting that Zap70 is integral to the CD19-CAR activation (Gudipati et al., 2020; Chu et al., 2023). These data, in combination with Figure 5.1, support the notion that pY induction is heavily dependent on the form of stimulation, and that the effectiveness of RDN and Soq treatment on pY signalling varies greatly with stimulation strength.

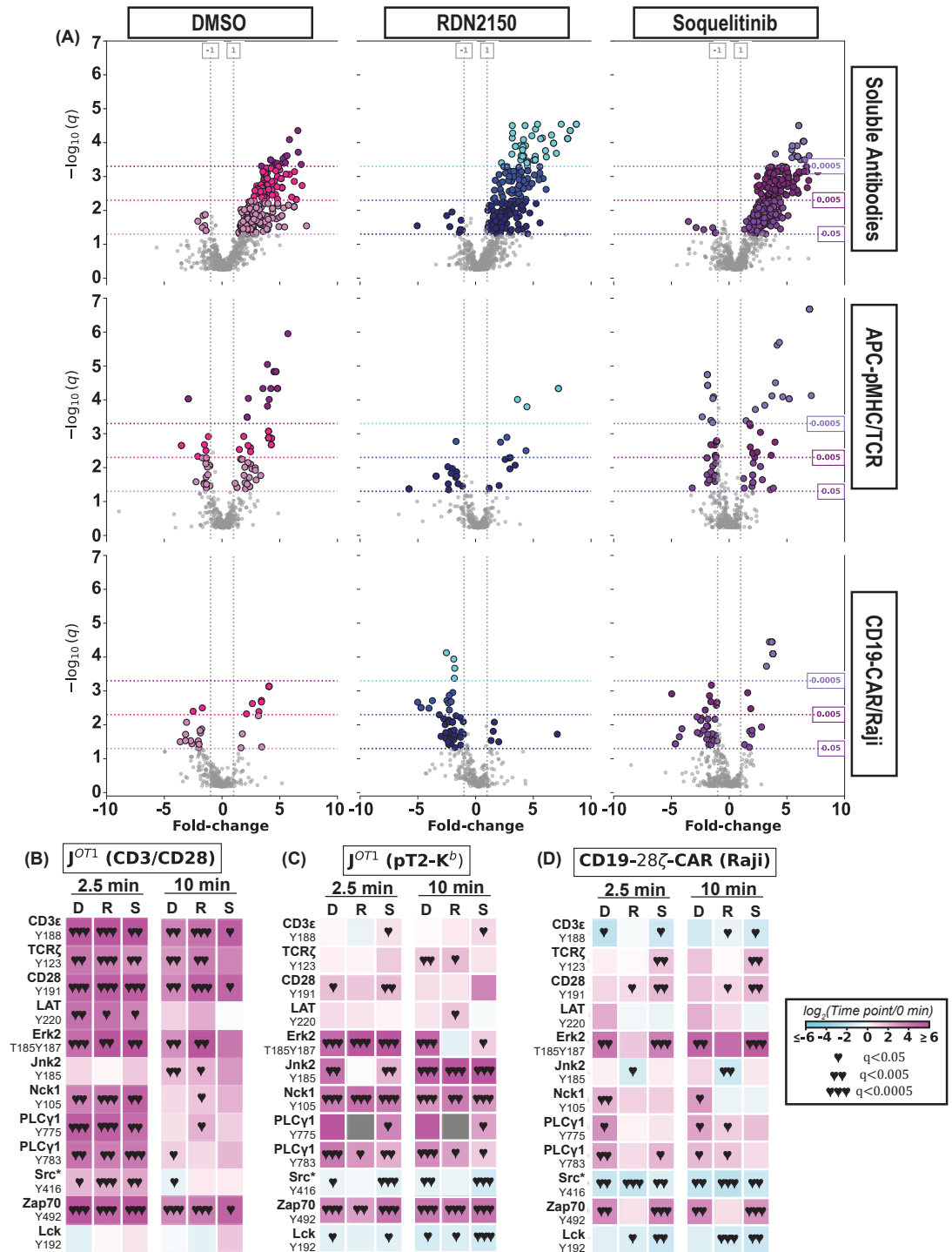


Figure 5.9: (Caption on next page)

Figure 5.9: Antibody/TCR stimulation strongly induces tyrosine phosphorylation irrespective of RDN or Soq treatment. (A) Volcano plot analysis of pY proteomics data comparing 2.5 minutes versus 0 minutes of antibody stimulation (top), APC-pMHC/TCR stimulation (middle), and CD19-CAR/Raji (bottom) in DMSO (left), RDN (middle), and Soq (right) treatment conditions. (B-D) Select site-specific heatmaps of time course pY proteomics data after antibody stimulation, APC-pMHC/TCR stimulation, and CD19-CAR/Raji stimulation. ♥ indicates $q < 0.05$, and ♥♥ indicates $q < 0.005$, and ♥♥♥ indicates $q < 0.0005$. $n \geq 3$ measurements from independent experiments for each unique pY site per treatment group/time point/stimulation method.

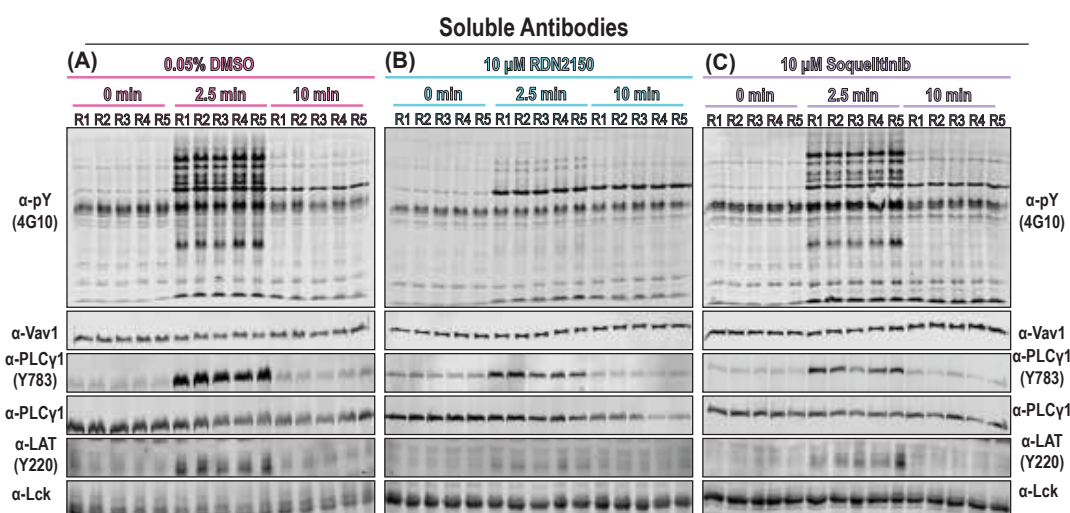


Figure 5.10: Western blot analysis of soluble antibody stimulation pY proteomics samples targeting: phosphotyrosine (pY, clone 4G10), Vav1 (loading control), PLC γ 1^{Y783}/PLC γ 1, and LAT^{Y220}/Lck in (A) DMSO control samples, (B) 10 μ M RDN2150 samples, and (C) 10 μ M Soquelitinib samples.

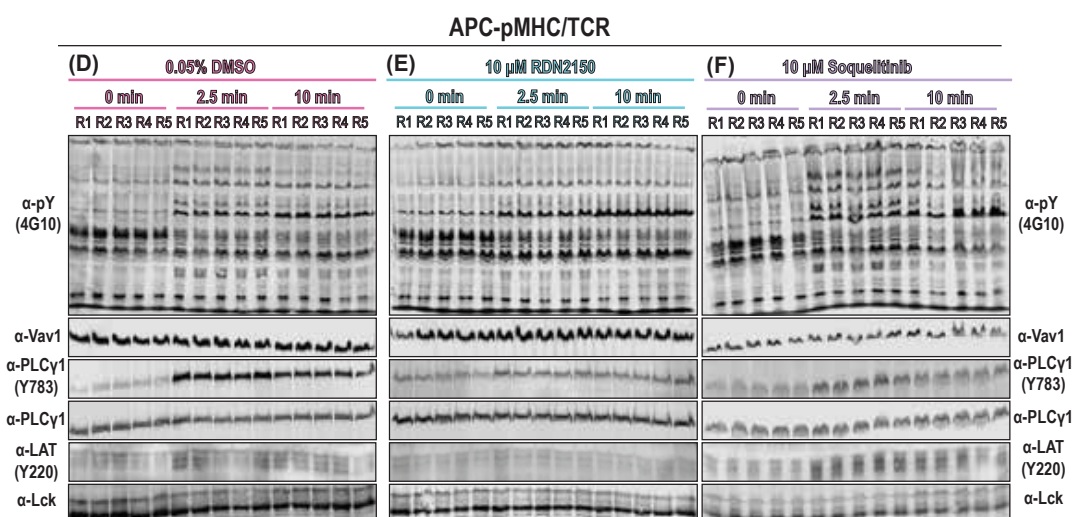


Figure 5.12: Western blot analysis of pMHC/TCR stimulation pY proteomics samples targeting: phosphotyrosine (pY, clone 4G10), Vav1 (loading control), PLC γ 1^{Y783}/PLC γ 1, and LAT^{Y220}/Lck in (A) DMSO control samples, (B) 10 μ M RDN2150 samples, and (C) 10 μ M Soquelitinib samples.

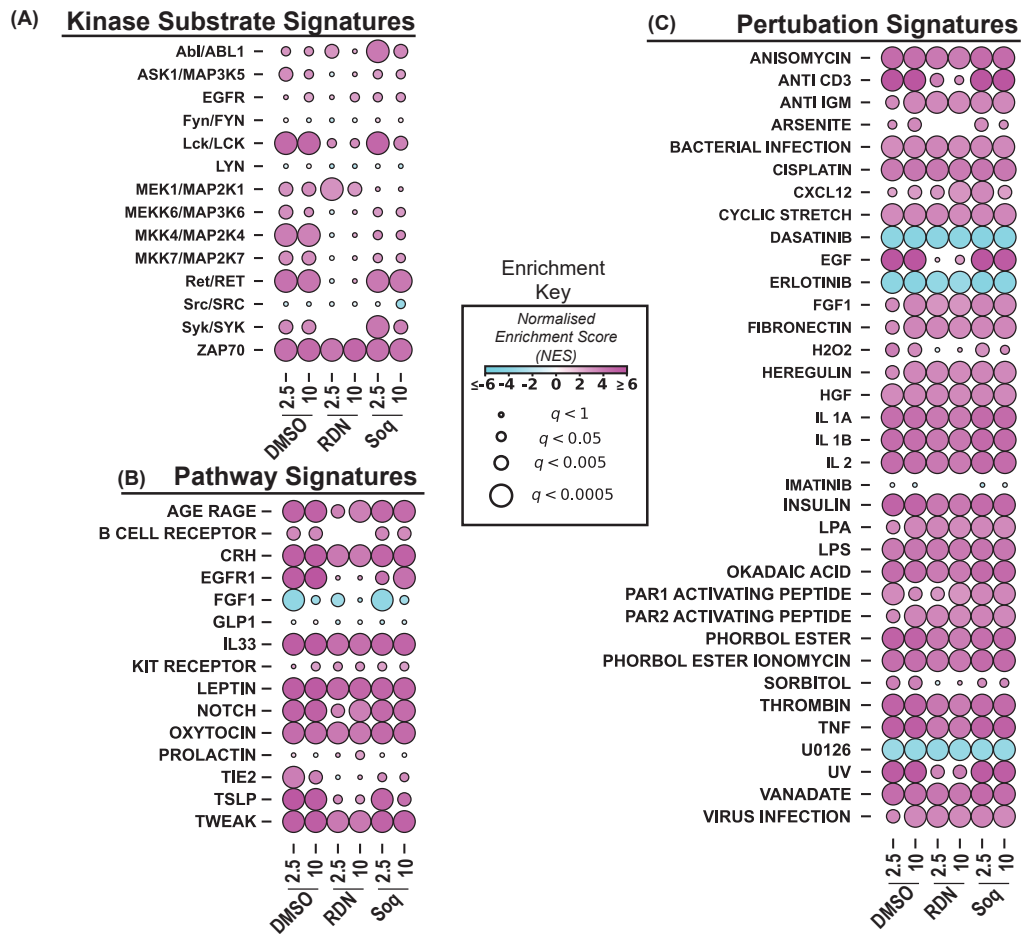


Figure 5.13: Post translational signature enrichment analysis (PTM-SEA) reveals pMHC/TCR stimulation induces pY signatures associated with increased kinase activity, signalling pathway engagement, and activating perturbations. (A) PTM-SEA on stimulation time course comparisons for PhosphoSitePlus Kinase Substrate Signatures. (B) PTM-SEA on stimulation time course comparisons for NetPath Pathway Signatures. (C) PTM-SEA on stimulation time course comparisons for PhosphoSitePlus Perturbation Signatures.

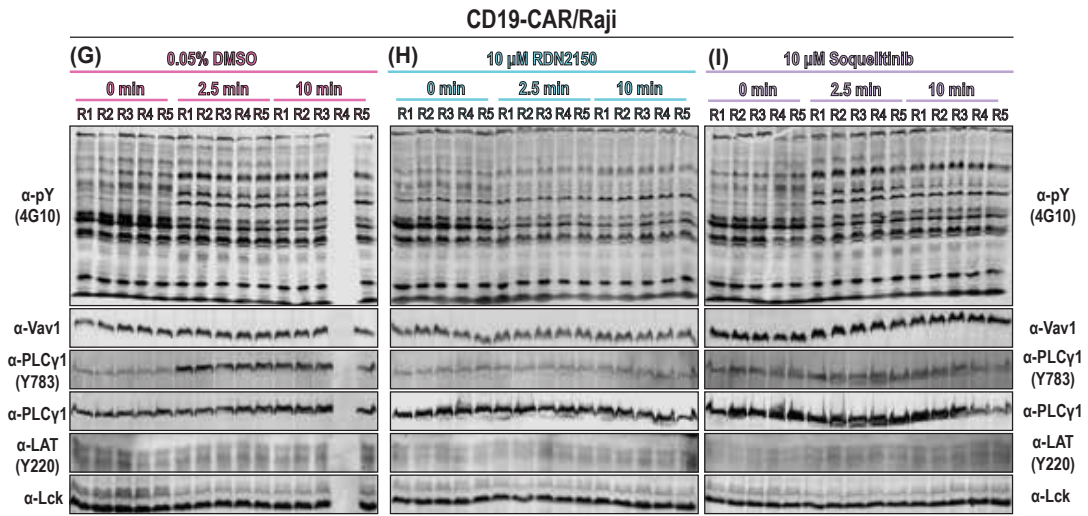


Figure 5.14: Western blot analysis of CAR/TAA stimulation pY proteomics samples targeting: phosphotyrosine (pY, clone 4G10), Vav1 (loading control), PLC γ 1^{Y783}/PLC γ 1, and LAT^{Y220}/Lck in (A) DMSO control samples, (B) 10 μ M RDN2150 samples, and (C) 10 μ M Soquelitinib samples.

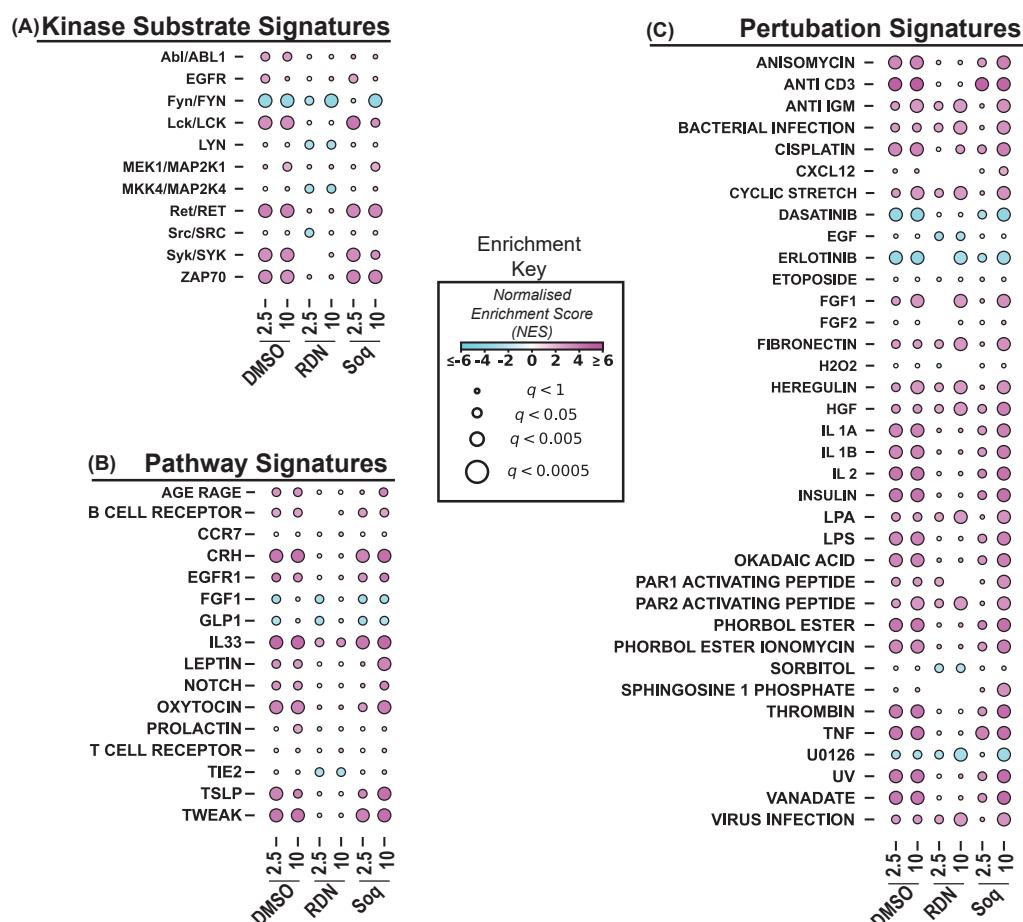


Figure 5.15: Post translational signature enrichment analysis (PTM-SEA) reveals CAR/TAA stimulation weakly engages pY signatures associated with increased kinase activity, signalling pathway engagement, and activating perturbations. (A) PTM-SEA on stimulation time course comparisons for PhosphoSitePlus Kinase Substrate Signatures. (B) PTM-SEA on stimulation time course comparisons for NetPath Pathway Signatures. (C) PTM-SEA on stimulation time course comparisons for PhosphoSitePlus Perturbation Signatures.

5.2.4 RDN treatment disrupts the TCR pathway more than Soq treatment

Given the ability to retain signalling capacity in the presence of each inhibitor, we sought to evaluate how inhibition affected pY-site abundance relative to the DMSO control. As expected, treatment with either 10 μ M RDN or 10 μ M Soq uniformly reduced pY abundance in soluble antibody-treated samples, with the majority of pY sites being significantly reduced at 0, 2.5, and 10 minutes of stimulation when compared to the DMSO control (Figures 5.16A, 5.10). pY sites along the TCR signalling pathway, including the Erk1/2 activation sites, Jnk2/3 activation sites, and multiple sites on PLC γ 1 and Zap70 were reduced during RDN treatment. Interestingly, 10 μ M RDN treatment increased Zap70^{Y493} abundance at the basal and 10-minute stimulation time points, whereas it reduced various TCR ITAM sites, such as TCR ζ ^{Y64}/TCR ζ ^{Y72} and CD3 ϵ ^{Y188} (Figure 5.16B). PTM-SEA showed downregulation of activating perturbation signatures like ‘ANTI CD3’ and ‘IL 2’ (Figure 4C), and upregulation of inhibitor signatures like ‘ERLOTINIB’ and ‘U0126’ (Figure 5.16D), suggesting that the phosphoproteomic signature of RDN and Soq mimicked that of pre-established inhibitors. Our results for RDN treatment in APC-pMHC/TCR and CD19-CAR/Raji stimulations were similar, showing that the majority of significantly changing pY sites were reduced (Figures 5.17A, 5.18A), those sites often were located on TCR signalling pathway proteins (Figures 5.17B, 5.18B), and that the PhosphositePlus Perturbation signatures and NetPath Pathway signatures were, in general, significantly downregulated during inhibitor treatment (Figures 5.17C-D, 5.18C-D). In contrast, pY site abundance changes during Soq treatment were more evenly distributed in APC-pMHC/TCR and CD19-CAR/Raji co-cultures, with very few TCR signalling proteins showing reduced pY site abundance (Figures 5.17, 5.18). Importantly, we consistently observed PLC γ 1^{Y783} to be significantly reduced relative to the DMSO control in APC-pMHC/TCR and CD19-CAR/Raji co-cultures (Figures 5.12, 5.14, 5.17, 5.18), indicating that Soq and RDN were present and active. Together, these data suggest that RDN, more than Soq, reduces pY abundance in T cells across stimulation methods.

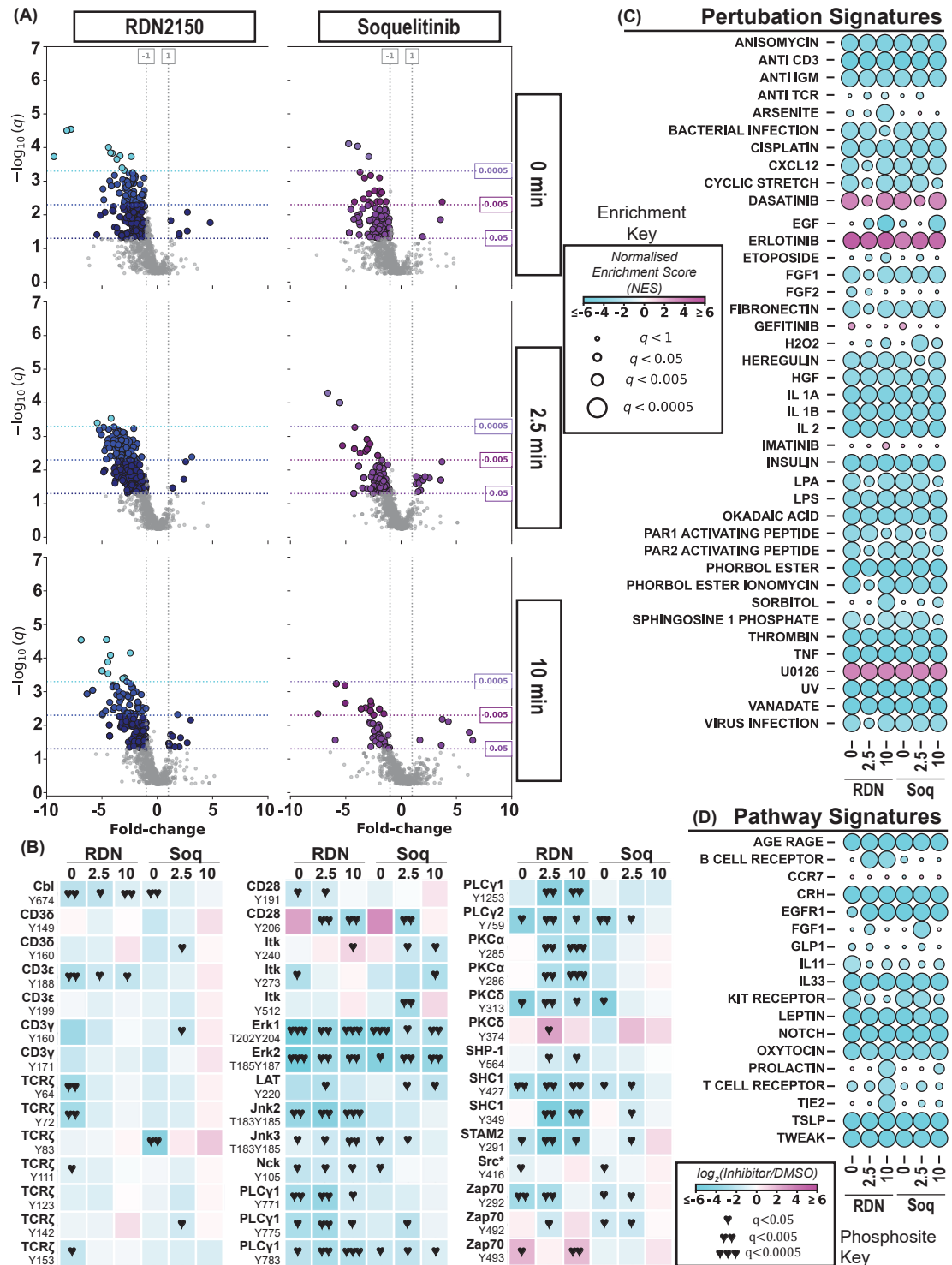


Figure 5.16: (Caption on next page)

Figure 5.16: 10 μ M RDN treatment downregulates global pY abundance and the TCR signalling pathway more than Soq treatment. (A) Volcano plot analysis of pY proteomics data comparing RDN and Soq treatment to a DMSO control in samples stimulated with soluble antibodies for 0 (top row), 2.5 (middle row), and 10 (bottom row) minutes. (B) Heat maps comparing RDN and Soq treatment to a DMSO control for specific pY sites in the T cell signalling pathway. ♥ indicates $q < 0.05$, and ♥♥ indicates $q < 0.005$, and ♥♥♥ indicates $q < 0.0005$. (C-D) PTM-SEA analysis of phosphoproteomic data showing PhosphoSitePlus Perturbation Signatures and NetPath Pathway Signatures, respectively. $n \geq 3$ measurements from independent experiments for each unique pY site per treatment group/time point/stimulation method.

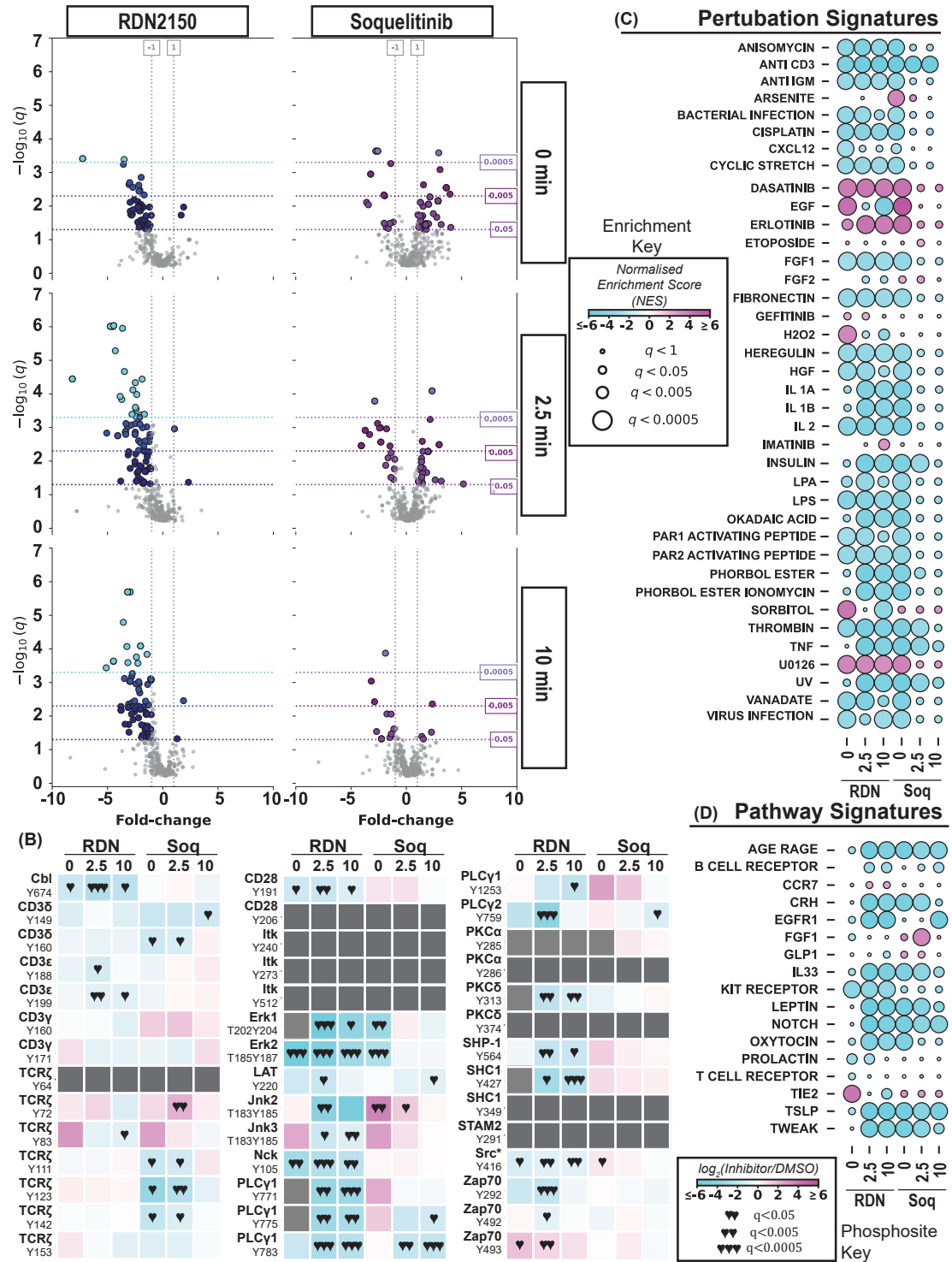


Figure 5.17: (Caption on next page)

Figure 5.17: 10 μ RDN2150 treatment downregulates global pY abundance and the TCR signalling pathway more than Soquelitinib treatment. (A) Volcano plot analysis of pY proteomics data comparing RDN2150 and Soquelitinib treatment to a DMSO control in pMHC/TCR stimulated samples for 0 (top row), 2.5 (middle row), and 10 (bottom row) minutes. (B) Heat maps comparing RDN2150 and Soquelitinib treatment to a DMSO control for specific pY sites in the T cell signalling pathway. ♥ indicates $q < 0.05$, and ♥♥ indicates $q < 0.005$, and ♥♥♥ indicates $q < 0.0005$. (C-D) Post-translational modification signature enrichment analysis (PTM-SEA) on DMSO comparison data showing PhosphoSitePlus Perturbation Signatures and NetPath Pathway Signatures, respectively.

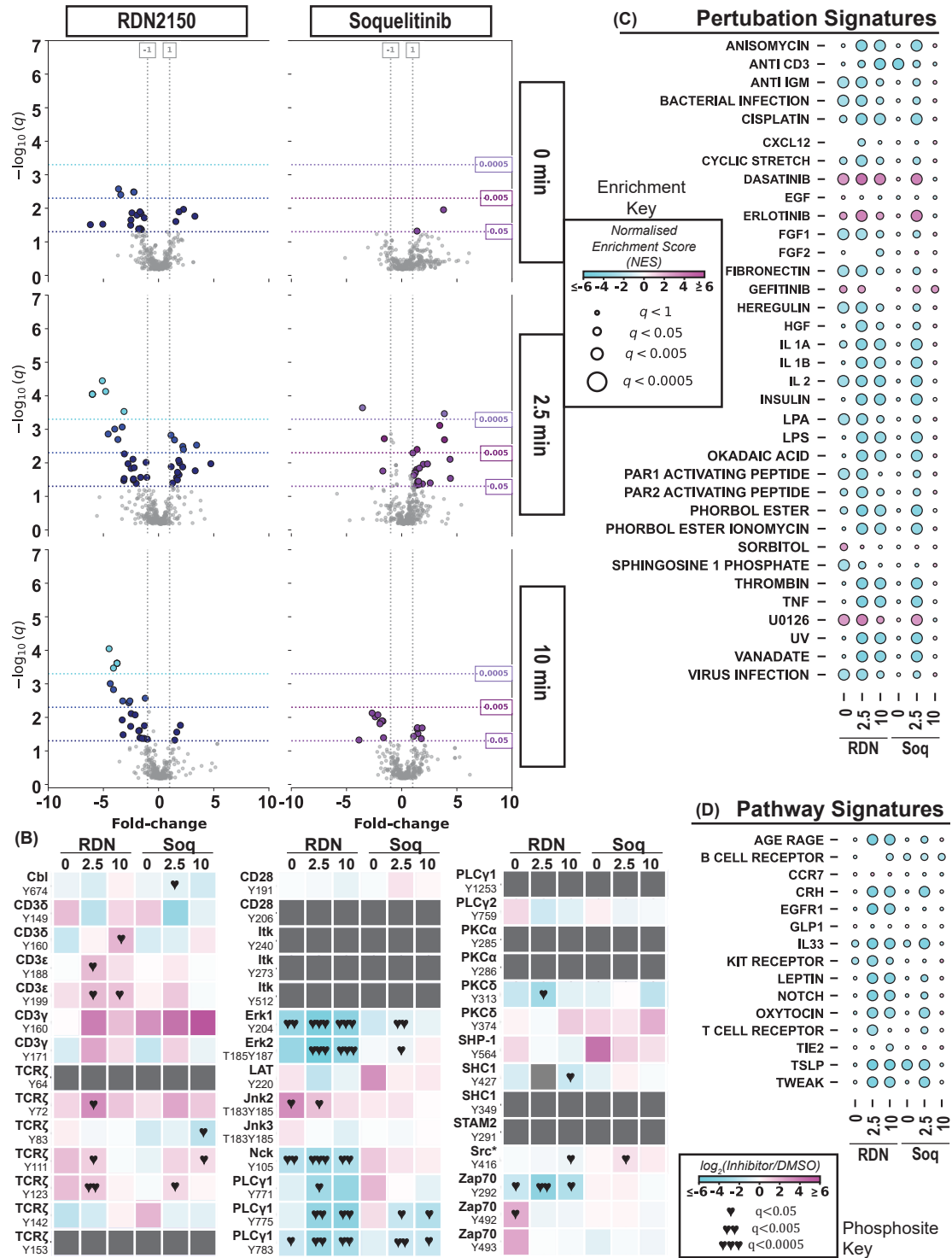


Figure 5.18: (Caption on next page)

Figure 5.18: RDN2150 treatment downregulates global pY abundance and the CAR signalling pathway more than Soquelitinib treatment. (A) Volcano plot analysis of pY proteomics data comparing RDN2150 and Soquelitinib treatment to a DMSO control in CAR/TAA stimulated samples for 0 (top row), 2.5 (middle row), and 10 (bottom row) minutes. (B) Heat maps comparing RDN2150 and Soquelitinib treatment to a DMSO control for specific pY sites in the T cell signalling pathway. ♥ indicates $q < 0.05$, and ♥♥ indicates $q < 0.005$, and ♥♥♥ indicates $q < 0.0005$. (C-D) Post-translational modification signature enrichment analysis (PTM-SEA) on DMSO comparison data showing PhosphoSitePlus Perturbation Signatures and NetPath Pathway Signatures, respectively.

5.2.5 Stimulation method-dependent effects on pY abundance during RDN and Soq treatment

Considering that RDN and Soq both significantly reduced pY abundance on T cell signalling proteins (Figures 5.16, 5.17, 5.18), yet had different effects on CD69 expression and IL-2 secretion (Figure 5.8), we sought to characterise the broader impact of RDN and Soq treatment. Treatment with RDN led to the greatest number of significant differentially expressed pY sites ($q < 0.05$ compared to DMSO control) at all time points in our soluble antibody stimulation model (219 vs 118, 277 vs 137, 174 vs 48 for 0-minute, 2.5-minute, and 10-minute, respectively). Most of those sites were unique to RDN treatment (Figure 5.19A-C). This trend held for APC-pMHC/TCR (Figures 5.19D-F) and CD19-CAR/Raji (Figures 5.19G-H) stimulations, particularly post-stimulation. Fewer than 50% of differential pY sites identified in RDN treatment were shared with Soq treatment (except at 0 minutes of antibody stimulation; Figures 5.19A-I). These data suggest that, despite similar trends of pY abundance in TCR signalling proteins compared with the DMSO control (Figures 5.16, 5.17, 5.18), RDN and Soq control unique subsets of pY sites in Jurkat T cells. In soluble antibody stimulation samples, we observed differential, non-canonical pY sites on key TCR signalling proteins, which were significantly reduced in both RDN and Soq, such as Lck^{Y470} and Fyb^{Y803}. Some TCR pY sites were significantly reduced only in RDN-treated samples, such as Cofilin-1^{Y85}, PAG^{Y317}, PAG^{Y387}, SHP-1^{Y536}, and Zap70^{Y164}, whereas others, including Itk^{Y588}, were significantly reduced during Soq treatment. Notably, we observed that pY sites on heterogeneous nuclear ribonucleoproteins (hnRNP) F^{Y82}, hnRNP K^{Y280}, and hnRNP H1^{Y82} were significantly reduced during RDN treatment. The hnRNP family of proteins is involved in stabilising pre-mRNA during mRNA maturation, and thus directly influences protein translation (Geuens et al., 2016). While the function of these pY sites is unknown, hnRNP F^{Y82} flanks a quasi-RNA recognition motif (RRM), hnRNP H1^{Y82} lays inside a qRRM, and hnRNP K^{Y280} is within an arginine-rich patch, which may be involved in phase separation of hnRNPs (Geuens et al., 2016; Martin et al., 2021; Lorton and Shechter, 2019; Moritz et al., 2014; Ryan et al., 2018). Similarly, many non-canonical pY sites on TCR signalling proteins were reduced more in RDN treatment than Soq

treatment during APC-pMHC/TCR stimulation (Figure 5.19K), and some pY sites were even increased in Soq treatment compared to the DMSO control, including Lck^{Y470}, Zap70^{Y164}, and Cofilin-1^{Y68}. Notably, the pY sites hnRNP 2H9^{Y296}, hnRNP M^{Y64}, and hnRNP A2/B1^{Y174} were significantly reduced by RDN treatment in APC-pMHC/TCR treatment. However, hnRNP K^{Y280} and hnRNP 2H9^{Y296} were significantly increased during Soq treatment (Figure 5.19K). While the trends for RDN treatment reducing non-canonical pY sites on signalling proteins held for CD19-CAR/Raji co-culture, we observed an elevation of hnRNP M^{Y64} in RDN treatment and hnRNP K^{Y280} (2.5 min), hnRNP M^{Y64} (2.5 min), and hnRNP C1/C2^{Y137} (0 min) during Soq treatment (Figure 5.19L). Altogether, our data confirm that Zap70 is the major regulator of main-line T cell signalling and is necessary for CAR signalling. Zap70 and Itk control unique, stimulation-dependent branches of the non-canonical T cell pY proteome.

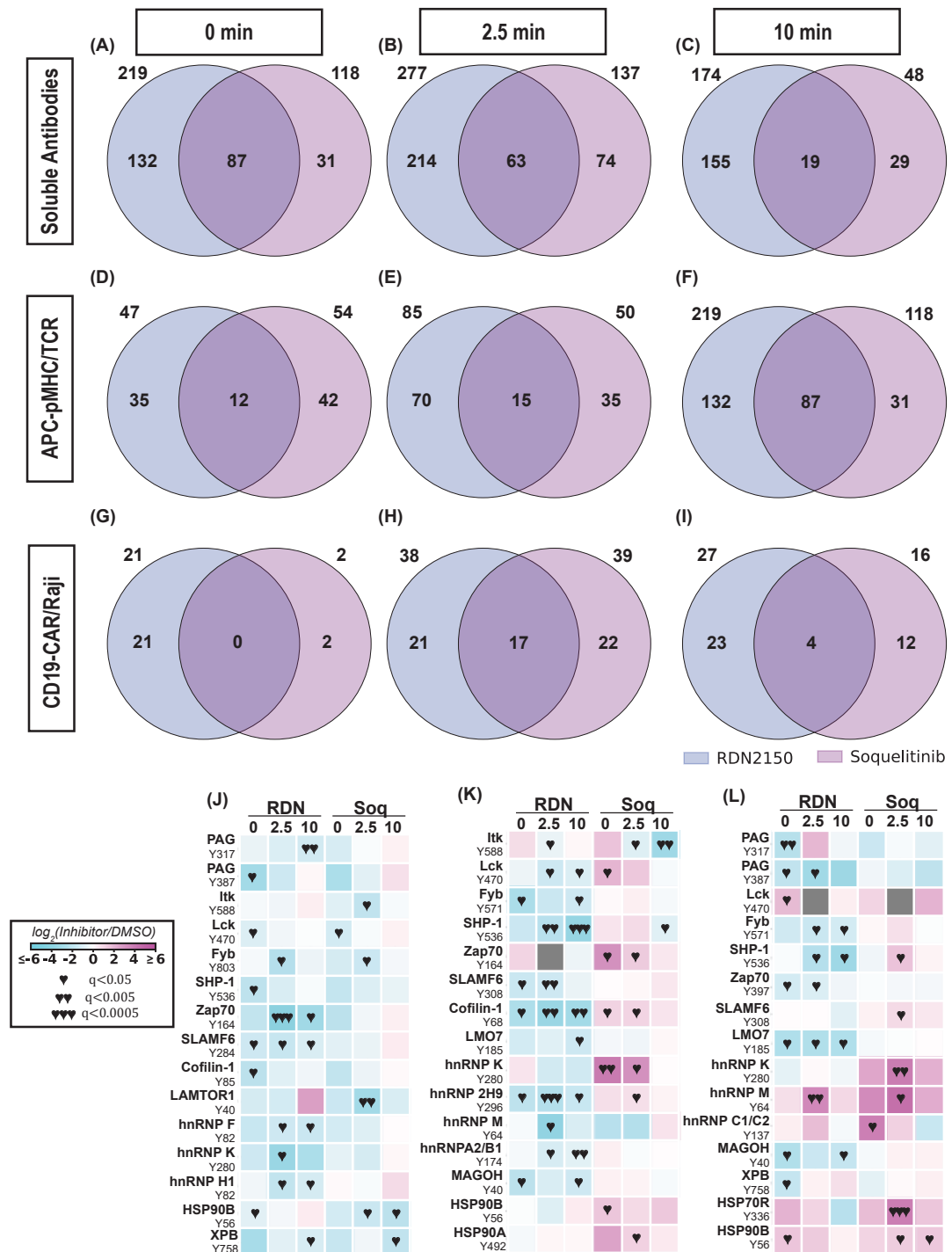


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Figure 5.19: Treatment with Soq increases pY abundance of non-canonical sites in response to APC-pMHC/TCR and CD19-CAR/Raji stimulation. (A-C) Venn diagrams showing overlap of unique pY sites observed as significantly changing ($q < 0.05$) compared to DMSO for soluble antibody stimulated samples at 0 minutes, 2.5 minutes, and 10 minutes, respectively. (D-F) As in A-C, except for APC-pMHC/TCR stimulated samples. (G-I) As in A-C, except for CD19-CAR/Raji stimulated samples. (J-L) Heat maps comparing RDN and Soq treatment to a DMSO control for specific, non-canonical pY sites in antibody, APC-pMHC/TCR, and CD19-CAR/Raji stimulation, respectively. ♥ indicates $q < 0.05$, and ♥♥ indicates $q < 0.005$, and ♥♥♥ indicates $q < 0.0005$. $n \geq 3$ measurements from independent experiments for each unique pY site per treatment group/time point/stimulation method.

5.3 Discussion

Activation via the T cell receptor and, more recently, chimeric antigen receptors has been extensively studied in Jurkat cells and primary T cells. Although Jurkat T cells are known to be deficient in PTEN, resulting in constitutive Itk localisation to the membrane(Shan et al., 2000), and show genetic instability across research groups(Wilson et al., 2025), researchers use Jurkat T cells because they show relatively consistent activation/deactivation within a research group and are amenable to genetic modification. Consequently, many fundamental aspects of T cell signalling have translated from Jurkat T cells to primary cells. Using Jurkats and primary T cells, researchers demonstrate that the basic TCR/CAR pathway involves Lck/Fyn-mediated phosphorylation of ITAMs on CD3 and/or TCR ζ , Zap70 phosphorylation, and activation. Subsequently, the production of a signalosome, including LAT/SLP76 in traditional T cells or CAR/SLP76 in CAR T cells, leads to signal diversification(Gaud et al., 2018; Lindner et al., 2020). However, while T and CAR T cell signalling is well studied, many integral aspects of TCR/CAR signalling remain elusive. For example, researchers have only recently decoded the role of Zap70 in the kinetic gatekeeping of T cell activation(Voisinne et al., 2022; Lo et al., 2019) how Lck and Zap70 coordinate to promote LAT phosphorylation(Lo et al., 2018), and how the Itk substrate site GADS^{Y45} integrates CD28 costimulation with TCR stimulation(Hallumi et al., 2021). Similarly, recent construction of optimized CARs that include unintuitive intracellular domains identified through high-throughput screens in Jurkat cells(Goodman et al., 2022; Gordon et al., 2025, 2022; Kang et al., 2020; Rydzek et al., 2019; Zhang et al., 2023a) and point mutations in CARs that can improve function(Salter et al., 2018, 2021; Boucher et al., 2021) are the first steps toward bringing this promising immunotherapy to more patients. Notably, signalling studies of CARs and TCRs are often confounded by the choice of stimulation method (e.g., antibodies, reconstituted MHCs, cell-to-cell contact), making results difficult to compare and impossible to determine stimulation-method-dependent effects on activation. Together, while we have made significant advances in understanding TCR and CAR signalling, our fundamental understanding of the processes underlying T cell activation through these receptors remains limited.

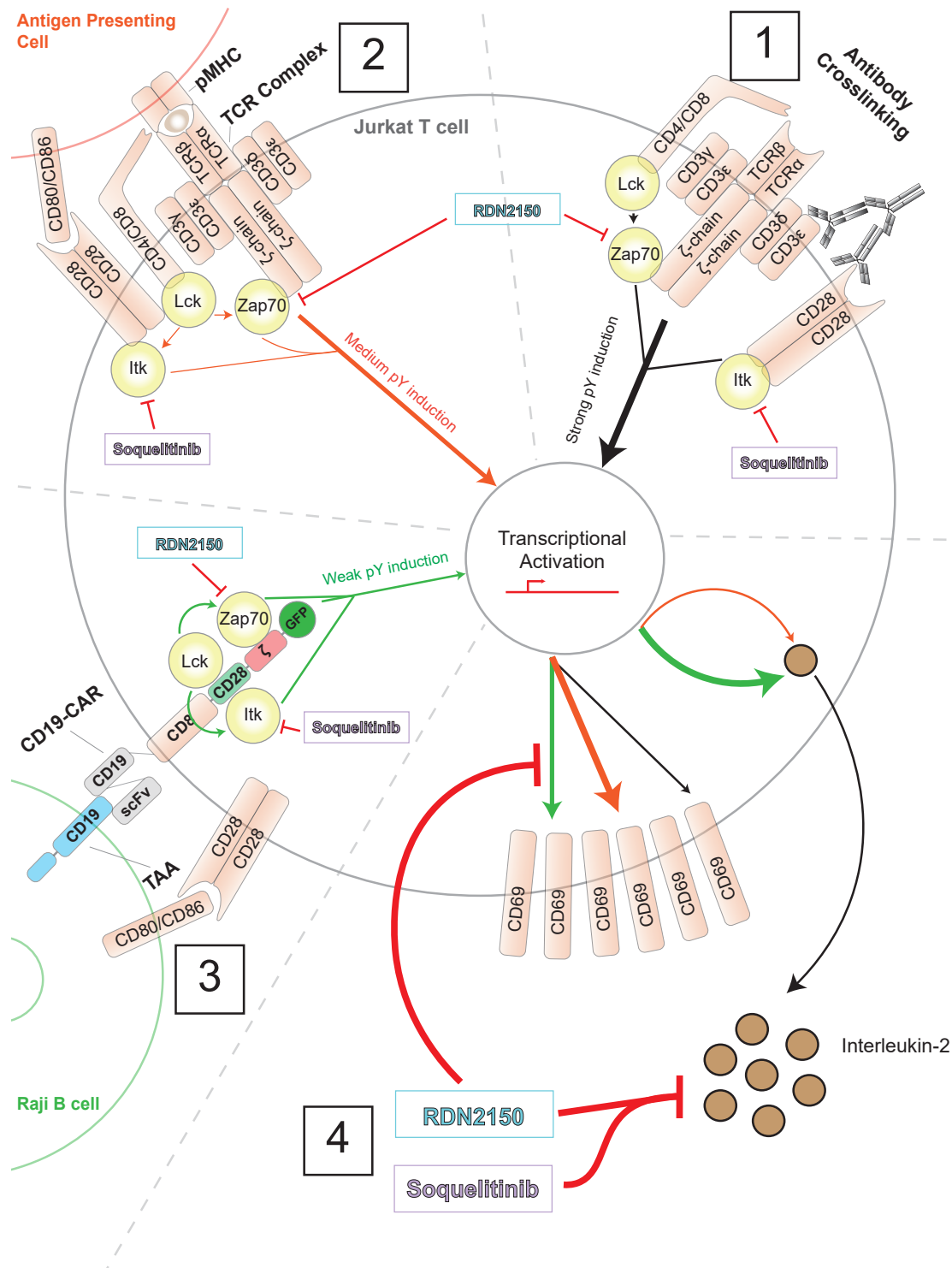


Figure 5.20: (Caption on next page)

Figure 5.20: pY induction and the T cell activation markers CD69/IL-2 are inversely related. [Part 1] Soluble antibody crosslinking of CD3 ϵ and CD28 induces very strong pY signalling down the T cell signalling cascade. Treatment with RDN or Soq reduces pY abundance at 0, 2.5, and 10 minutes post-stimulation (Figures 5.1, 5.9, 5.16); however, pY signalling remains induced. [Part 2] CD19-CAR/Raji binding through co-culture induces TCR signalling to a milder degree than antibody crosslinking, and RDN largely impairs pY induction (Figure 5.1, 5.16). [Part 3] CD19-CAR/Raji binding induces much weaker pY signalling than either antibody crosslinking or APC-pMHC/TCR binding. Treatment with RDN blunts pY signalling entirely (Figures 5.1, 5.16). [Part 4] APC-pMHC/TCR and CD19-CAR/Raji co-culture induce high CD69 expression, whereas soluble antibody crosslinking does not, and soluble antibody crosslinking is unable to induce IL-2 production (Figures 5.8A, 5.8C, 5.8E). RDN, but not Soq, blocks CD69 expression; however, both RDN and Soq block IL-2 production (Figure 5.8).

Here, we leveraged the sensitivity of mass spectrometry-based phosphotyrosine proteomics to determine the roles of Zap70 and Itk kinase activity in Jurkat T and CAR Jurkat T cell activation using two novel T cell inhibitors. The first, RDN, was described by Rao et al. (2023) and shown to covalently modify Zap70^{C346}, which lies at the N-terminus of Zap70's catalytic domain. Rao et al. showed that RDN suppressed *in vitro* kinase activity more than 98% at concentrations of 1 μ M, suppressed LAT^{Y220} phosphorylation in Jurkats at concentrations as low as 400 nM with 48 hours of CD3 stimulation (albeit without replicates), and suppressed CD69, CD25, IFN- γ , and IL-17A production at concentrations between 100-400 nM in primary murine CD4+ T cells (Rao et al., 2023). While our functional activation data agree, showing that 100-1000 nM is effective for suppressing CD69 and IL-2 production (Figure 5.8), we found no significant decrease in LAT^{Y220} phosphorylation until 10 μ M RDN after less than 20 minutes of CD3/CD28 crosslinking (Figure 5.1), the biologically relevant timescale for LAT phosphorylation. Furthermore, we found that treatment with low concentrations of RDN could significantly enhance PLC γ 1Y783 induction after soluble antibody stimulation (Figure 5.1C) and Erk1^{T202Y204}/Erk2^{T185Y187} induction after any stimulation method (Figures 5.1B, 5.8, 5.9, 5.16). These data are consistent with previous findings showing that the kinase domain of Zap70 mediates basal signalling and negative feedback on the TCR to ensure proper T cell signalling and activation (Sjölin-Goodfellow et al., 2015). With a treatment of 10 μ M RDN, our pY proteomics data showed that the majority of pY sites were significantly decreased when compared to a DMSO control, many of which are on integral T cell proteins like Cbl, CD28, Erk1, Erk2, LAT, Jnk2, Jnk3, Nck, PLC γ 1, PKC α , PKC δ , SHP-1, SHC1, and STAM2. Further, the molecular signatures in the data resembled that of other kinase inhibitors and showed significant downregulation of signatures associated with cell signalling pathways (Figure 5.16). Despite this, soluble antibody stimulation induced widespread pY in the presence of 10 μ M RDN. In contrast, stimulation with APC-pMHC/TCR or CD19-CAR/Raji co-culture appeared muted in the presence or absence of RDN (Figure 5.9). The residual pathway stimulation observed in the presence of either Soq or RDN (Figure 5.9) could reflect partial inhibition of these kinases at the concentrations tested or the activation of compensatory pathways via parallel kinases such as Lck or Fyn. Together, these data in Jurkat cells support a model

by which Zap70 is a primary driver of pY signalling from the TCR, is more effective in systems with weaker pY induction, and inhibits the production of activation markers even when insufficient to block pY signalling completely (Figure 5.20), in line with the current model where Zap70 acts as a kinetic proofreader of TCR signalling.

Next, we evaluated the Itk inhibitor Soquelitinib (Soq; formerly CPI-818), developed by Corvus Pharmaceuticals and in clinical trials for T cell lymphoma treatment (Corvus, 2023). Recently, Hsu et al. (2024) described the synthesis and characterisation of Soq, which covalently modifies Itk^{C442}, a residue at the ♥ of the Itk kinase domain. Hyu et al. showed that Itk prevented CD3ε-induced PLCγ1^{Y783} phosphorylation at concentrations as low as 100 nM, impaired IL-2 production at 0.1-1 μM, significantly decreased primary cell viability at concentrations greater than 1 μM, and skewed T cell differentiation towards a Th1 phenotype (Hsu et al., 2024). Our data largely agreed with Hsu et al., showing that low concentrations of Soq impaired PLCγ1^{Y783} and Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation in response to CD3ε/CD28, TCR, and CAR stimulation (Figures 5.1, 5.4, 5.5, 5.6), as well as IL-2 production (Figure 5.8). Interestingly, we found that with APC-pMHC/TCR and CD19-CAR/Raji stimulation, all tested concentrations of Soq were insufficient to reduce activation-induced CD69 expression, whereas under soluble-antibody stimulation, it did significantly reduce CD69 upregulation. (Figure 5.8). Previous literature has shown that the kinase activity of Itk is important for IL-2 production, although the mechanism by which Itk controls IL-2 production is unclear⁵⁸, and that CD69 expression is an ‘all or nothing’ threshold that depends on ligand strength (von Bonin et al., 2011). Thus, while our data in Jurkat cells showed that Itk is involved in CD69 production, Itk was less critical than Zap70, which was necessary for both CD69 and IL-2 production (Figure 5.8). Using a treatment of 10 μM Soq, we found that global pY abundance decreased compared to a DMSO control; however, pY sites along the T cell signalling pathway did not appear to be heavily affected. Notably, PLCγ1^{Y783} and Erk1^{T202Y204}/Erk2^{T185Y187} were reduced, in line with our titration Western blot data, and some pY sites on Zap70 were significantly reduced. The molecular signatures associated with Soq treatment reflected its inhibitory activity (Figure 5.16). In the context of pY induction, Soq appeared to have little effect, regardless of the stimulation method (Figure 5.9A). Our data support the previously established

model of Itk function, whereby Itk acts as a rheostat to tune T cell activation, with control over the IL-2 production pathway (Figure 5.20).

Lastly, our data provide a comprehensive comparison of T cell activation methods frequently used in the literature, including soluble antibody stimulation of the TCR, APC-pMHC/TCR co-culture, and CD19-CAR/Raji co-culture. We found that fold induction of key TCR signalling pY sites by Western blot differed between soluble antibody, APC-pMHC/TCR, and CD19-CAR/Raji stimulations (Figure 5.1), and this difference did not correlate with CD69 expression or IL-2 secretion (Figure 5.8). We found that soluble antibody stimulation led to a large increase in tyrosine phosphorylation that was not seen in either APC-pMHC/TCR or CD19-CAR/Raji stimulations (Figures 5.1, 5.9), however, soluble antibody stimulation was unable to sufficiently promote CD69 expression or IL-2 secretion compared to APC-pMHC/TCR or CD19-CAR/Raji co-culture. While RDN and Soq reduced pY abundance on T cell signalling proteins irrespective of stimulation method (Figure 5.16), they had unique effects on non-canonical TCR pY sites. In particular, RDN treatment reduced phosphorylation of multiple heterogeneous nuclear ribonucleoproteins (hnRNPs) independent of stimulation method; however, Soq treatment increased pY abundance on multiple hnRNP pY sites in APC-pMHC/TCR and CD19-CAR/Raji co-culture experiments. While many of these pY sites are uncharacterised, hnRNPs play a significant role in T cell physiology. In particular, Chang et al. (2009) showed that hnRNP K was a direct pathway target of the TCR, was required for effective IL-2 production, and promoted Vav1 proteolysis (Chang et al., 2009). Meininger et al. (2016) showed that hnRNP U suppressed exon 7 inclusion in *MALT1* mRNA splicing, thereby regulating *MALT1A* expression and T cell activation (Meininger et al., 2016). White et al. (2025) showed that hnRNP L controlled differentiation of peripheral T cells, particularly into T follicular helper cells (White et al., 2025). Finally, Shankarling et al. (2014) used crosslinking and immunoprecipitation sequencing to identify alternative splicing events of TCR signalling mRNAs in response to TCR signalling (Shankarling et al., 2014). Although the signalling pathway linking T cell signalling and hnRNP regulation remains unclear in many cases, our data suggest that Zap70 and Itk play stimulation-dependent roles on the abundance of hnRNP pY sites.

Here, we provide a thorough, site-specific resource on the activation pathway of Jurkat T and CAR T cells in the presence of two T cell inhibitors, RDN and Soq. We confirmed that Zap70 is the dominant regulator of TCR signal initiation and T cell activation, while Itk selectively regulates IL-2 induction through a more limited branch of T cell signalling. While our studies directly inform the interpretation of high-throughput Jurkat CAR engineering screens, future targeted studies with primary human T cells will define the translational potential of these findings to improve CAR T therapies and identify the possible benefits of combination therapies.

5.4 Materials and Methods

5.4.1 Materials

For flow cytometry, the following antibodies were used: α -CD69 APC (20 μ L/2e6 T cells; BD Pharmingen #560967), α -CD45 (0.06 μ g/2e6 cells; Thermo Fisher Scientific #17-0459-42) α -CD3 ϵ PE (20 μ L/2e6 T cells; BD Pharmingen #561808), α -TCR $\alpha\beta$ PE (20 μ L/2e6 T cells; BD Pharmingen #561674), α -CD28 PE (0.25 μ g/2e6 T cells; Thermo Fisher Scientific #12-0289-41), α -CD80 (0.5 μ g/2e6 Raji or T2-K^b cells; Thermo Fisher Scientific #12-0809-42), α -CD86 (0.5 μ g/2e6 Raji or T2-K^b cells; Thermo Fisher Scientific #12-0869-41). For Western blotting, the following antibodies were used: α -phospho-PLC γ 1^{Y783} (1:2000; Cell Signaling Technologies #2821), α -phospho-LAT^{Y220} (1:2000; Cell Signaling Technologies #3584), α -phospho-SFK^{Y416} (1:2000; Cell Signaling Technologies #2101), α -phospho-Erk1^{T202Y204}/Erk2^{T185Y187} (1:2000; Cell Signaling Technologies #9101), α -PLC γ 1 (1:10000; Millipore Sigma #05-163), α -LAT (1:1000; Thermo Fisher Scientific #MA1-19307), α -Lck (1:5000; Cell Signaling Technologies #2657), α -Erk1 (1:2000; Cell Signaling Technologies #9107), IRDye 680RD Goat α -Mouse IgG (1:20000; LI-COR #926-68070), IRDye 800CW Goat α -Mouse IgG (1:20000; LI-COR #926-33210), IRDye 680RD Donkey α -Rabbit IgG (1:20000; LI-COR #926-68073), IRDye 800CW Donkey α -Rabbit IgG (1:20000; LI-COR #926-32213)

5.4.2 Cell culture and SILAC labelling

Jurkat T cells (clone E6.1), J^{OT1} (Jurkat T cells clone E6.1 expressing murine OT1 TCR, human CD8, and cytosolic GFP; gift from Dr. Arthur Weiss), 28 ζ -CAR (Jurkat T cells clone E6.1 expressing a FMC63 scFv, CD8 hinge/transmembrane, CD28 costimulatory domain, TCR ζ stimulation domain containing CAR with a terminal superfold GFP tag. In the J^{OT1} system, CD8/MHC recognition is present because the human CD8 expressed by J^{OT1} functionally engages murine MHC class I (H-2Kb) on T2-K^b antigen-presenting cells^{50,60}. Characterised in Callahan et al. (2025)), T2-K^b cells (T2 cells expressing the Kb class 1 MHC; gift from Dr. Arthur Weiss), and Raji B cells (gift from Dr. Xiaolei Su), were maintained in RPMI 1640 supplemented with 10% FBS (Peak Serum #PS-FB3 Lot 01Z2233), 1X penicillin streptomycin glutamine (Cytiva #SV30082.01), and 2.5 μ g/mL plasmocin (InvivoGen #ant-mpp) in a humidified incubator at 37 C, 5% CO₂. For co-culture phosphoproteomics, Jurkat T cell derivatives were grown initially in supplemented RPMI 1640 as described above, before transitioning to SILAC RPMI (Thermo #A33823) supplemented with 10% dialysed FBS, 1X penicillin streptomycin glutamine, and 2.5 μ g/mL plasmocin, 0.38 mM ¹³C₆ ¹⁵N₄ Arginine (Millipore #608033), and 0.22 mM ¹³C₆ ¹⁵N₂ Lysine (Cambridge Isotopes #CNLM-291-H-1) for at least 7 days, as previously described(Griffith et al., 2022; Callahan et al., 2025c).

5.4.3 Inhibitor treatment

For inhibitor treatment experiments, Jurkat T cells were collected by centrifugation at 400 xg for 5 minutes, washed once in unsupplemented RPMI 1640 (or SILAC RPMI 1640 for phosphoproteomics), then resuspended at a concentration of 1x10⁷ cells/mL in RPMI 1640 supplemented with the indicated concentration of RDN (MedChemExpress #HY-155978), Soq (MedChemExpress #HY-150298), or DMSO and were allowed to incubate at 37 C, 5% CO₂ in a humidified incubator for 3 hours. After inhibitor treatment, the cells were collected and washed using 1X DPBS supplemented with inhibitor, then resuspended at a concentration of 2x10⁸ cells/mL for stimulation.

5.4.4 T cell receptor/Chimeric Antigen Receptor stimulation

Soluble antibody stimulation of the T cell receptor was performed as previously described(Callahan et al., 2025a). Briefly, Jurkat T cells were suspended in RPMI 1640 at a concentration of 2×10^8 cells/mL (1×10^8 cells for pY proteomics, 1×10^7 cells for Western samples), then allowed to rest at 37 C for 30 minutes. T cell receptor stimulation was initiated by addition of a stimulating antibody solution (25 μ L for Western, 250 μ L for pY proteomics) containing α -CD3 ϵ (Thermo #16-0037-85, clone OKT3; 2 μ g/mL final concentration) and α -CD28 (Thermo #16-0288-81, clone CD28.6; 2 μ g/mL final concentration) for 30 seconds, followed by addition of α -Mouse IgG (Jackson ImmunoResearch #115-005-062, 22 μ g/mL final concentration, 25 μ L for Western, 250 μ L for pY proteomics). Stimulation was stopped by the addition of 9 M Urea Lysis Buffer (9 M Urea, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM β -glycerophosphate in 20 mM HEPES) at the indicated time point.

Co-culture stimulation of Jurkats expressing the OT1 TCR was performed by loading T2-K^b cells with OVA²⁵⁷⁻²⁶⁴ (SIINFEKL; Rockland #000-001-M45) by incubation T2-K^b cells in plain RPMI 1640 supplemented with 1 μ M OVA²⁵⁷⁻²⁶⁴ peptide for 3 hours, then washing with 1X DPBS and resuspending at 200,000,000 cells/mL (1×10^8 cells for pY proteomics, 1×10^7 cells for Western samples). Stimulation was initiated by the 1:1 (v/v) addition of OVA²⁵⁷⁻²⁶⁴ loaded T2-K^b cells to J^{OT1} cells (1×10^8 cells for pY proteomics, 1×10^7 cells for Western samples) followed by a 30-second centrifugation at 500 xg to initiate cell-to-cell contact. Stimulation was stopped at the indicated time points by addition of 9 M Urea lysis buffer. Co-culture stimulation of 28 ζ -CAR T cells was performed as previously described(Callahan et al., 2025b,c) by 1:1 addition of Raji B cells to 28 ζ -CAR T cells (1×10^8 of each cell for pY proteomics, 1×10^7 of each cell for Western samples), centrifugation at 500 xg for 30 seconds, and lysis with 9 M Urea Lysis Buffer. Samples for Western blotting were then sonicated and diluted 1:1 in 2x Laemmli sample buffer, as previously described(Callahan et al., 2025a).

5.4.5 Flow cytometry

Assessment of GFP or surface expression using flow cytometry was performed by collecting 2×10^6 cells by centrifugation (400 xg, 5 min) from log-phase growth, resuspending cells in 100 μ L of ice cold flow buffer (2% FBS in 1x DPBS), then treating cells with fluor-conjugated antibodies for 30 minutes at 4 °C (or no antibody for GFP samples). Cells were washed 3 times with 1 mL of ice-cold flow buffer (centrifugation at 400 xg, 5 min), then resuspended in 100 μ L of ice-cold flow buffer. Cells were fixed via addition of 100 μ L of ice cold 4% paraformaldehyde and incubated at room temperature in the dark for 15 minutes. After fixing, cells were washed 3 times in 1 mL of ice-cold flow buffer (centrifugation at 1000 xg, 2 min). After the final wash, cells were resuspended in 100 μ L of ice-cold flow buffer and stored at 4 °C in the dark until acquisition on a Cytex Aurora flow cytometer. Output files were analysed using Floreada.io, a free online tool for flow cytometry analysis, as previously described(Callahan et al., 2025b).

T cell activation was assessed by measuring surface expression of CD69 after TCR stimulation, as previously described(Callahan et al., 2025b). Briefly, cells were treated with the inhibitor as described in the “Inhibitor treatment” section, except for one hour, then activated as described in the “T cell receptor/Chimeric Antigen Receptor stimulation” section using soluble antibodies or PFA fixed target cells, then incubated at 37 °C, 5% CO₂ in 1.5 mL of complete RPMI containing inhibitor in a 6-well plate at a concentration of 7×10^5 cells/mL in a humidified incubator for 24 hours. After 24 hours, about 1×10^6 T cells (2×10^6 total cells) were collected by centrifugation at 400 xg for 5 minutes, then resuspended in flow cytometry buffer and treated with APC-conjugated α -CD69 antibody for 30 minutes at 4 °C. After flow antibody binding, cells were washed 3 times in 1 mL flow cytometry buffer, fixed with 2% PFA for 15 minutes in the dark, then washed 3 times in 1 mL flow cytometry buffer before analysis on a Cytex Aurora flow cytometer. Output files were analysed using Floreada.io. T cells were determined by first gating on high GFP expression, then determining CD69 positivity (Figure 5.21). Statistical analysis was performed by first using an ANOVA to determine if any groups were significantly different, then using Fisher’s least significant difference with the Holm-Sidak FWER correction to determine which groups were significantly different. Samples

represent individual stimulation experiments, and each sample was only measured once. The cell culture supernatant from these experiments was saved and assessed for IL-2 by ELISA (section 5.4.7).

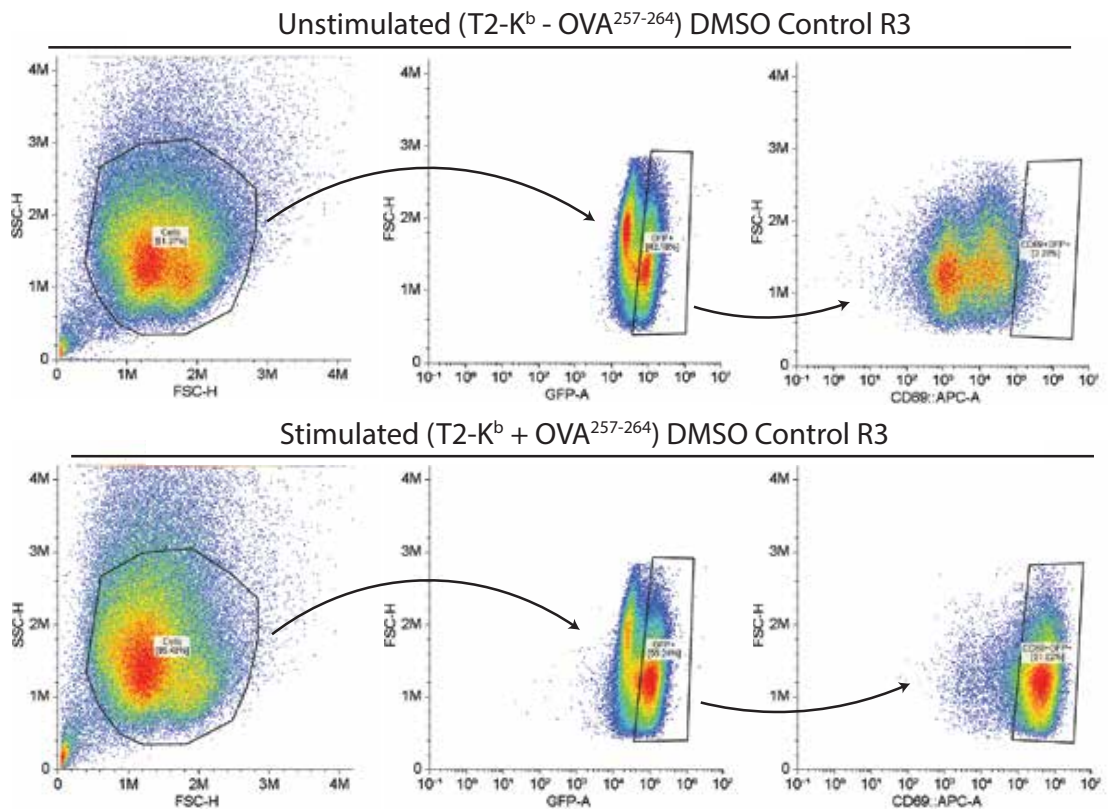


Figure 5.21: Gating scheme for co-culture flow cytometry analysis. First, the bulk cell population is selected. Then, the GFP high population (T cells) is selected, and finally the CD69 positive population is selected. Unstimulated and stimulated DMSO control are shown separately as an example, however all samples for one stimulation method were gated simultaneously in the actual analysis.

5.4.6 Western blotting

Western blotting was performed as previously described(Callahan et al., 2025a,b; Griffith et al., 2022; Callahan et al., 2025c). Briefly, 15 μ L of lysates were separated on 10% polyacrylamide gels poured in house at 100 V for 120 minutes, then transferred to PVDF membranes (Millipore #IPFL00010) at 100 V for 100 minutes in a Wet Transfer apparatus using Tris-Glycine running and transfer buffers. Membranes were blocked in Intercept TBS Blocking Buffer (LI-COR #927-60003) for 1 hour at room temperature then incubated with primary antibodies diluted in 5% BSA in 1x TBST overnight at 4 °C. Primary antibodies solution was removed, the membranes were washed three times with 1x TBST, then incubated with secondary antibodies diluted in 5% BSA in 1x TBST for 1 hour at room temperature. After secondary incubation, the membranes were washed three times with 1x TBST, once with 1x TBS, then imaged on a LI-COR OdysseyM imager. Quantification of Western blots was performed as previously described(Griffith et al., 2022), by first correcting the signal in the phosphorylation channel using the total protein as a control, then calculating ‘Fold-change’ by dividing all corrected intensity values by the mean of the 0 minute samples for that inhibitor treatment group. Therefore, all treatment groups start at a fold change of 0, and the observed changes are relative to the basal state for each group. Statistical analysis was performed as described in the “Flow cytometry” section. Samples represent individual stimulation experiments, and each sample was only measured once. The complete uncropped Western blots for Figures 5.1B, 5.1E, and 5.1H are provided as Figures 5.22-5.24.

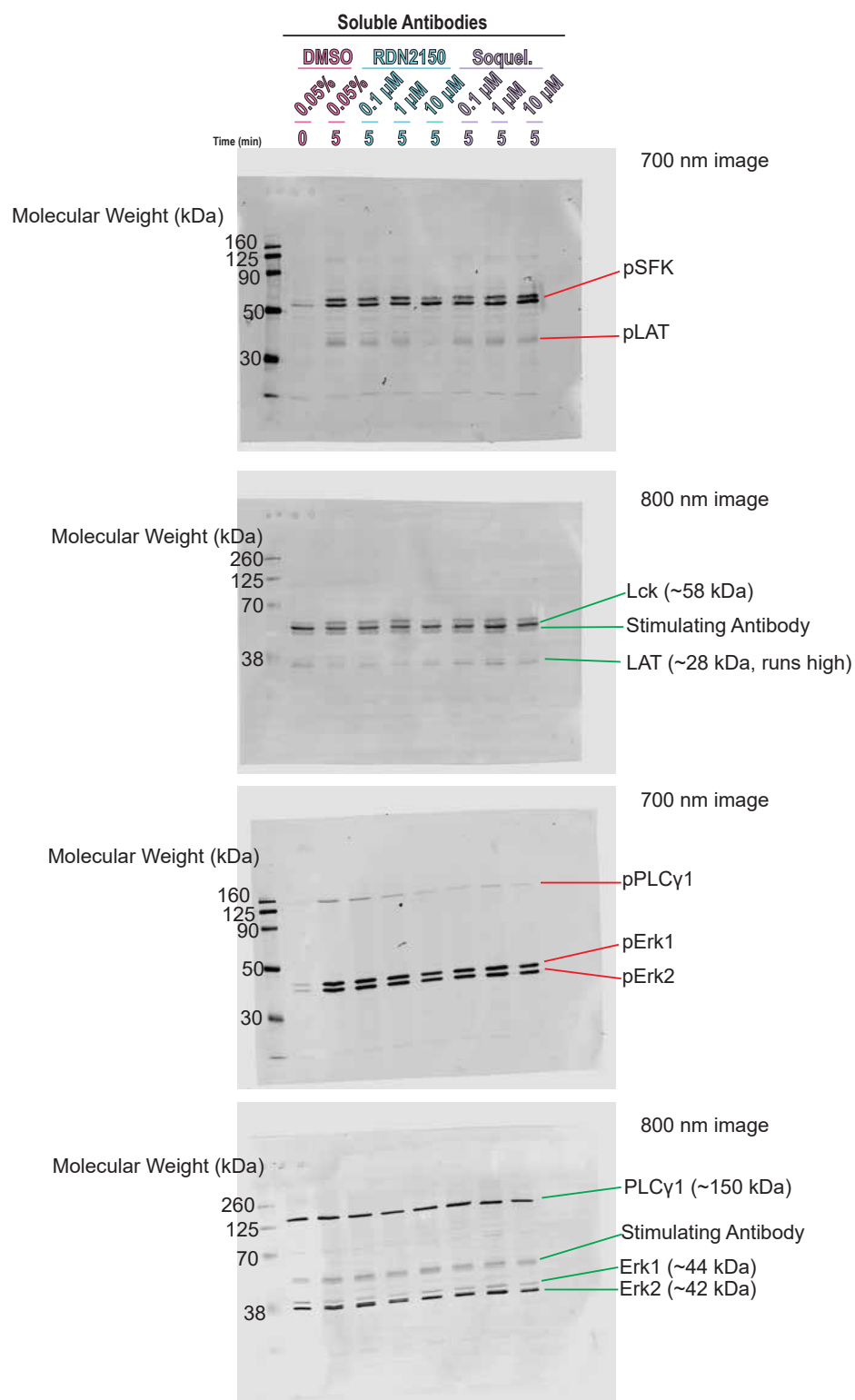


Figure 5.22: Uncropped, annotated Western blots for Figure 5.1B.

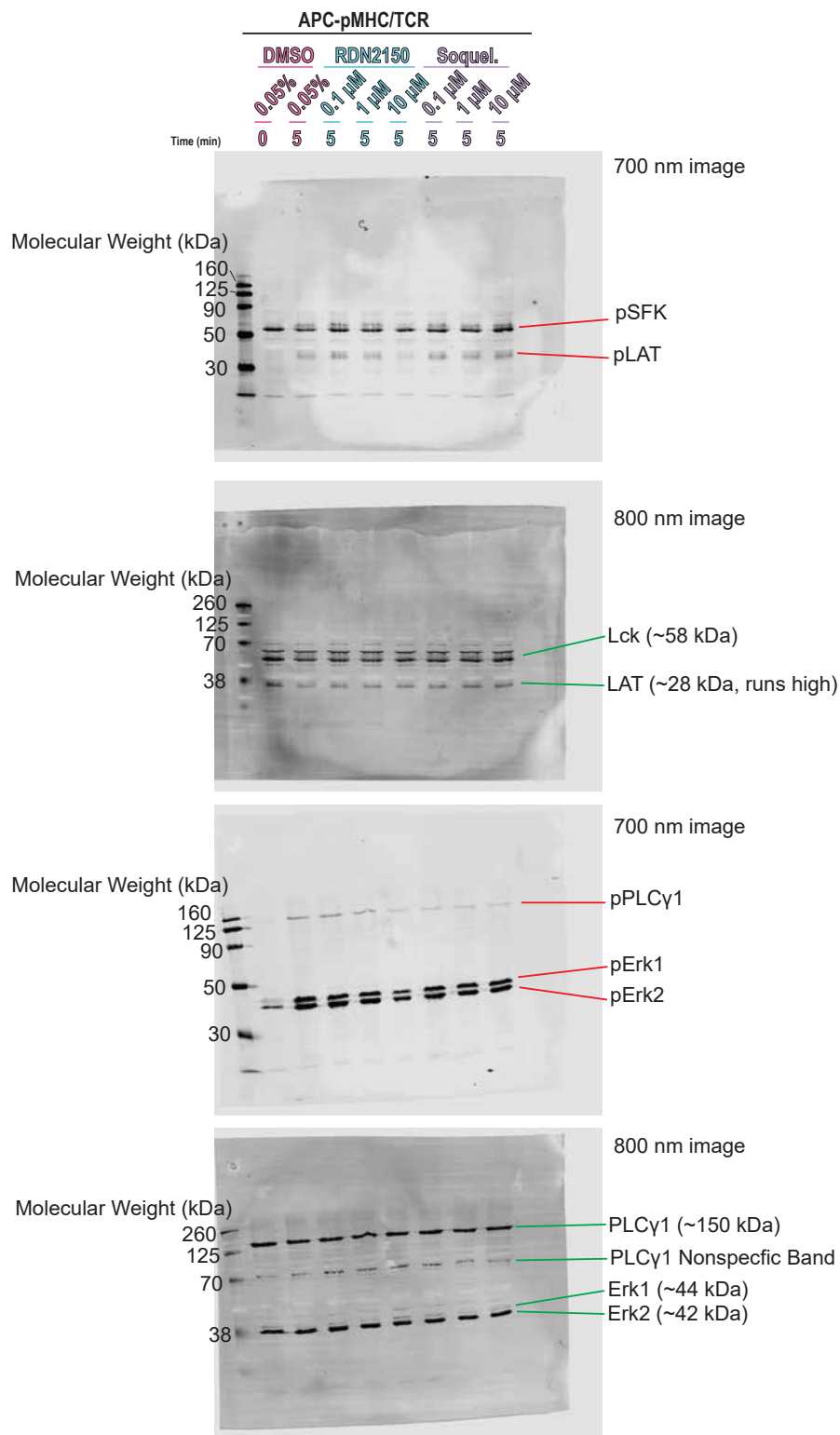


Figure 5.23: Uncropped, annotated Western blots for Figure 5.1E.

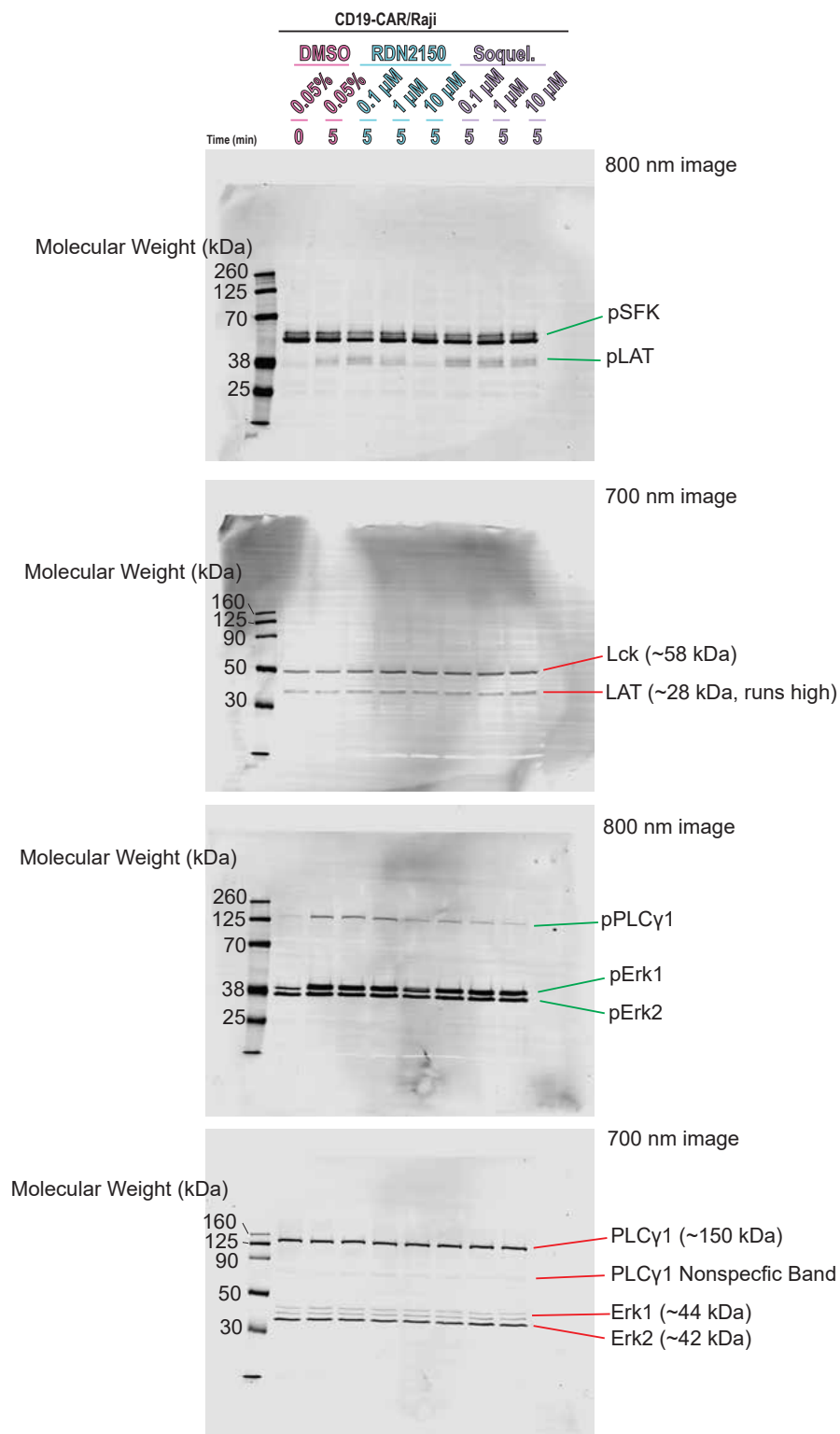


Figure 5.24: Uncropped, annotated Western blots for Figure 5.1H.

5.4.7 Interleukin-2 ELISA

Samples for IL-2 ELISAs were taken from the cell culture supernatant produced during flow cytometry preparation and frozen at -80 C until use. Interleukin-2 abundance was assessed using the Human IL-2 Sandwich ELISA Kit (proteintech #KE00017) per the manufacturer’s instructions. Briefly, 100 uL of samples diluted 1:4 in complete growth media were incubated in the ELISA plate for 2 hours at room temperature and washed four times in Wash Buffer before addition of 100 uL Detection Antibody solution. The plate was incubated at room temperature for 1 hour with Detection Antibody Solution, then washed four times with Wash Buffer. Next, 100 uL of Streptavidin-HRP solution was added to each well and incubated at room temperature for 40 minutes before washing four times with Wash Buffer. For signal development, 100 uL of TMB was added to each well and incubated for 15 minutes before quenching with 100 uL of Stop Solution. Absorbance was read at 450 nm with correction at 630 nm. Absolute quantitation was determined by using a standard curve of Human IL-2. Statistical analysis was performed as described in the “Flow cytometry” section. Samples represent individual stimulation experiments and each sample was only measured once.

5.4.8 Sample preparation and phosphoproteomic sample acquisition

Reduction, alkylation, trypsinization and desalting

Eleven milligrams of total protein were taken for each sample, then samples were adjusted to an equivalent volume (4.45 mL, ~1.6 M Urea final) with 20 mM HEPES, then reduced with 10 mM dithiothreitol (final) for 30 minutes at 37 C, then alkylated with 10 mM iodoacetamide (final) for 15 minutes in the dark. In solution trypsinisation was initiated with the addition of Sequencing Grade Trypsin (Promega #V5113) at a ratio of 1:100 trypsin:protein. After trypsinisation, samples were acidified to 1% trifluoroacetic acid (TFA), then cleared by centrifugation. Samples were desalted using Waters C18 vacuum columns as previously described³⁴. Under low vacuum, columns were activated using 100% acetonitrile (ACN), then equilibrated with 2 washes of 0.1% TFA. Samples were then passed through the columns, trapping peptides, and washed three times with

1, 5, and 6 mL of 0.1% TFA, before elution with 40% TFA and 0.1% ACN. After elution, 10 pmol of a phosphotyrosine standard (LIEDpYTAK) was added to each sample, then each sample was diluted to 20% ACN before freezing and 48-hour lyophilisation.

pY enrichment with Src SH2 superbinder

Phosphotyrosine enrichment using recombinant Src SH2 superbinder (sSH2) was performed as previously described(Callahan et al., 2024, 2025a,b; Griffith et al., 2022; Callahan et al., 2025c). Briefly, ~11 mg of dried peptide was resuspended in ice-cold IAP buffer (10 mM sodium phosphate monobasic monohydrate, 50 mM sodium chloride, 50 mM morpholinopropanesulfonic acid) at room temperature for 30 minutes with brief, interspersed vortexing. One hundred micrograms of recombinant sSH2 conjugated to agarose beads in-house was aliquoted into microcentrifuge tubes, then washed 3 times (1,500 xg, 2 min to collect beads) with 1 mL of ice-cold IAP buffer before addition of sample for 2 hours at 4 °C on an end-over-end rotator. After pY peptide binding, beads were washed 2 times with 1 mL ice-cold IAP buffer, then 3 times with ice-cold HPLC water. After the final water wash, all liquid was removed from the beads using an insulin syringe. Phosphotyrosine peptides were eluted from the beads using 0.15% TFA on a thermomixer at 1,150 rpm for 10 minutes, twice. Eluted pY peptides were then desalted using Empore C18 StageTips as previously described(Callahan et al., 2025a) and dried by speed-vac before LC-MS/MS acquisition.

LC-MS/MS acquisition

Samples were separated as previously described^{34,35} on a Vanquish Neo uHPLC system with an Acclaim PepMap C18 Trap column (7 cm, 2 cm bed length, 3 μ m particle size, 100 Å) and IonOpticks Aurora Ultimate (25 cm long x 75 μ m ID, 1.7 μ m particle size) and acquired on an Orbitrap Ascend mass spectrometer (Thermo Fisher). The separation gradient started at 5% Solvent B (80% ACN, 0.1% Formic Acid [FA])/95% Solvent A (0.1% FA) and increased to 30% Solvent B over 50 minutes, then spiked to 99% Solvent B for the remaining 5 minutes of acquisition. After acquisition, the flow path was washed with 4 alternating zebra washes, alternating between 100% Solvent A and 100% Solvent B, before being reequilibrated for the next run. The spray voltage was

2050 V, the ion transfer tube temperature was 275 °C, and the lock mass was 445.12003. For MS1 acquisition, wide quant isolation on the Orbitrap detector at a resolution of 120,000 with a mass range of 300-1500 m/z was used. The maximum injection time was 50 ms, the normalised AGC target was 250%, and the RF lens was set to 30%, and data collection was in centroid mode. The following filters were included: monoisotopic peak and peptide mode, Charge state (+2-+7), Precursor Selection Range (375-1,500 m/z), Dynamic Exclusion (exclude after 1 time, 15 sec exclusion time, 10ppm low and high mass tolerance, excluding isotopes), Intensity Threshold (intensity range of 20000 (min) – 1E20 (max) with relative intensity threshold of 20%). MS2 spectra were acquired in data-dependent mode with a cycle time of 3 seconds, normalised HCD fragmentation energy of 30%, and a 0.7 m/s quadrupole isolation window. Peptide fragments were detected using the ion trap detector with Turbo Mode active (scan range 150-2000 m/z) in centroid mode with the following settings: 35 ms maximum injection time, 100% normalised AGC target, dynamic maximum injection time active.

Data analysis, database searching and relative abundance quantification

.RAW files from the mass spectrometer were searched as previously described using the High Throughput Autonomous Proteomics Pipeline(Yu and Salomon, 2010) and PeptideDepot(Yu and Salomon, 2009). MS2 spectra were searched using the Mascot search engine against the complete UniProt proteome dataset (downloaded August 2019, 98,300 unique forward sequences) with a 0.1% FDR cutoff against a decoy database. The following parameters were used for peptide spectrum matching: trypsin (up to 2 missed cleavages), precursor mass tolerance of 7 ppm, 500 mmu fragment ion mass tolerance, phosphorylation as a variable modification (S/T/Y + 79.9963 Da), oxidation as a variable modification (M +15.9949), and SILAC labelling (K: +8.0142 Da, R: +10.0083 Da) and carbamidomethylation (C +57.0215) as a static modification. Relative peptide abundance was determined using peak area integration and retention time alignment of selected ion chromatograms. The abundance of pY peptides was normalised using the spike in standard peptide LIEDpYTAK, and any samples without LIEDpYTAK identified were omitted. Statistical significance between the log2 mean abundance for each comparison was determined using Welch's T test and corrected using the method of Storey(Storey

and Tibshirani, 2003) as previously described. Post-translational modification signature enrichment analysis was performed as previously described (Krug et al., 2019).

All post-PeptideDepot data processing was performed using Python 3.10 in the Windows Subsystem for Linux 2 in JupyterLab environments with the following dependencies: matplotlib (3.10.1), scipy (1.15.2), numpy (2.2.5), scikitlearn (1.6.1), pandas (2.2.3), and a custom ‘helpers’ module for improving matplotlib graph quality. The Holm Sidak algorithm for FWER correction was performed using a self-coded version of the algorithm as described by GraphPad Prism. All code used for the analysis of the data are publicly available on GitHub (<https://github.com/Aurdeegz/Zap70-Itk-Inhibitor-Profiling>).

5.4.9 Data availability

All uncropped LICOR full Western blot images, Floreada workspaces, and all code used for the analysis and presentation of our mass spectrometry data are publicly available on GitHub (<https://github.com/Aurdeegz/Zap70-Itk-Inhibitor-Profiling>). Mass spectrometry data are available from the ProteomeXchange Consortium via the PRIDE repository (Dataset ID: PXD069485, Reviewer Username: reviewer__pxd069485@ebi.ac.uk; Reviewer Password: bNHndQDrwzdD).

5.5 Acknowledgements

The authors would like to thank Dr. Xiaolei Su and his research team for providing the Jurkat CAR T cells for these experiments, as well as the group of Dr. Arthur Weiss for providing T2-K^b cells and Jurkat cells expressing the murine OT1 TCR & CD8. We would also like to wish Dr. Weiss a happy retirement and thank him for our years of enjoyable and productive collaborations.

Funding Declaration

This work was supported by NIH grants P01AI091580, T32GM136566, and 1S10OD036295.

Additional Information

The authors declare no competing interests. Generative AI was not used in the writing process, nor in the generation of figures for the manuscript.

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CHAPTER 6

The phosphatases TCPTP, PTPN22, and SHP1 play unique roles in T cell phosphotyrosine maintenance and feedback regulation of the TCR

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Abstract The protein tyrosine phosphatases (PTPs) TCPTP, PTPN22, and SHP1 are critical regulators of the activating phosphotyrosine (pY) site on the initiating T cell kinase, Lck^{Y394}. Still, the broader implications of these phosphatases in T cell receptor (TCR) signalling and T cell biology remain unclear. By combining CRISPR/Cas9 gene editing and mass spectrometry, we evaluate the protein- and pY-level effects of TCPTP, PTPN22, and SHP1 in the Jurkat T cell model system. We find that deletion of each phosphatase corresponds to unique changes in the proteome of T cells, with few large-scale changes to TCR signalling proteins. Notably, PTPN22 and SHP1 deletions have opposing effects on pY abundance globally, while TCPTP deletion modestly elevates pY levels. Finally, we show that TCPTP is indirectly involved in Erk1/2 positive feedback to the TCR. Overall, our work provides evidence for alternative functions of three T cell phosphatases long thought to be redundant.

6.1 Introduction

T cell activation is a delicate signalling process, and mutations that affect the timing or strength of T cell activation often result in autoimmune disorders (Gaud et al., 2018; Ohashi, 2002). T cell activation is achieved through binding of a peptide-loaded major histocompatibility complex (pMHC) to the α - and β -subunits of the T cell receptor (TCR), promoting dephosphorylation of the Src-family tyrosine kinase Lck at tyrosine 505 (Lck^{Y505}) and subsequent autophosphorylation and activation of Lck^{Y394}. Lck then phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) on TCR subunits CD3 ϵ / δ / γ , which act as a docking site for the Syk-family kinase Zap70. After Zap70 phosphorylation and activation by Lck, Lck and Zap70 phosphorylate the scaffolding proteins LAT and SLP76 and their associated binding partners, forming the TCR signalosome. Signal diversifies in the TCR signalosome to coordinate gene expression, rearrangement of the actin cytoskeleton, and release of proteins with immune system activating or cytotoxic function (T cell signalling has been reviewed extensively in Gaud et al. (2018)). Despite a large body of literature describing the feed-forward activation of T cells from TCR/pMHC binding, much less is known about the feedback mechanisms involved in maintaining homeostasis and regulation along the TCR signalling pathway.

Our current understanding of TCR signalling regulation focuses on the initiating kinase, Lck, and three phosphorylation sites known to play a role in activation or substrate specificity: Lck^{Y505}, Lck^{Y394}, and Lck^{Y192}. When phosphorylated by Csk, Lck^{Y505} locks Lck in an inactive conformation, preventing Lck^{Y394} autophosphorylation and Lck activation. Thus, its dephosphorylation by CD45 is a necessary step in Lck activation (Bougeret et al., 1996; McNeill et al., 2007; Takeuchi et al., 1993). Phosphorylation of Lck^{Y192} is known to prevent Lck/CD45 interactions, promote interactions with Zap70, TCR ζ , and PLC γ 1, and regulate the PLC γ 1-dependent activation of Erk1/2 (Borowicz et al., 2021; Granum et al., 2014; Kästle et al., 2020, 2022; Courtney et al., 2017). Phosphorylation of Lck^{Y394} increases its kinase activity, which can be dampened by dephosphorylation. Five protein tyrosine phosphatases (PTPs) are known to dephosphorylate Lck^{Y394}: CD45, T cell protein tyrosine phosphatase (TCPTP), PTP non-receptor type 22 (PTPN22), SH2

domain-containing tyrosine phosphatase 1 (SHP1), and JNK-associated phosphatase (JKAP)(Abraham and Veillette, 1990; Chiang and Sefton, 2001; D’Oro and Ashwell, 1999; Gjörloff-Wingren et al., 1999; Li et al., 2014; Wiede et al., 2011; Wu et al., 2006). The recruitment of TCPTP and PTPN22 to Lck and the early T cell receptor components is ambiguous, with no known recruitment strategy for TCPTP and conflicting literature for Csk-dependent and TRAF3-dependent recruitment of PTPN22(Cloutier and Veillette, 1996; Vang et al., 2012; Wallis et al., 2017). Thus, the biological significance of TCPTP and PTPN22 dephosphorylation of Lck^{Y394} remains unclear. In contrast, JKAP is anchored in the plasma membrane by myristoylation, similarly to Lck, which is thought to be necessary for JKAP-dependent dephosphorylation of Lck^{Y394}(Li et al., 2014; Schwertassek et al., 2010). Lastly, SHP1 can bind directly to the C-terminal region of Lck, which is disrupted by Lck^{S59} phosphorylation by Erk1/2. However, SHP1 also associates with GRB2 and GRB2-associated proteins via SH2 domain-mediated binding to shared or adjacent phosphotyrosine-containing motifs on adapter proteins such as LAT(Helou et al., 2013; Pani et al., 1996; Paster et al., 2015). Regardless, TCPTP, PTPN22, SHP1, and JKAP dysregulation are implicated in numerous autoimmune disorders for their role in maintaining T cells’ homeostasis(Gaud et al., 2018; Li et al., 2014; Bottini et al., 2006; Croker et al., 2008; Cubas et al., 2020; Gomez et al., 2005; Han et al., 2021; Ladner et al., 2005; Li et al., 2019; Markovics et al., 2020; Nielsen et al., 2007; Puerta et al., 2013; Rieck et al., 2007; Salmond et al., 2015; Sharma et al., 2016; Sinthuwiwat et al., 2022; Smyth et al., 2004; Takeuchi et al., 2020; Zheng and She, 2005; Zhernakova et al., 2005; Zhou and Li, 2022; Zhu et al., 2021; Zikherman and Weiss, 2011).

Our laboratory and others have previously defined a positive feedback mechanism connecting Erk1/2 to TCR signal transduction(Helou et al., 2013). Loss of Erk1/2 activation through small molecule inhibition of MEK1/2 leads to a global decrease in tyrosine phosphorylation, which mutes TCR signalling responses to soluble antibody or pMHC ligation(Helou et al., 2013; Paster et al., 2015; Štefanová et al., 2003). The leading hypothesis, in which Erk1/2 phosphorylates Lck^{S59} to prevent SHP1 docking and Lck^{Y394} dephosphorylation, remains ambiguous as Lck^{S59A} knock-in mice fail to show overt defects in T cell activation and ligand discrimination(Paster et al., 2015). Due to

the redundancy of Lck^{Y394} dephosphorylation, we hypothesise that Lck-regulating PTPs may be involved in Erk1/2 positive feedback. Using the Jurkat T cell model system, CRISPR/Cas9 gene editing, and mass spectrometry-based proteomics, we evaluate the roles of TCPTP, PTPN22, and SHP1 in maintaining proteomic homeostasis, maintaining pY homeostasis, and Erk1/2 positive feedback. We find that T cell signalling proteins were largely unaffected by the knockout of each phosphatase. However, J.PTPN22 shows significant deenhancement of KEGG T cell signalling proteins. TCPTP, PTPN22, and SHP1 play opposing roles in pY maintenance globally, with PTPN22 negatively regulating, SHP1 positively regulating, and TCPTP modestly negatively regulating pY abundance. Unlike SHP1 and PTPN22, TCPTP knockout rendered global phosphorylation largely insensitive to U0126 inhibition of Mek1/2 both in the basal and even more strikingly in the stimulated state suggesting a role of TCPTP downstream of Erk in the Erk to Lck positive feedback loop. Overall, each phosphatase TCPTP, PTPN22, and SHP1 appear to play unique roles in phosphotyrosine regulation in Jurkat T cells, with TCPTP likely participating in Erk positive feedback.

6.2 Results

6.2.1 *PTPN2* (TCPTP), *PTPN22* (PTPN22), and *PTPN6* (SHP1) knockouts do not affect the abundance or retention of key TCR signalling proteins

To evaluate the specific effects of TCPTP, PTPN22, and SHP1 on T cell homeostasis and feedback regulation of the TCR, we generated genomic KOs of *PTPN2*, *PTPN22*, and *PTPN6* in Jurkat T cells using CRISPR/Cas9-based gene editing (Figure 6.1A). Clonal KOs were screened for TCPTP, PTPN22, and SHP1 expression by Western blot, and one clone was selected for use (Figure 6.1A,B). The resulting clones, J.TCPTP- (T2 1C10), J.PTPN22- (P1 1C1), and J.SHP1- (S1 2C4) displayed surface levels of CD3 ϵ and CD45 similar to WT Jurkats (clone E6.1), while J.SHP1- and J.TCPTP- showed varying levels of surface TCR α by flow cytometry (Figure 6.1C). Using LC -MS/MS-based protein profiling, we evaluated the influence of PTPN2, PTPN22, and PTPN6 knockout

on global protein expression. We sequenced the proteome of each cell line deeply and reproducibly, with few imputed values per replicate, cell line-dependent clustering, and high linearity (Figure 6.1D, Figure 6.2A). Two samples, J.TCPTP- R5 and J.PTPN22-R4, show markedly reduced protein abundance compared with the other replicates (Figure 6.2B) and were removed from further analysis. We found that genomic deletions of PTPN2 and PTPN22 had a minimal effect on global protein abundance (Figure 6.1D,E), with few T cell signalling proteins showing significantly reduced abundance compared to WT Jurkats (Figure 6.1F). Interestingly, PTPN2 and PTPN22 deletion increased the abundance of MAGE-A9, a protein of completely unknown function implicated in several cancers (Gu et al., 2014; Meyer et al., 2018; Xu et al., 2014, 2015). In contrast, PTPN6 deletion broadly influenced non-TCR protein expression, including upregulation of TUBA4A and CD1b, and downregulation of galectin-9 and RPL15 (Figure 6.1E,F). While single-sample gene set enrichment analysis (ssGSEA) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa and Goto, 2000) revealed a significant deenhancement of TCR signalling proteins in J.PTPN22- (Figure 6.1G), individual TCR signalling proteins were not significantly altered. In contrast, J.TCPTP- showed deenhancement of Cell Cycle and DNA Replication pathways, and J.SHP1- showed enhancement of multiple metabolic pathways and disease-associated pathways (Figure 6.1G). Together, our data show that PTPN2, PTPN22, and PTPN6 knockouts are successful and do not broadly impact individual proteins in the TCR pathway. However, they do appear to have specific effects on the proteome.

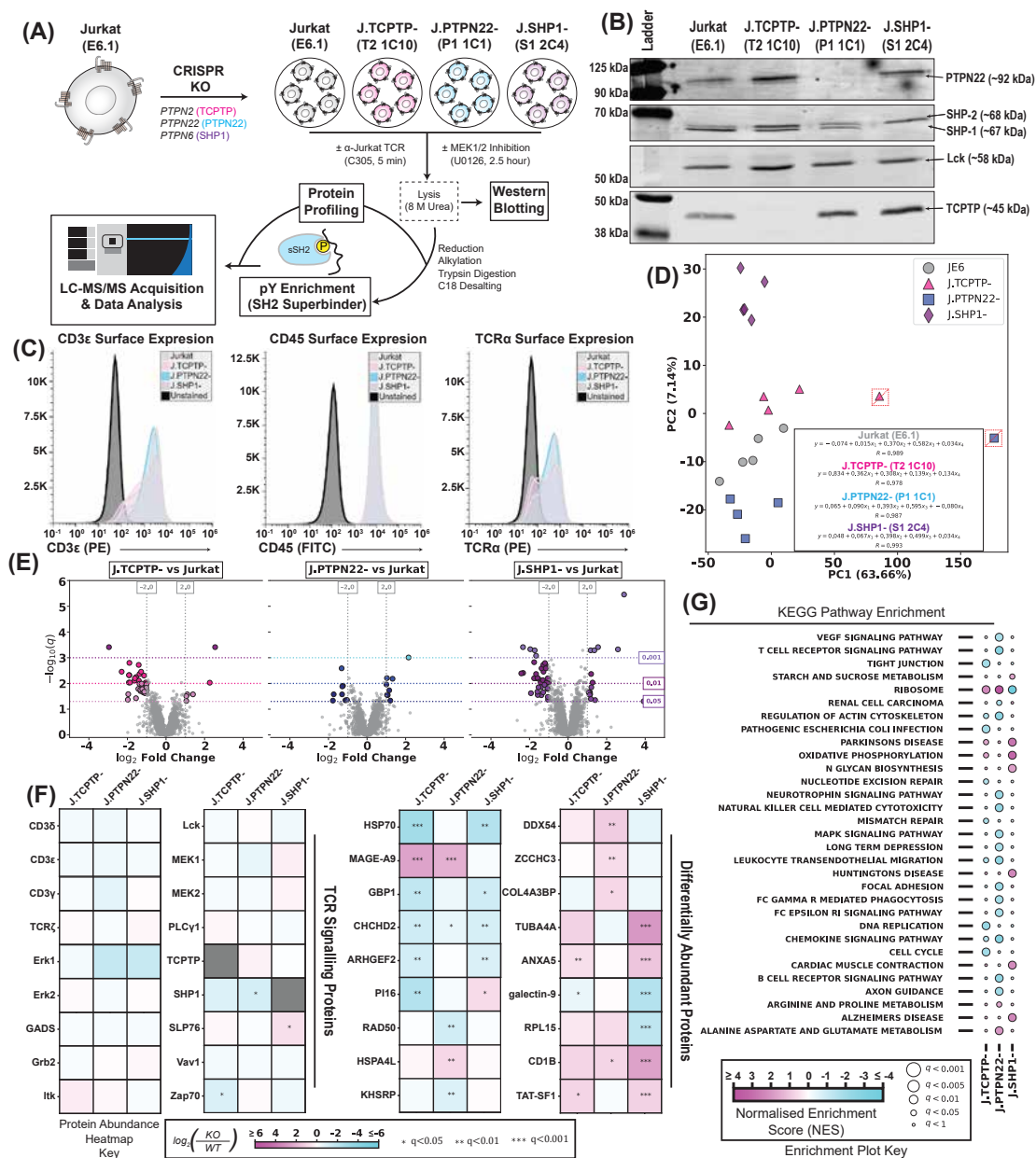


Figure 6.1: Generation and proteomic characterisation of J.TCPTP-, J.PTPN22-, and J.SHP1-. (A) Schematic representation of CRISPR Cas9 gene editing of Jurkat T cells and subsequent signalling analysis. Jurkat T cells are depicted with a TCR and CD4 coreceptor. (B) Western blot showing loss of PTPN22 (row 1), SHP1 (row 2), and TCPTP (row 4) in the selected clones. (C) Flow cytometry analysis of CD3 ϵ (left), CD45 (middle), and TCR α (right) surface expression in the phosphatase KO clones. (D) Principal component analysis of LC-MS/MS protein profiling data for the selected clones. J.TCPTP- Replicate 5 and J.PTPN22- Replicate 4 are discarded due to sample quality (See Figure 6.2). (E) Volcano plot analysis showing differentially abundant proteins in J.TCPTP- (left), J.PTPN22- (middle), and J.SHP1- (right) relative to Jurkats. (F) Heatmaps showing specific protein abundance for T cell proteins (left) and differentially abundant proteins (right). (G) Single sample gene set enrichment analysis using the KEGG database for differentially abundant proteins.

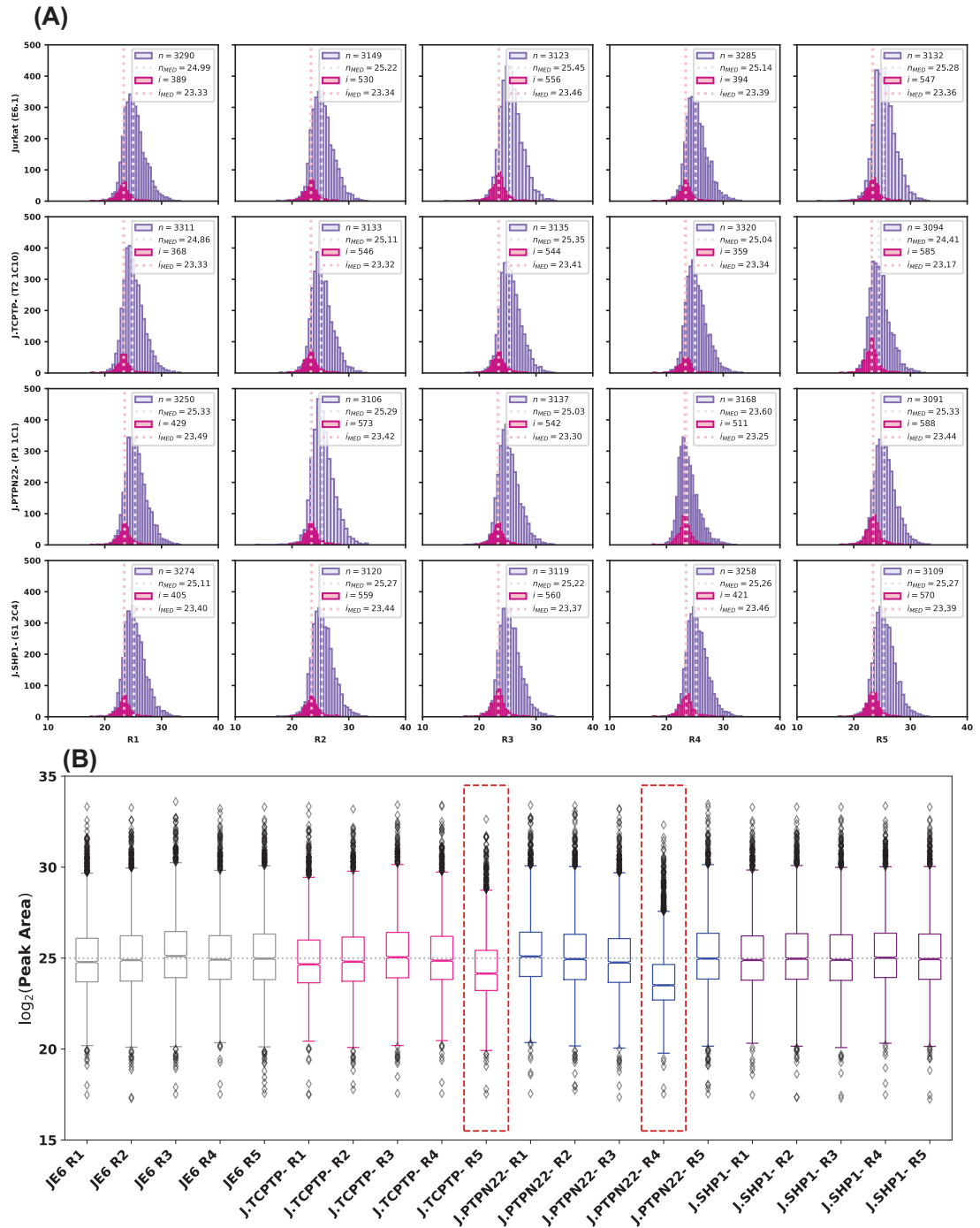


Figure 6.2: Quality control of Jurkat, J.TCPTP-, J.PTPN22-, and J.SHP1- protein profiling mass spectrometry data. (A) Histogram of $\log_2$ transformed abundances in purple with imputed distributions in pink. (B) Box and whisker plots showing the intensity distribution for each protein profiling sample. J.TCPTP- R5 and J.PTPN22- R4 are circled in red. Due to the notably lower protein abundance in these replicates, they were discarded from further analysis.

6.2.2 *PTPN2* (TCPTP), *PTPN22* (PTPN22), and *PTPN6* (SHP1) knockouts respond to TCR stimulation and, to some degree, Erk positive feedback

To determine whether the knockouts remained responsive to T cell receptor stimulation and Erk positive feedback, we inhibited MEK1/2 using U0126 and stimulated cells with α -Jurkat TCR (clone C305) followed by Western blot analysis of various pY sites known to be TCR responsive (Figure 6.3A). Titration of U0126 from 0.2 μ M to 50 μ M showed competent Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation until a U0126 concentration of 20 μ M (Figures 6.3B-C, 6.4), as seen previously (Helou et al., 2013). Positive feedback from Erk1/2 to the TCR is well described and includes a phenotype of global pY depletion (Helou et al., 2013). Using α -pY (clone 4G10), an antibody that robustly detects tyrosine phosphorylated T cell proteins (Courtney et al., 2017), we visualised changes in a small subset of pY proteins during TCR stimulation and U0126 treatment. We observed that U0126 reduced pY levels to the untreated basal state at 20 μ M in all cell lines (Figures 6.3D, 6.5). Since 20 μ M U0126 appeared to completely ablate Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation, we evaluated any changes in the kinetics of TCR signalling proteins with or without 20 μ M U0126 treatment. As expected, Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation was significantly reduced with U0126 treatment in all cell lines at all time points (Figures 6.3E-F, 6.6). J.TCPTP- showed the highest recovery of Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation during U0126 treatment of all cell lines after 5 min of TCR stimulation ($p = 0.0016$ vs 0 min), with all lines except J.SHP1- showing significant Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation after 10 min (Figure 6.3E-F, 6.6). All cell lines except J.PTPN22- showed significant pY induction after 2 and 5 min of TCR stimulation ($p < 0.05$), and no cell line showed significant global pY induction after U0126 treatment despite clear increases in specific pY bands in J.TCPTP- and J.SHP1- (Figures 6.3G, 6.7, 6.8). Similarly, phosphorylation of the Src family kinase activation site (peptide NEpYTAR) at a molecular weight consistent with Lck (Figure 6.9) was induced at 2 min ($p < 0.05$; Jurkat, J.TCPTP-, J.SHP1-), 5 min ($p < 0.05$; Jurkat, J.PTPN22-, J.SHP1-) and 10 min ($p < 0.05$; Jurkat) post stimulation, and this induction was ablated entirely in all cell lines after U0126

treatment (Figures 6.3H, 6.9). Lastly, the activation site PLC γ 1^{Y783} was significantly more responsive in non-U0126-treated J.TCPTP- cells at 2 min after TCR stimulation compared to all other cell lines ($p < 0.05$). J.TCPTP- cells treated with U0126 showed significant phosphorylation of PLC γ 1^{Y783} at all time points, whereas Jurkat and J.SHP1- only showed PLC γ 1^{Y783} induction after 5- and 10-min, and J.PTPN22- showed no significant PLC γ 1^{Y783} response (Figures 6.3I, 6.10). Taken together, our selected clones J.TCPTP- (T2 1C10), J.PTPN22- (P1 1C1), and J.SHP1- (S1 2C4) are suitable for evaluating the regulatory role of TCPTP, PTPN22, and SHP1 in the context of pY homeostasis and TCR signalling.

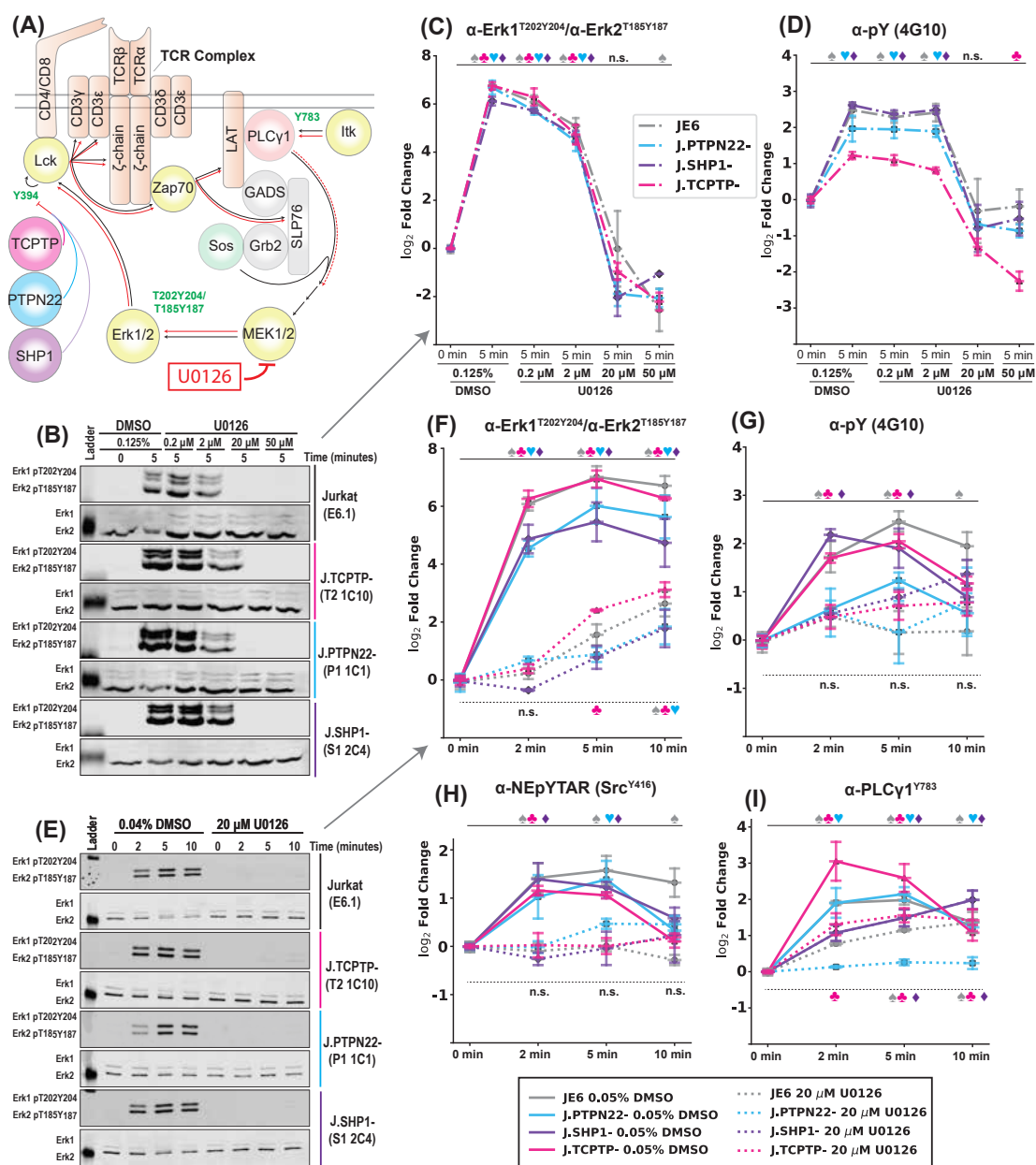


Figure 6.3: (Caption on next page)

Figure 6.3: Knockout of PTPN2 (TCPTP), PTPN22 (PTPN22), or PTPN6 (SHP1) retain TCR signalling and responsiveness to U0126 treatment. (A) Truncated schematic of TCR signalling, with black lines/arrows showing forward signalling and red lines/arrows showing the effect of U0126 treatment to reduce flux through the TCR signalling pathway. (B) Representative Western blots targeting Erk1^{T202Y204}/Erk2^{T185Y187} during a U0126 titration. Quantification of U0126 titration Western blots targeting (C) Erk1^{T202Y204}/Erk2^{T185Y187} and (D) pY. (E) Representative Western blots targeting Erk1^{T202Y204}/Erk2^{T185Y187} during a TCR stimulation time course in the presence or absence of U0126. Quantification of time course Western blots targeting (F) Erk1^{T202Y204}/Erk2^{T185Y187}, (G) pY, (H) Src family kinase activation site, sequence NEpYTAR, and (I) PLC γ 1^{Y783}. For all Western blots, statistical significance between 0-min normalised, log₂ transformed abundances is determined by Fisher's LSD with the Holm Sidak correction. ♠♣♥♦ indicate $p < 0.05$ when comparing the given time point to the 0-min sample for Jurkat, J.TCPTP-, J.PTPN22-, and J.SHP1-, respectively. Upper symbols are for 0.04% DMSO treatment, and lower symbols are for U0126 treatment time courses. 'n.s.' indicates no statistical significance in that comparison for any cell line.

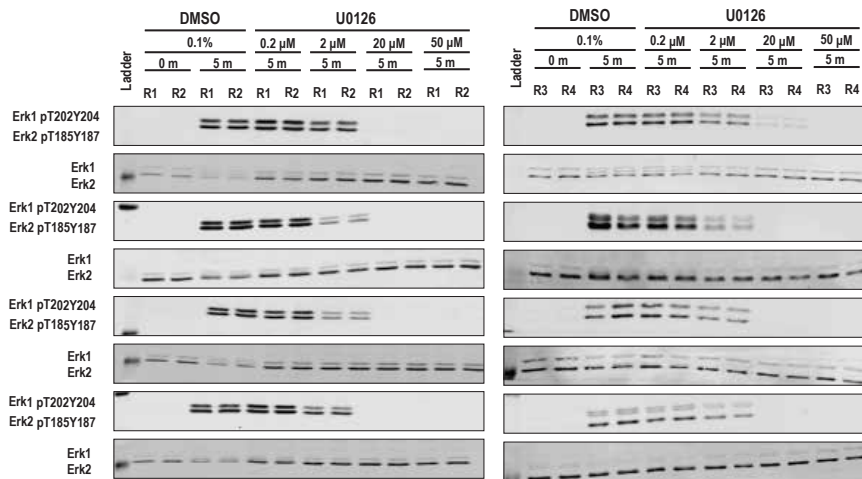


Figure 6.4: Western blots used for quantification of $\text{Erk1}^{\text{T202Y204}}/\text{Erk2}^{\text{T185Y187}}$ during a U0126 titration.

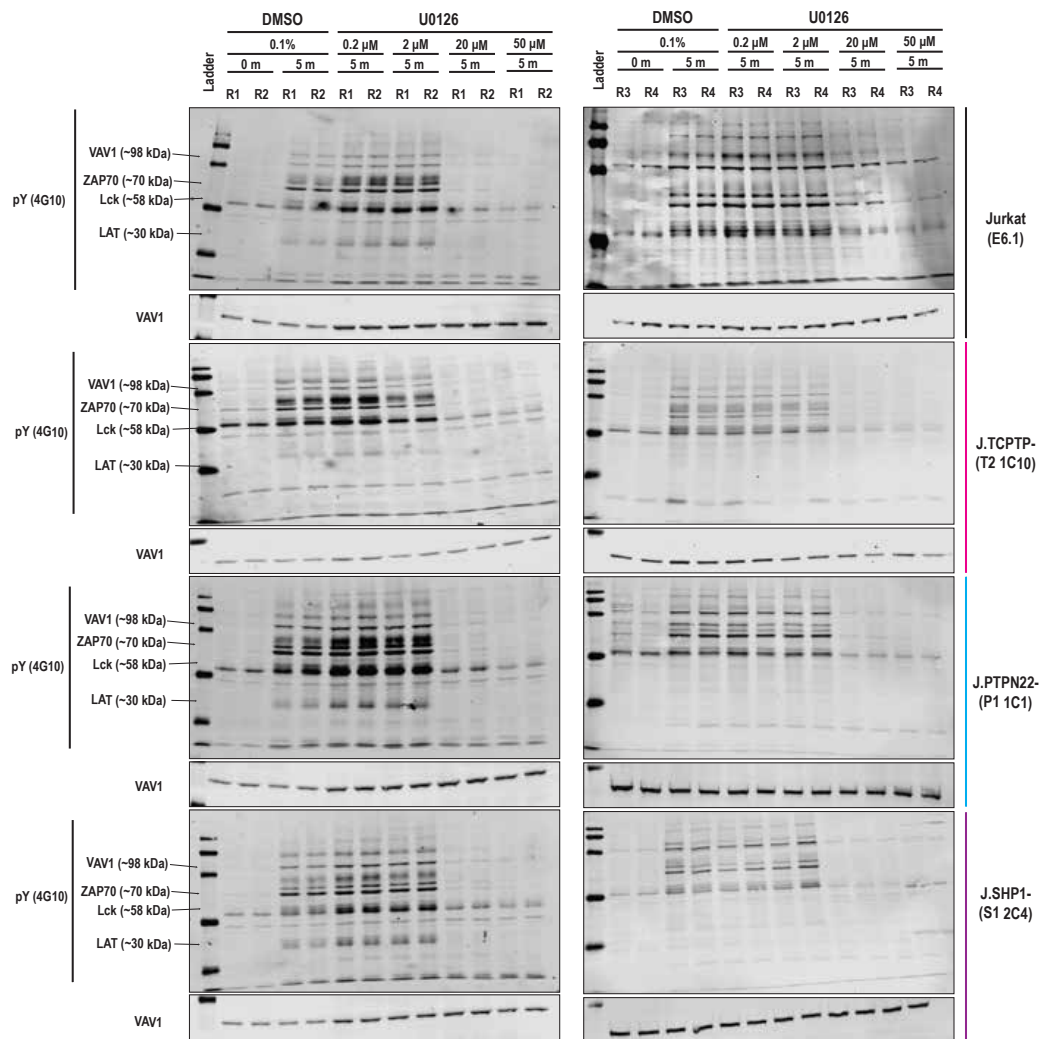


Figure 6.5: Western blots used for quantification of global pY abundance during a U0126 titration.

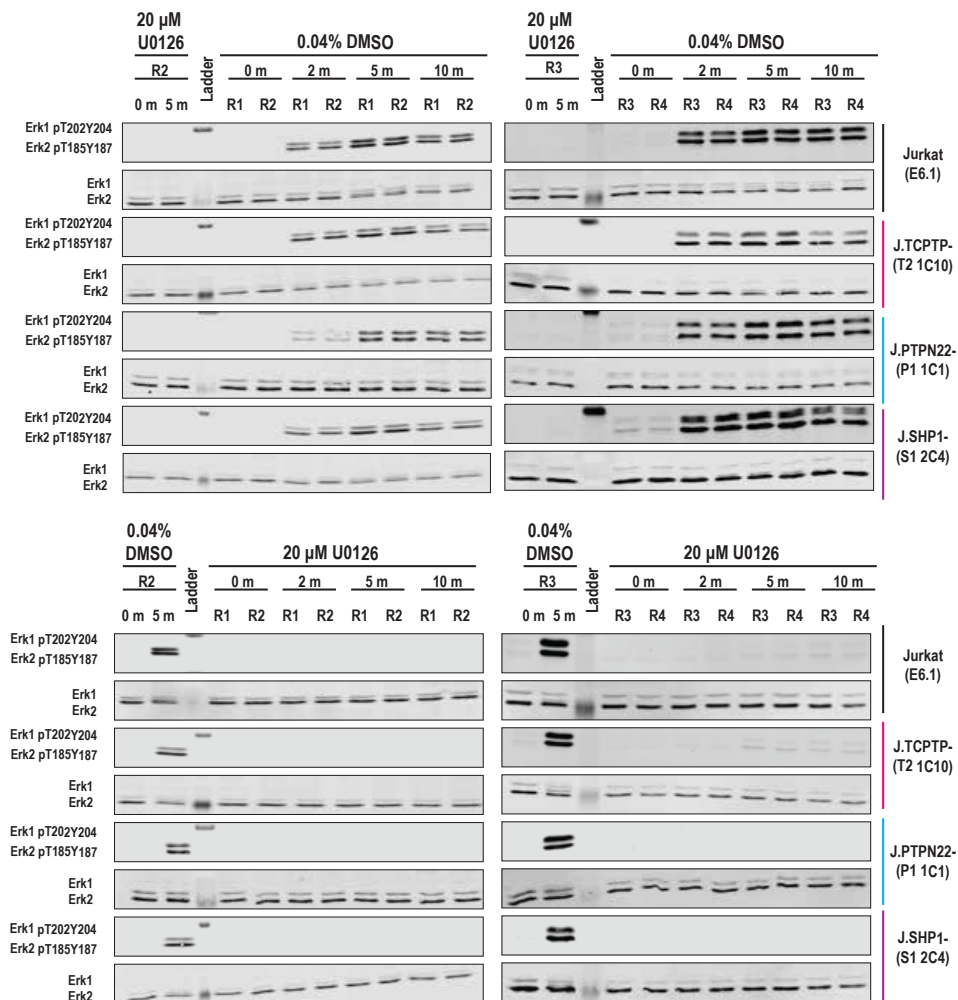


Figure 6.6: Western blots used for quantification of Erk1T202Y204/Erk2T185Y187 during a TCR stimulation time course in the absence (top) or presence (bottom) of 20 μ M U0126.

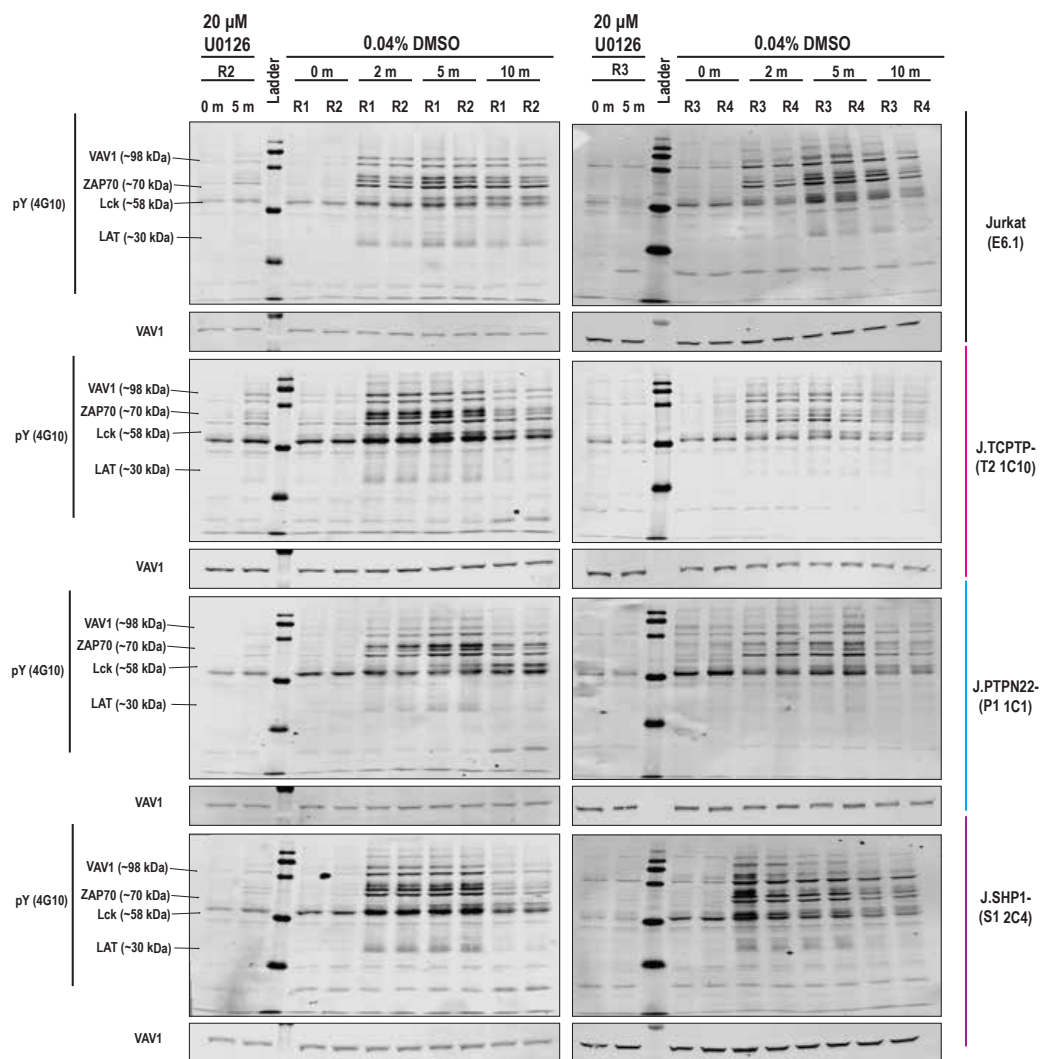


Figure 6.7: Western blots used for quantification of global pY abundance during a TCR stimulation time course in the presence of 0.04% DMSO.

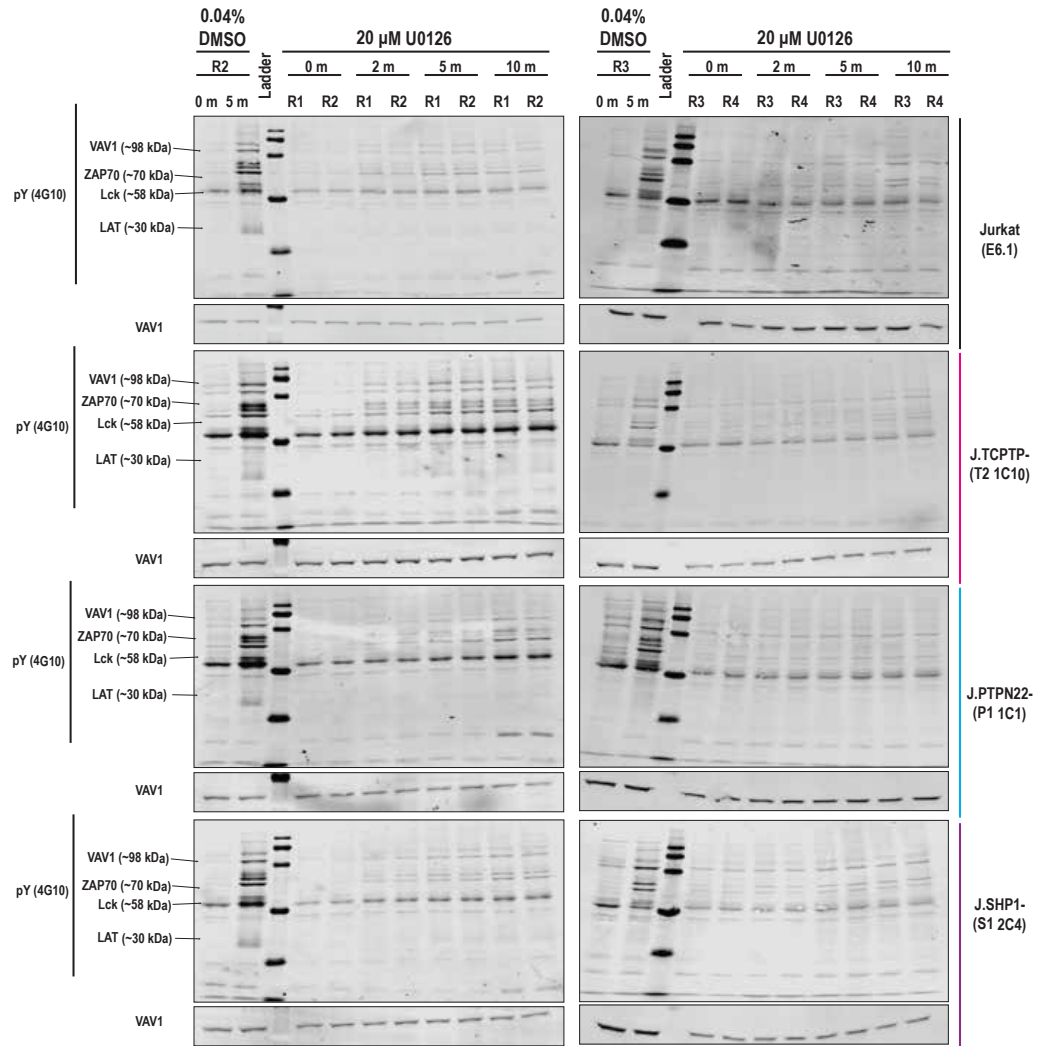


Figure 6.8: Western blots used for quantification of global pY abundance during a TCR stimulation time course in the presence of 20 μ M U0126.

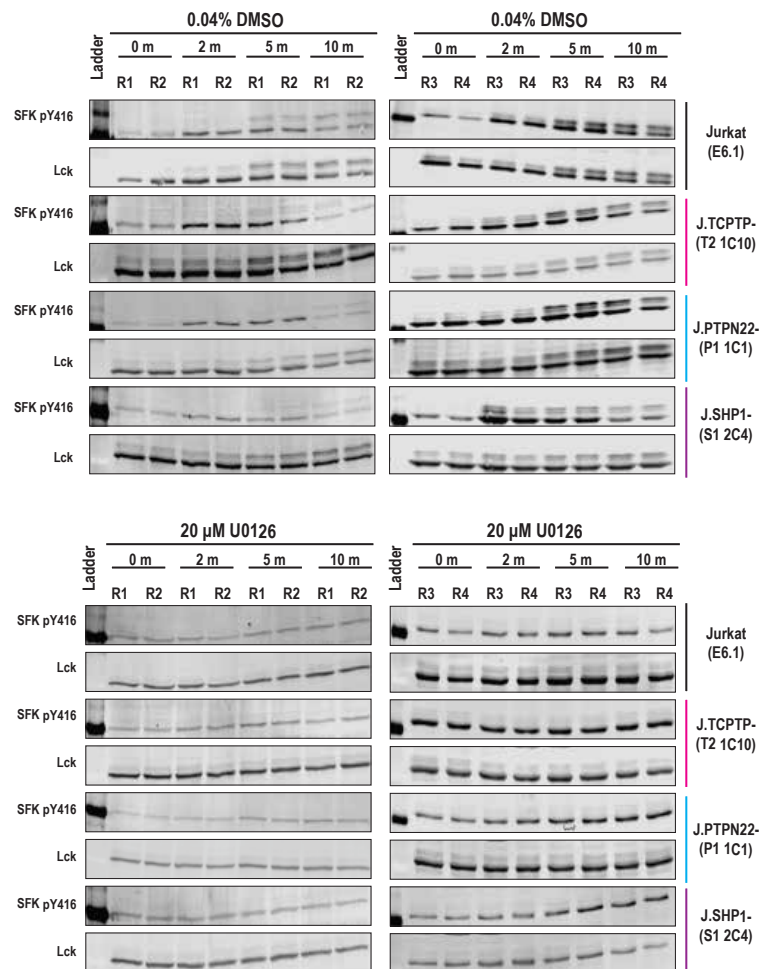


Figure 6.9: Western blots used for quantification of the Src family kinase activation site (SFK^{Y416}) during a TCR stimulation time course in the absence (top) or presence (bottom) of 20 μ M U0126.

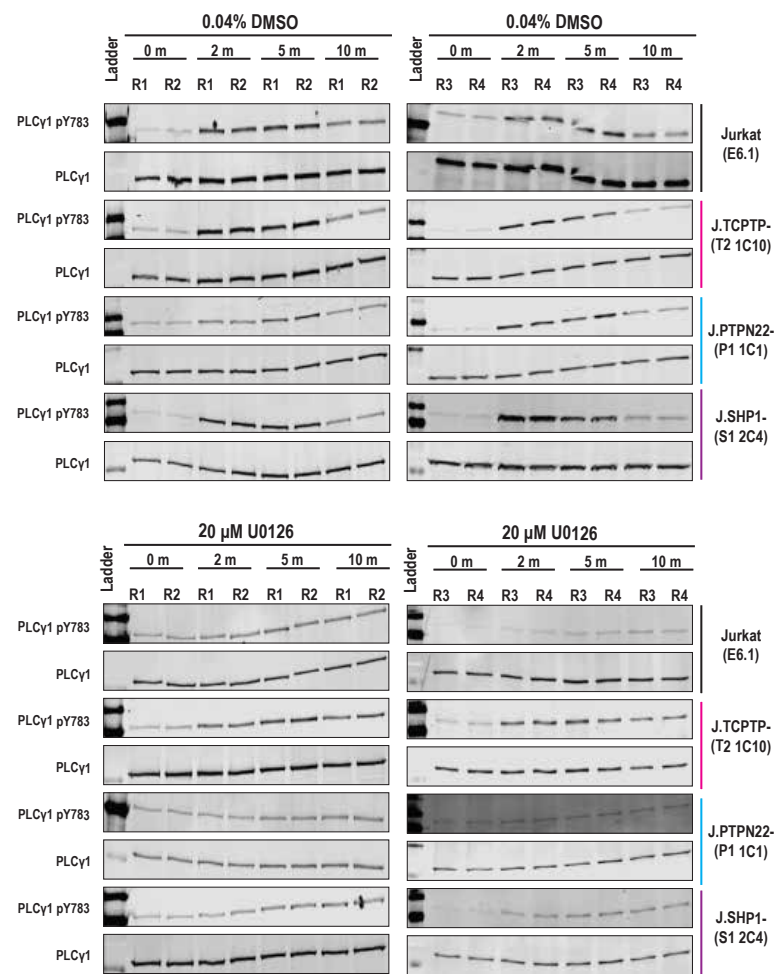


Figure 6.10: Western blots used for quantification of PLCγ1^{Y783} during a TCR stimulation time course in the absence (top) or presence (bottom) of 20 μM U0126.

6.2.3 TCPTP, PTPN22, and SHP1 play different regulatory roles on global pY abundance

Since PTPs are responsible for tyrosine dephosphorylation, which can activate or inactivate target proteins, we used the Src SH2 superbinder (sSH2) pY enrichment strategy (Callahan et al., 2024; Griffith et al., 2022) to evaluate global levels of site-specific tyrosine phosphorylation in Jurkat, J.TCPTP-, J.PTPN22-, and J.SHP1- in the basal state and an antibody-stimulated state. PCA clustering showed cell line clustering and, to a lesser extent, clustering of the stimulated and unstimulated states for each cell line (Figure 6.11A). In the basal state, we found that few significantly changing pY sites were uniquely decreased in J.TCPTP- (15 sites) or J.PTPN22- (13 sites), and J.SHP1- showed a marked reduction in pY site abundance overall (123 sites) compared to WT Jurkats. In contrast, J.PTPN22- showed 260 unique pY sites significantly elevated, with few unique sites upregulated in J.TCPTP- (21) and J.SHP1- (25) (Figure 6.11B -E top row). In the stimulated state, 106, 51, and 3 pY sites were uniquely downregulated, and 52, 19, and 256 pY sites were uniquely upregulated in J.SHP1-, J.TCPTP-, and J.PTPN22-, respectively (Figures 6.11D-F, bottom row, 6.12A-B). PTPN22 KO significantly upregulated many canonical T cell signalling protein pY sites in both the basal and stimulated states, including ITAMs on TCR subunits ($CD3\delta^{Y160}$, $CD3\epsilon^{Y188}$, $CD3\gamma^{Y160}$, $TCR\zeta^{Y142}$, and $TCR\zeta^{Y153}$), Lck^{Y192} , activating sites on Zap70 ($Zap70^{Y492}$, $Zap70^{Y493}$, $Zap70^{Y492Y493}$), the activating site Itk^{Y512} , docking site LAT^{Y220} and $GADS^{Y45}$. TCPTP KO displayed a milder yet similar phenotype as PTPN22, with specific upregulation of $TCR\zeta$, Zap70, and $PLC\gamma1$ phosphorylation sites that are no longer significantly upregulated after TCR stimulation. Interestingly, SHP1 KO led to a significant reduction in some canonical TCR phosphorylation sites, including $TCR\zeta^{Y153}$, the Src family activation site (peptide sequence LIEDNEpYTAR, SFK^{Y416}), and $PLC\gamma1^{Y783}$, while elevating other sites, including $Erk1^{T202Y204}/Erk2^{T185Y187}$, $Vav1^{Y791}$, and Fyn^{Y440} . After stimulation, most pY sites on PAG1 – a negative-feedback adaptor that, once phosphorylated, recruits the Src-family-kinase inhibitor Csk – switched from significantly down-regulated to up-regulated in SHP1-deficient cells but not in the PTPN22- or TCPTP-knockouts (Figure 6.11G). This rise in PAG1 phosphorylation would be expected to bring Csk to

the membrane, promote phosphorylation of Lck^{Y505}, and thereby lock Lck in its inactive, closed conformation, providing an additional brake on TCR signalling uniquely evident when SHP1 is absent (Figure 6.11G). Using post-translational modification signature enrichment analysis (PTM-SEA), an algorithm that determines whether specific signatures are overrepresented or underrepresented in PTM-based proteomics data (Krug et al., 2019), Fyn substrate signatures were elevated by TCPTP and PTPN22 KO, T cell receptor signatures were elevated after stimulation in J.TCPTP-, and α -CD3 signatures were elevated in all KOs after stimulation (Figures 6.12C -E). Together, our data suggest that TCPTP, PTPN22, and SHP1 each regulate unique aspects of T cell pY homeostasis, with differential influence on TCR signalling pY sites, contrary to the view that they are essentially redundant.

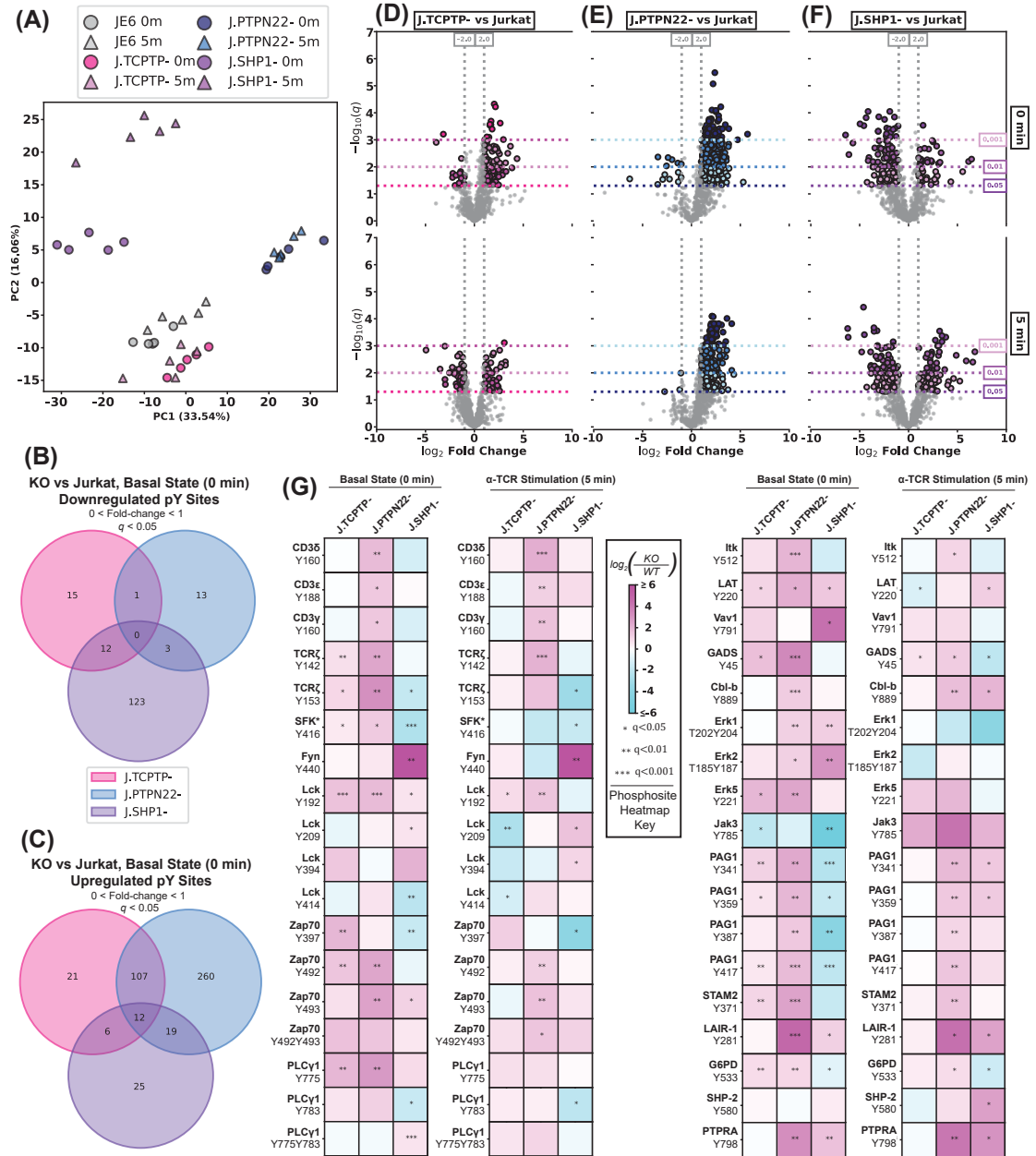


Figure 6.11: TCPTP, PTPN22, and SHP1 differentially maintain global pY abundance in Jurkat T cells. (A) PCA clustering on pY proteomics data. Venn diagram showing the overlap between (B) downregulated and (C) upregulated pY sites in J.TCPTP-, J.PTPN22-, and J.SHP1- in the basal state samples. (D,E) Volcano plots comparing unique pY sites in J.TCPTP-, J.PTPN22-, and J.SHP1-, respectively, to Jurkats in the basal state (0 min—top row) and stimulated state (5 min—bottom row). (G) Heatmaps showing individual pY sites compared with Jurkats for each phosphatase KO.

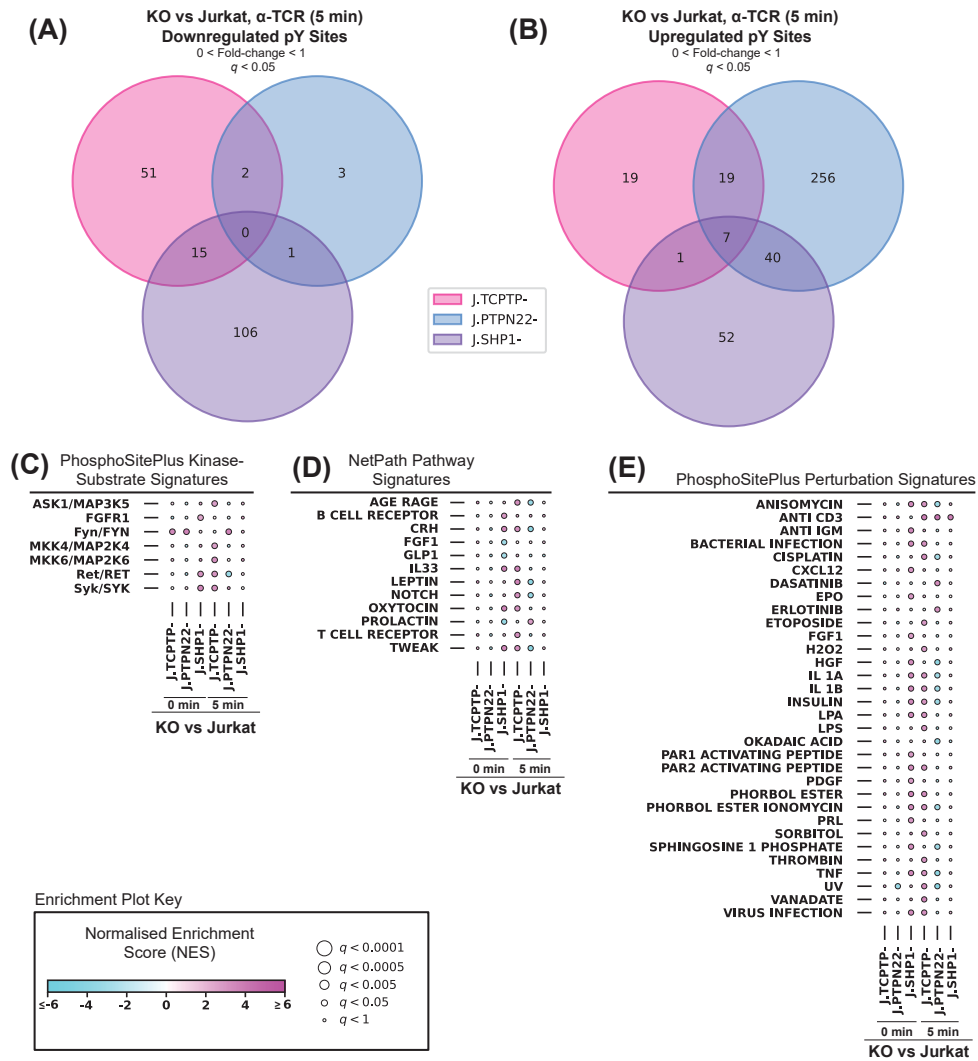


Figure 6.12: Comparison of significantly changing, unique pY sites and pathway enrichment in KO compared to Jurkats. (A-B) Venn diagrams showing overlap of unique pY sites that are downregulated and upregulated, respectively, in J.TCPTP-, J.PTPN22-, and J.SHP1- compared with Jurkats during TCR stimulation. (C-E) Bubbleplots showing PTM-SEA of PhosphoSitePlus Kinase Signatures, NetPath Pathway Signatures, and PhosphoSitePlus Perturbation Signatures, respectively, for KO vs WT comparisons.

6.2.4 SHP1 and PTPN22 play opposing roles in pY induction after TCR stimulation

Signalling from the TCR, whether through soluble antibody stimulation, soluble pMHC conjugation, or cell-based TCR-pMHC ligation, consists of characteristic pY site induction across many TCR signalling proteins, which dampen over time (> 10 min after ligation) due to engagement of negative feedback pathways (Gaud et al., 2018). After stimulation with α -Jurkat TCR α (clone C305), we observed very few significantly downregulated pY sites in all cell lines, with J.TCPTP- having the most uniquely downregulated pY sites (38; Figure 6.13A). In contrast, J.SHP1- had a robust response to stimulation, with 121 pY sites significantly upregulated, compared with 12 and 6 pY sites unique to J.PTPN22- and J.TCPTP-, respectively (Figure 6.13B). Stimulation of J.SHP1- cells led to a massive wave of pY induction, far greater than Jurkats or J.TCPTP-, while induction and significance of pY sites in J.PTPN22- was globally muted (Figures 6.13C -F), suggesting that SHP1 plays a uniquely inhibitory role in TCR signalling. We observed characteristic induction patterns for ITAMs on CD3 $\delta/\epsilon/\gamma$ and TCR ζ , the activation site Zap70^{Y492Y493}, an inhibitory site PLC γ 1^{Y771}, an activating site PLC γ 1^{Y775}, and GADS^{Y45}, a site necessary for signal integration between the TCR and CD28, with SHP1-deficient cells showing the most potent induction. In J.SHP1- cells, PAG1 became uniquely responsive to TCR signalling across multiple pY sites, suggesting that SHP1 plays a unique role in preventing stimulation-dependent negative feedback to the TCR (Figure 6.13G). PTM-SEA revealed that, after receptor stimulation, Fyn, Lck, and Zap70 substrate signatures were significantly elevated in all cell lines after stimulation, with J.TCPTP- having the smallest enrichment for Fyn signatures (Figure 6.13H). T cell receptor signatures were significantly enriched in Jurkats, J.TCPTP-, and J.PTPN22- but not in J.SHP1- after receptor stimulation (Figure 6.13I), suggesting that loss of SHP1 increases the TCR responsiveness of typically non-TCR responsive pY sites. Finally, small molecule perturbation signatures consistent with activating agents, including α -CD3, were significantly elevated after stimulation in all cells, whereas inhibitor signatures, including U0126, were significantly decreased (Figure 6.13J). Overall, these results suggest that genomic KO of TCPTP, PTPN22, or SHP1 does not prevent

the forward progression of TCR signalling. However, PTPN22 KO slightly impairs TCR stimulation responsiveness, and SHP1 KO enhances it, consistent with the preliminary Western data (Figure 6.13).

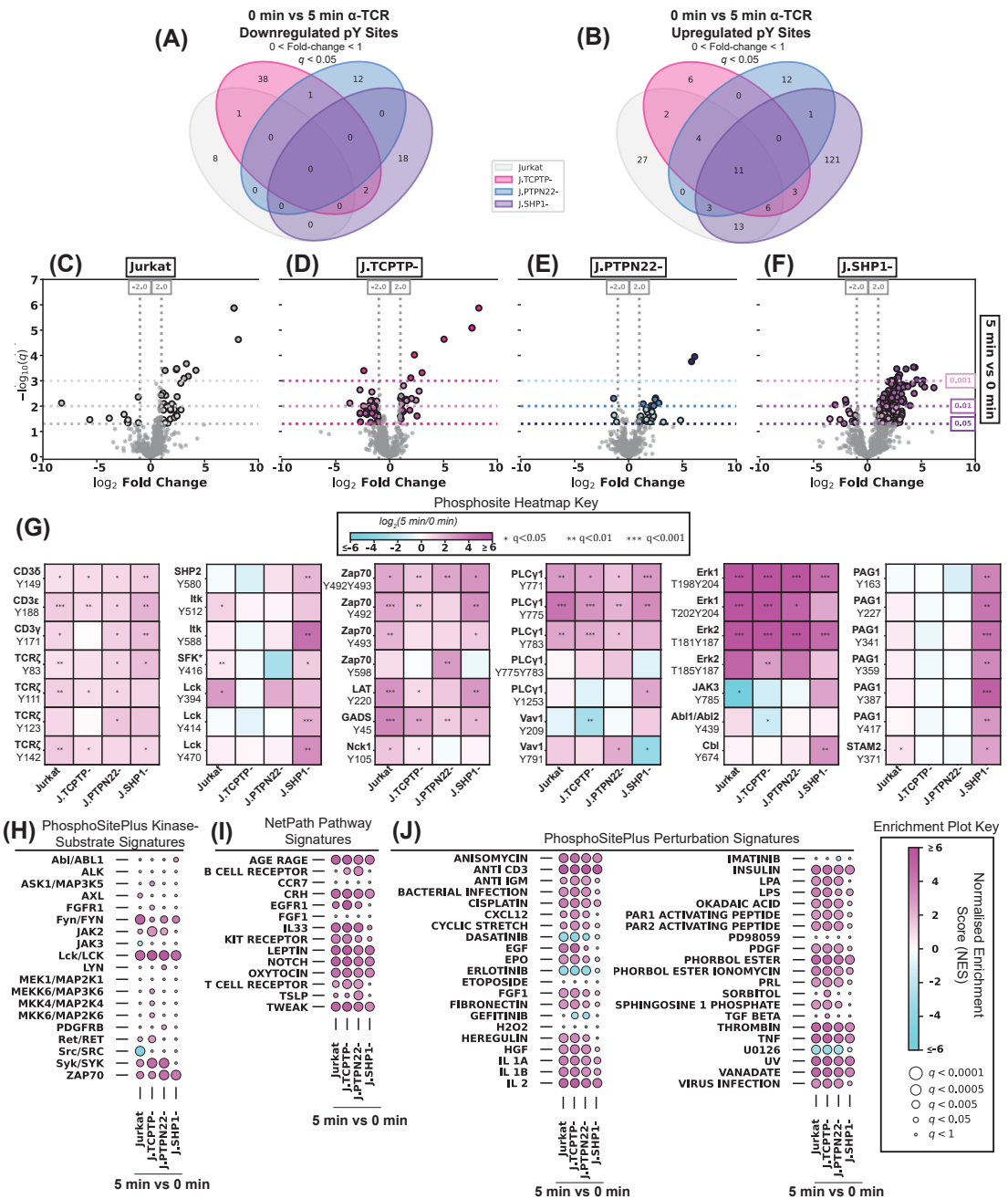


Figure 6.13: PTPN22 knockout mutes TCR signalling, while SHP1 knockout enhances TCR signalling. Venn diagram showing the overlap between (A) downregulated and (B) upregulated pY sites in Jurkat, J.TCPTP-, J.PTPN22-, and J.SHP1- after TCR stimulation. (C -F) Volcano plots comparing unique pY abundance in TCR stimulated (5 min) and unstimulated samples in Jurkat, J.TCPTP-, J.PTPN22, and J.SHP1-. (G) Heatmaps showing fold-change and significance of individual pY sites in the TCR signalling pathway. (H -J) Bubbleplots showing post-translational modification signature enrichment analysis of PhosphoSitePlus Kinase Signatures, NetPath Pathway Signatures, and PhosphoSitePlus Perturbation Signatures, respectively, for comparisons between 5 and 0 min samples.

6.2.5 TCPTP knockout elevates pY abundance and decreases U0126 efficacy during TCR stimulation

U0126 treatment is known to interfere with TCR signalling due to positive feedback from Erk1/2. To evaluate the potential role of TCPTP, PTPN22, and SHP1 in Erk1/2 positive feedback, we treated Jurkats, J.TCPTP-, J.PTPN22-, and J.SHP1- with 20 μ M U0126 before TCR stimulation with α -Jurkat TCR α (clone C305) and sSH2 pY enrichment proteomics, as previously described(Callahan et al., 2024; Griffith et al., 2022). Treatment with U0126 reduced the distinction in PCA clustering between basal state and stimulated samples while generally maintaining cell line clustering (Figure 6.14A). Compared to Jurkats, U0126-treated J.TCPTP- and J.PTPN22- had elevated pY levels in the basal and stimulated states, whereas J.SHP1- had reduced pY abundance in both (Figures 6.14B -D). The number of pY sites observed as significantly downregulated or significantly upregulated in the knockouts was similar to those observed without U0126 (Figures 6.11B-C, 6.12A-B, 6.14A -D), which included many pY sites along the TCR signalling pathway (Figure 6.14E). During U0126 treatment, α -Jurkat TCR α (clone C305) induced mild TCR stimulation in all cell lines, in line with our previous work(Helou et al., 2013), and PTPN22 deletion did not affect the pY induction (Figure 6.15A -G). J.SHP1- cells showed no engagement along the TCR signalling pathway (Figure 6.15F -G), suggesting that the decrease of global phosphorylation due to SHP1 and U0126 treatment is muted after 5 min of stimulation compared to in the absence of U0126 (Figure 6.13). Meanwhile, α -Jurkat TCR α (clone C305) stimulation of J.TCPTP- showed few significantly changing pY sites, but higher fold changes than any other cell line (Figure 6.15D,G). Many of the significantly changing pY sites were on T cell signalling proteins, including Zap70^{Y492Y493}, GADS^{Y45}, PLC γ 1^{Y783}, and Erk1^{T202Y204}. We observed enrichment of Fyn, Lck, and Zap70 kinase substrate signatures in all but J.SHP1- after stimulation (Figure 6.15G -H). We observed enrichment of various NetPath Pathway Signatures in J.TCPTP- and J.PTPN22-, however, TCR pathway signatures were downregulated in Jurkats and not enriched in J.TCPTP-, J.PTPN22-, or J.SHP1- after TCR stimulation, despite significant upregulation of α -CD3 signatures in J.TCPTP- and J.PTPN22- (Figure 6.15I,J). Together, our data show that even with the protective

effect of TCPTP and PTPN22 knockouts on pY abundance, the loss of TCPTP is the most influential concerning U0126 treatment.

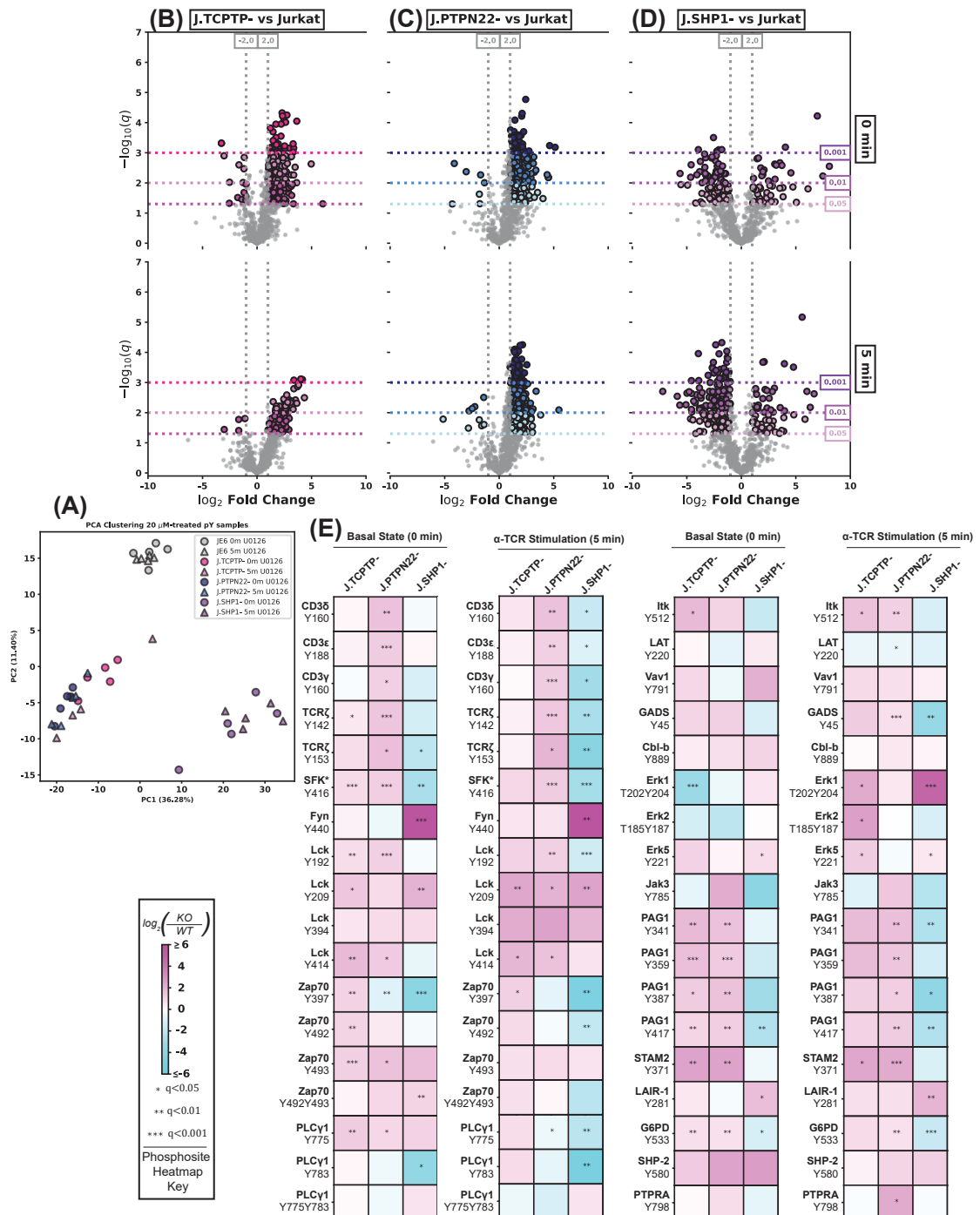


Figure 6.14: (See next page for caption)

Figure 6.14: TCPTP recovers the highest fold-change induction during TCR stimulation with U0126 treatment. Venn diagram showing the overlap between (A) downregulated and (B) upregulated pY sites in Jurkat, J.TCPTP-, J.PTPN22-, and J.SHP1- after TCR stimulation during 20 μ M U0126 treatment. (C -F) Volcano plots comparing unique pY abundance in TCR stimulated (5 min) and unstimulated samples in Jurkat, J.TCPTP-, J.PTPN22, and J.SHP1- during 20 μ M U0126 treatment. (G) Heatmaps showing fold-change and significance of individual pY sites in the TCR signalling pathway during 20 μ M U0126 treatment. (H -J) Bubbleplots showing PTM-SEA of PhosphoSitePlus Kinase Signatures, NetPath Pathway Signatures, and PhosphoSitePlus Perturbation Signatures, respectively, for comparisons between 5 and 0 min samples during 20 μ M U0126 treatment.

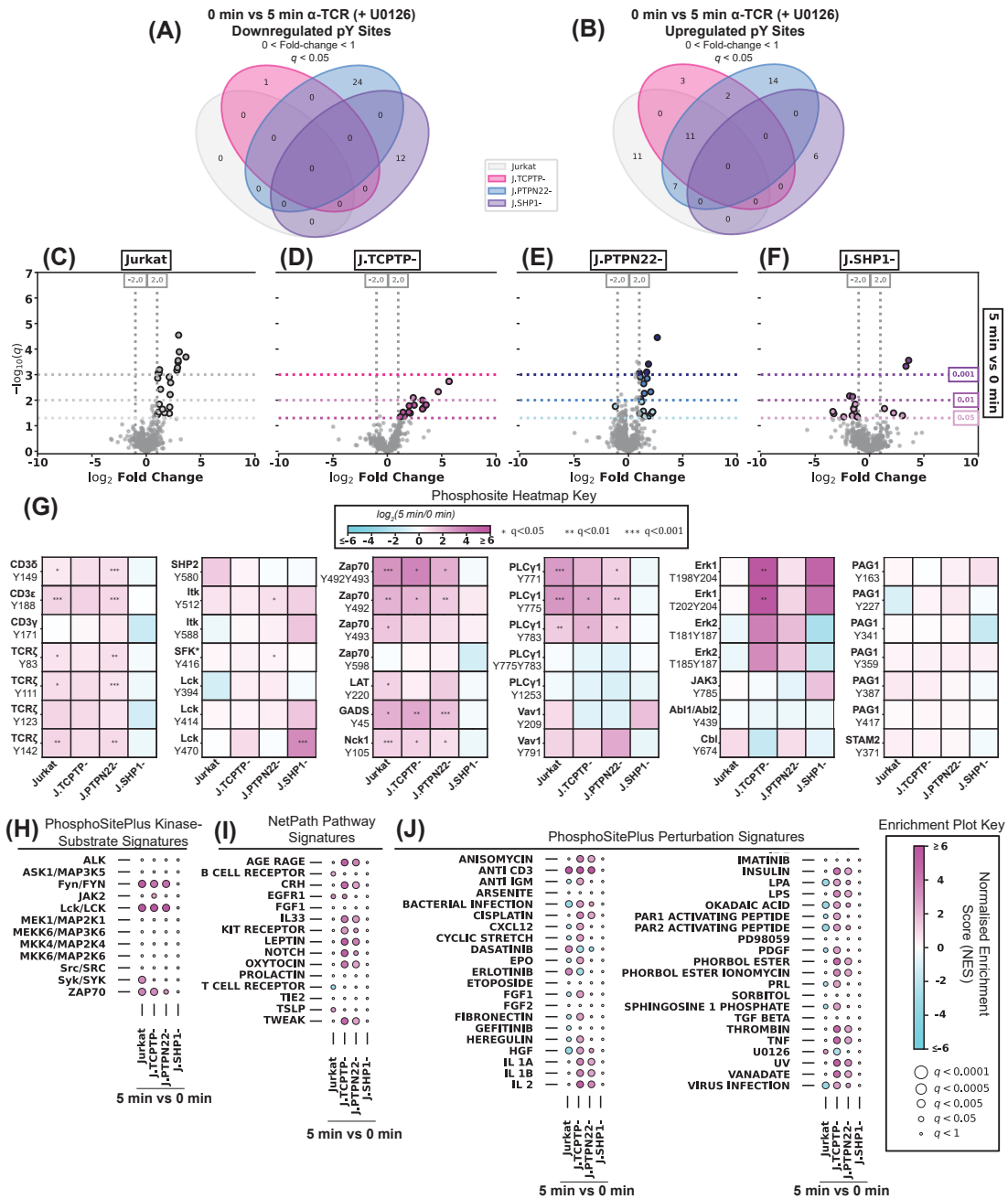


Figure 6.15: U0126 treatment does not change the distribution of pY abundance when comparing knockouts to Jurkats. (A) PCA showing clustering of samples treated with U0126. (B-D) Volcano plots comparing unique pY sites in J.TCPTP-, J.PTPN22-, and J.SHP1-, respectively, to Jurkats in the basal state (0 minute - top row) and stimulated state (5 minute - bottom row) during 20 μ M U0126 treatment. (E) Heatmaps showing individual pY sites compared with Jurkats for each phosphatase KO during 20 μ M U0126 treatment.

6.2.6 TCPTP knockout protects against U0126-dependent pY depletion

Our laboratory previously reported that MEK1/2 inhibition with U0126 leads to global depletion of tyrosine phosphorylation, a primary phenotype of Erk1/2 positive feedback in T cells (Helou et al., 2013). We found the same results here; treatment with U0126 decreased pY abundance in Jurkat T cells in both the basal (140/8 down/up-regulated pY sites) and stimulated (200/3 down/up-regulated pY sites) states (Figures 6.16A, 6.17A -C). The severe loss of pY abundance in Jurkats was phenocopied in J.PTPN22- and J.SHP1-, with few upregulated sites in the basal or stimulated states (Figures 6.16C-D, 6.17A -C). Treatment of J.TCPTP- with U0126 led to a modest loss of pY abundance in the basal state (87/28 down/up-regulated pY sites) that was almost completely neutralised by TCR stimulation (14/17 down/up-regulated pY sites), suggesting that TCPTP loss protects against U0126-induced pY loss on a global level (Figures 6.15E, 6.16B, 6.17A -C). In the basal state, U0126 treatment significantly lowered pY abundance on ITAMs ($CD3\delta^{Y160}$, $CD3\epsilon^{Y199}$, $TCR\zeta^{Y142}$), $Erk1^{T202Y204}$, $Zap70^{Y492}$, and other TCR signalling proteins. In general, pY sites on TCR signalling proteins were the most impacted in J.PTPN22-, with the additional downregulation of Itk^{Y512} , $GADS^{Y45}$, $PLC\gamma1^{Y775}$, $STAM2^{Y371}$, $PAG1^{Y163}$, and $GIT1^{Y545}$ (Figure 6.16F). In the stimulated state, the poor responsiveness to α -Jurkat TCR α (clone C305) exacerbated the pY abundance loss, with the exception of J.TCPTP-. We observed upregulation of non-canonical pY sites on Lck (Lck^{Y209} , Lck^{Y414}), Itk (Itk^{Y273}), and $Zap70$ ($Zap70^{Y248}$), while observing very few pY sites on TCR signalling proteins with significant downregulation after U0126 treatment (Figure 6.16F). Using PTM-SEA, we found that U0126 treatment uniformly downregulated Fyn substrates with minimal effects on Lck substrates, and we observed no significant enrichment of Zap70 substrates (Figure 6.16G). As expected, U0126 treatment uniformly downregulated NetPath Pathway signatures, with significant downregulation of pY sites associated with TCR signalling in the basal and stimulated state of J.TCPTP- (Figure 6.15H). Finally, U0126 treatment led to significant downregulation of α -CD3 signatures across all cell lines and states and upregulation of U0126 signatures in the basal state and stimulated state of J.TCPTP- (Figure 6.16I). Together, these data suggest the

existence of a pathway involving feedback from Erk1/2 through TCPTP that maintains pY balance in Jurkat T cells.

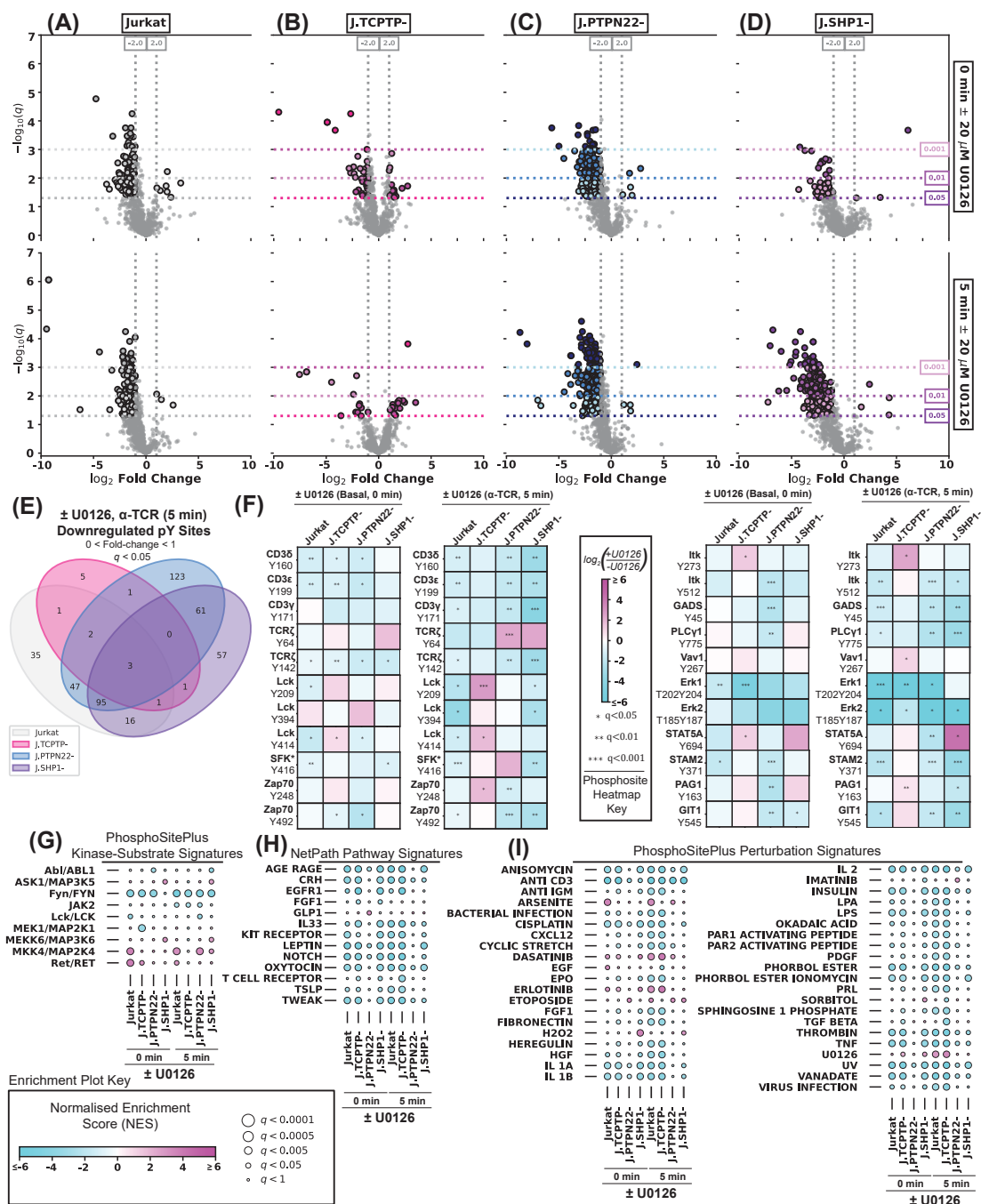


Figure 6.16: TCPTP plays a protective effect on pY abundance in the TCR-stimulated state during U0126 treatment. (A -D) Volcano plots comparing unique pY sites sequenced in Jurkat, J.TCPTP-, J.PTPN22-, and J.SHP1-, respectively, treated with 0.04% DMSO or 20 μM U0126. (E) Venn diagram showing the overlap between downregulated pY sites in Jurkat, J.TCPTP-, J.PTPN22-, and J.SHP1- in the 5 min stimulation condition with or without 20 μM U0126 treatment. (F) Heatmaps showing fold-change and significance of individual pY sites in the TCR signalling pathway with or without 20 μM U0126 treatment. (G -I) Bubbleplots showing PTM-SEA of PhosphoSitePlus Kinase Signatures, NetPath Pathway Signatures, and PhosphoSitePlus Perturbation Signatures, respectively, for basal state and stimulated state comparisons with or without 20 μM U0126 treatment.

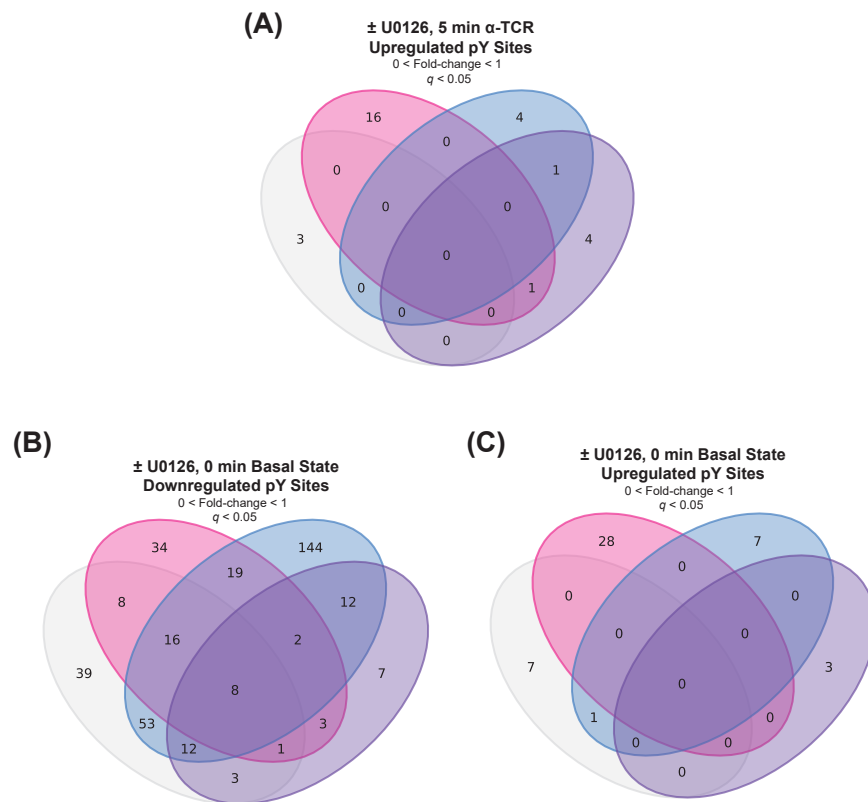


Figure 6.17: Effects of U0126 treatment on Jurkats, J.TCPTP-, J.PTPN22-, and J.SHP1-. (A) Venn diagram showing overlap of unique pY sites that are upregulated in response to U0126 treatment in the stimulated state. (B) Venn diagram showing overlap of unique pY sites that are downregulated in response to U0126 treatment in the basal state. (C) Venn diagram showing overlap of unique pY sites that are upregulated in response to U0126 treatment in the basal state.

6.3 Discussion

Regulation of cellular signalling pathways is necessary for effective and timely responses to external factors, including small molecule perturbation, alterations in nutrients, or influx of particular ligands. In the context of immune cell signalling, alterations in the kinetics, overall duration, or magnitude of signalling pathways cause autoimmune disorders(Ohashi, 2002; Takeuchi et al., 2020). Forward propagation of T cell receptor signalling, in which a pMHC complex on an antigen-presenting cell ligates a TCR for sufficiently long, is a well-studied process that requires the coordinated activation and deactivation of several enzymes, namely tyrosine kinases(Gaud et al., 2018). In particular, regulation of the initiating kinase, Lck, has been the subject of intense research and debate; five phosphatases (CD45 on Lck^{Y505}, CD45/TCPTP/PTPN22/SHP1/JKAP on Lck^{Y394}) and three kinases (Csk on Lck^{Y505}, Lck on Lck^{Y394}, Itk on Lck^{Y192}) are known to act on Lck and control the forward progression of TCR signalling(Bougeret et al., 1996; Takeuchi et al., 1993; Granum et al., 2014; Kästle et al., 2020; Courtney et al., 2017; Abraham and Veillette, 1990; D’Oro and Ashwell, 1999; Gjörlöf-Wingren et al., 1999; Li et al., 2014; Wiede et al., 2011; Wu et al., 2006; Vang et al., 2012; Carter et al., 1999; Cloutier and Veillette, 1999; Johnson et al., 1999; Lefebvre et al., 2003). CD45 and Csk compete for Lck deactivation, as Lck^{Y505} locks Lck in an inactive conformation, preventing autophosphorylation of Lck^{Y394} and subsequent activation of Lck. The four phosphatases TCPTP, PTPN22, SHP1, and JKAP have all been shown to dephosphorylate Lck^{Y394} in vitro. Conflicting evidence exists for the ‘primary’ phosphatase responsible for Lck^{Y394} dephosphorylation(Gjörlöf-Wingren et al., 1999; Li et al., 2014; Wiede et al., 2011; Štefanová et al., 2003; Carter et al., 1999). Complications arise from overwhelming evidence for a positive feedback loop involving the kinases Erk1/2 and Lck that maintains pY levels in T cells, which is thought to involve Lck^{S59} phosphorylation by Erk1/2 that disrupts SHP1 binding despite contradictory evidence(Helou et al., 2013; Paster et al., 2015; Wiede et al., 2011; Štefanová et al., 2003). Fewer studies have evaluated the regulation of the other TCR proximal kinases, Fyn, Zap70, and Itk/Tec, leading to ambiguities in the feedback mechanisms involved in accurate and timely TCR signalling that is crucial for an effective immune response. Our work shows that the phosphatases TCPTP, PTPN22, and SHP1 have distinct regulatory roles in the context of global pY

maintenance and regulation of the T cell receptor.

T cell protein tyrosine phosphatase was one of the first characterised phosphatases in T cells and exists in 48 kDa nuclear- and 45 kDa cytoplasmic-localising isoforms (Cool et al., 1989; Lorenzen et al., 1995; Mosinger et al., 1992). Early work described TCPTP-mediated Lck dephosphorylation (Wiede et al., 2011) and regulation of Janus kinase 1/3 (Perez-Quintero et al., 2024), implicating TCPTP as a key negative regulator of T cell activation pathways (Perez-Quintero et al., 2024). While work investigating the specificity of TCPTP was performed early (Ruzzene et al., 1993), signalling studies of TCPTP in T cells have largely ceased despite the immunological relevance of TCPTP. Recent work evaluating TCPTP has focused on TCPTP structure (Hao et al., 1997; Singh et al., 2022, 2021; Tun et al., 2022), disease-relevant mutations, and drugs (Zikherman and Weiss, 2011; Perez-Quintero et al., 2024; Dong et al., 2023; Flosbach et al., 2020; Tang et al., 2021; Zhang et al., 2024). Our work suggests that TCPTP is a modest negative regulator of T cell receptor signalling (Figure 6.11), and TCPTP loss allows for recovery of TCR induction through a PLC γ 1-dependent pathway (Figure 6.3). Further, loss of TCPTP attenuates the pY loss associated with Erk deactivation (Figure 6.16) and increases fold-induction after TCR stimulation (Figure 6.15). Four non-canonical pY sites on key TCR signalling proteins—Lck^{Y209}, Lck^{Y414}, Zap70^{Y248}, and Itk^{Y273}—are significantly elevated in the TCR stimulated state when J.TCPTP- are treated with U0126 (Figure 6.16F), providing possible phosphorylation sites regulated by TCPTP that are involved in Erk feedback. Notably, Y273 (Itk), Y248 (Zap70), and Y209 (Lck) lie adjacent to, or within, the SH2 domain of their respective kinases; analogous to the well-characterised Itk-dependent phosphorylation of Lck^{Y192}, these modifications could modulate SH2-mediated docking specificity and rewire protein-interaction networks within the T-cell signalosome (Andersen et al., 2019). Thus, TCPTP may be involved in positive feedback mechanisms involving MEK1/2 and Erk1/2 to maintain pY homeostasis and TCR responsiveness in T cells (Figure 6.18).

Protein tyrosine phosphatase non-receptor type 22 (PTPN22; also lymphoid tyrosine phosphatase Lyp) was first isolated in 1993 by Cohen *et al.* and later identified to interact with Csk and dephosphorylate various TCR signalling proteins (Wu et al., 2006;

Cloutier and Veillette, 1996; Vang et al., 2012; Ladner et al., 2005; Chien et al., 2003; Cohen et al., 1999; Hill et al., 2002) cementing the role of PTPN22 as a negative regulator of T cell receptor signalling. Mutations in PTPN22 were then increasingly documented in cases of autoimmune disorders (Bottini et al., 2006; Gomez et al., 2005; Nielsen et al., 2007; Rieck et al., 2007; Smyth et al., 2004; Zheng and She, 2005; Zhernakova et al., 2005), and PTPN22 is being investigated as a druggable target for cancer immunotherapies (Cubas et al., 2020; Ho et al., 2021). Our work is well aligned with previous studies: *PTPN22* knockout elevates TCR pY levels, mutes TCR responsiveness, and enhances the effects of MEK1/2 inhibitor (Figures 6.3, 6.11, 6.13, 6.15, 6.16 and 6.18). Interestingly, PTPN22 deletion elevates the abundance of LAIR-1^{Y281}, an ITIM site required for SHP1 binding (Park et al., 2020; Xu et al., 2000), indicating a potential method of phosphatase crosstalk in TCR regulation. However, our work demonstrates the broader, global effect of PTPN22 on resting pY levels in a TCR-independent context, suggesting that the role of PTPN22 may extend beyond just T cell signalling.

Src homology 2 domain-containing phosphatase 1 (SHP1) was first isolated in 1992 (Plutzky et al., 1992) and subsequently characterised as a negative regulator of various immune signalling pathways, including TCR signalling. In the TCR signalling context, SHP1 dephosphorylates Lck^{Y394} and can associate with unphosphorylated Lck^{S59} to downregulate TCR signalling activity (Chiang and Sefton, 2001; Štefanová et al., 2003; Brockdorff et al., 1999; Lorenz et al., 1994; Plas et al., 1996). SHP1 also binds directly to the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on PD-1 and CEACAM1 (Nagaishi et al., 2006; Sheppard et al., 2004), bringing negative TCR signalling regulators to the active TCR signalosome and preventing exhaustion (Fuertes Marraco et al., 2015). Our work agrees with previous studies that SHP1 acts as a negative regulator of TCR signalling, as *PTPN6* deletion results in upregulation of various TCR signalling phosphorylation sites Lck^{Y192}, Lck^{Y394} (trending but n.s. in all knockouts), Zap70^{Y493}, LAT^{Y220}, PLC γ 1^{Y775Y783}, and SHP2^{Y580} in the stimulated and/or basal states (Figure 6.11G). In contrast, global pY abundance appears to drop without SHP1 (Figure 6.11), and more pY sites become strongly TCR responsive (Figure 6.13). Some pY proteins, including PAG1 and Cbl, are known negative regulators of TCR signalling (Naramura

et al., 2002; Smida et al., 2013), suggesting that SHP1 may play a context-dependent role in suppressing negative regulators of the TCR. Although the role of SHP1 in T cell pY maintenance and TCR signalling is still evolving, SHP1 is implicated in many autoimmune disorders and cancers (Croker et al., 2008; Markovics et al., 2020; Sharma et al., 2016; Shultz et al., 1997; Snook et al., 2020; Varone et al., 2020), thus understanding the mechanisms of SHP1 regulation of T cell signalling is imperative.

Regulation of T cell activation is a critical process for maintaining a coordinated immune response. Loss-of-function mutations in the PTPs TCPTP, PTPN22, and SHP1 cause autoimmune disorders through immune system dysregulation, particularly in T cells and their responsiveness to antigens. The redundancy of these phosphatases, coupled with the small number of TCR-responsive pY sites, raises questions regarding the timing, specificity, and substrates of these proteins. Our work addresses the questions of timing and specificity: each phosphatase has a distinct influence on the phosphotyrosine proteome in T cells, and each controls a subpopulation of TCR-responsive pY sites. Further, we show that TCPTP has a protective effect on MEK1/2 inhibition, a role unique to TCPTP. To better understand the unique role each phosphatase plays in T cell biology, it is necessary to investigate the specific substrates for each phosphatase and their effects on those pathways.

Because SHP1 (Zhao and Jiang, 2024), TCPTP (Baumgartner et al., 2023) and PTPN22 (Ho et al., 2021; Cubas et al., 2020) are now being pursued as drug targets, our mechanistic insights have immediate translational relevance. Specifically, the observation that TCPTP loss blunts the global drop in tyrosine phosphorylation typically triggered by MEK1/2 inhibition implies that cancers or immune cells with diminished TCPTP activity may exhibit primary resistance to MAP-kinase -pathway drugs. Pairing MEK/ERK blockade with targeted restoration of TCPTP, through promoter demethylation, redox-protective stabilization of its catalytic cysteine, or vector-mediated re-expression, could resensitize these otherwise refractory cells. Conversely, the global pY elevation (Figure 6.11E) and muted TCR responsiveness (Figure 6.13E) we observed upon *PTPN22* deletion identify easily quantifiable phosphotyrosine signatures that could act as pharmacodynamic biomarkers for forthcoming PTPN22-directed therapies. Together,

these observations underscore the value of biomarker-guided combination strategies that account for the distinctive feedback circuits controlled by each phosphatase.

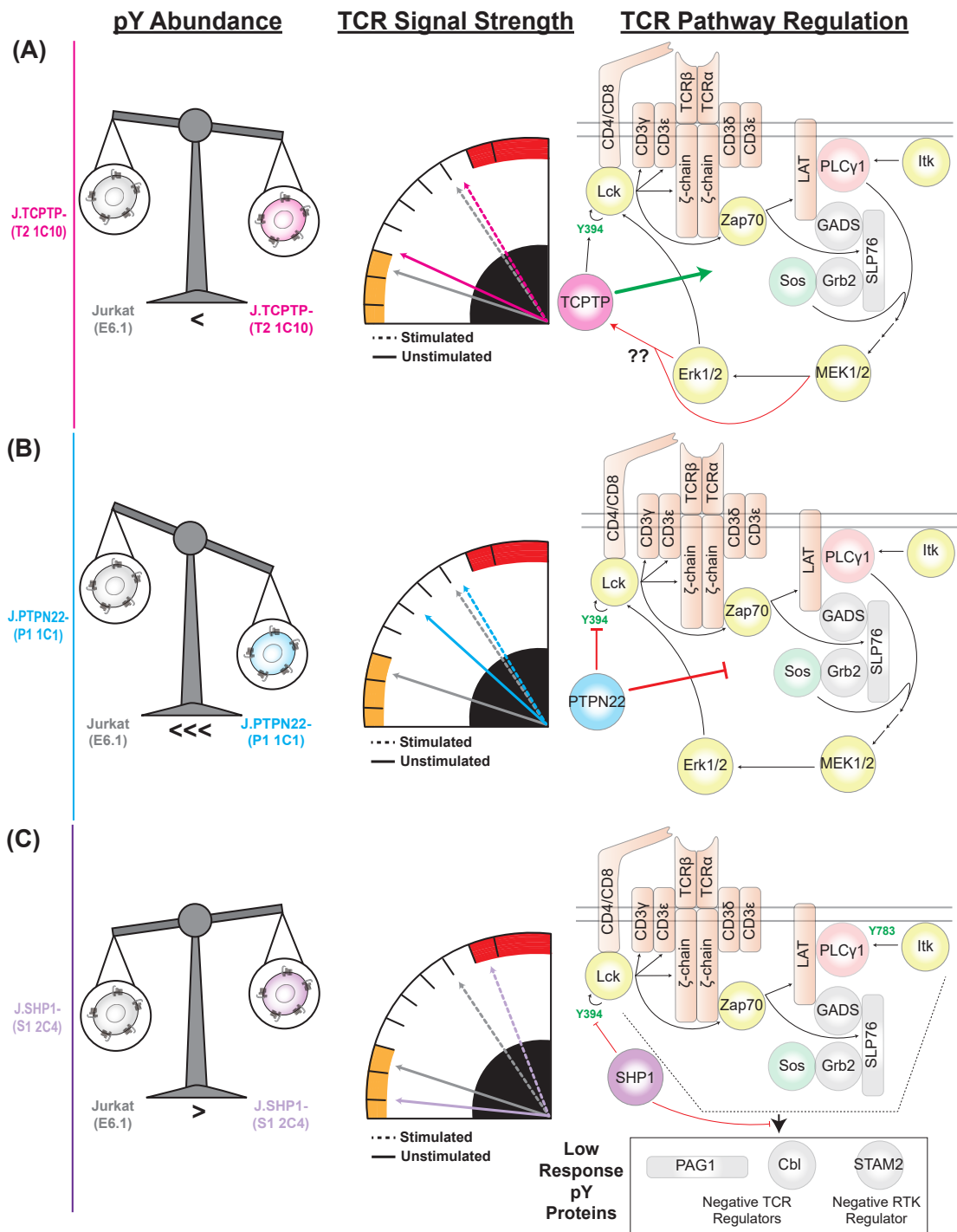


Figure 6.18: (See next page for caption)

Figure 6.18: A model depicting the effects of (A) TCPTP, (B) PTPN22, and (C) SHP1 on global pY abundance, TCR stimulation strength, and TCR pathway regulation. Global pY abundance increases slightly upon deletion of PTPN2, largely upon deletion of PTPN22, and decreases slightly upon PTPN6 deletion. J.TCPTP- cells respond similarly to Jurkats when stimulated with α -Jurkat TCR α , whereas J.PTPN22- cells have a muted response, and J.SHP1- cells have an elevated response. Loss of TCPTP ameliorates Erk positive feedback in the stimulated state, thus TCPTP is involved in a feedback pathway involving Erk1/2 and/or MEK1/2. Loss of PTPN22 elevates pY abundance globally and mutes TCR responsiveness, thus PTPN22 acts as a major negative regulator of the TCR signalling pathway. Loss of SHP1 slightly elevates TCR signalling pathway pY abundance while altering the regulation of negative TCR regulators and globally reducing pY abundance, thus SHP1 regulates other forms of negative feedback to the TCR.

6.4 Methods

6.4.1 Materials

Flow antibodies included CD3 ϵ PE (1:50, BD Pharmingen #555333), TCR α PE (1:50, BD Pharmingen #555548), CD45 FITC (1:50, eBioscience #11-0459-41). Western Primary Antibodies included pY clone 4G10 (1:2000, Cell Signalling Technologies #96215), PLC γ 1 (1:10,000, Millipore Sigma #05-163), phosphorylated PLC γ 1^{Y783} (1:2000, Cell Signalling Technologies #2821), Erk1/2 (Cell Signalling Technologies #9107), phosphorylated Erk^{T202Y204} (1:2000, Cell Signalling Technologies #9101), Lck (1:5000, Cell Signalling Technologies #2657S), phosphorylated Src Family Kinase Y416 (1:2000, Cell Signalling Technologies #59548S, 2101S), TCPTP (1:2000, Cell Signalling Technologies #58935S), PTPN22 (1:1000, Thermo #11783 -1-AP), SHP1 (1:500, Santa Cruz Biotechnology #sc-287), and Vav1 (1:2000, Cell Signalling Technologies #4657S, 2505S). Western secondary antibodies included IRDye 680RD Goat anti-Mouse IgG (LI-COR #926-68070) IRDye 800CW Goat anti-Mouse IgG (LI-COR #926-33210), IRDye 680RD Donkey anti-Rabbit IgG (LI-COR #926-68073), and IRDye 800CW Donkey anti-Rabbit IgG (LI-COR #926-32213).

6.4.2 Cell culture and generation of PTP knockout cells

Jurkat T cells (clone E6.1, ATCC #TIB-152) and all JE6 derivatives were maintained in RPMI 1640 with 10% FBS (PeakSerum #PS-FB3), 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin (PSQ; HyClone #SV30082.01), and 10 mM HEPES, pH 7.4, at 5% CO₂ in a humidified incubator. Jurkat T cells with a PTPN22 (J.PTPN22-), PTPN2 (J.TCPTP-), or PTPN6 (J.SHP1-) genomic deletion were generated by CRISPR Cas9 by using guide RNAs targeting *PTPN22* (Forward = CACCGTCTTGCTTTGGGCCTCATCC, Reverse = AAACGGATGAGGCCCAAAGCAAGAC), *PTPN2* (Forward = CACCGTTTCGAACCTCCCGCTCGATGG, Reverse = AAACCCATCGAGCGGGAGTTTGAAC), and *PTPN6* (Forward = GTGAGTTCTGGATCCGAATAT, Reverse = AAACATATTTCGGATCCAGAACTCAC). Guide RNAs were cloned into the pSpCas9(BB)-2A-GFP vector or the PX458 vector before transfection by electroporation (260 V, 1250 μ F) and a recovery period of 48 h in RPMI 1640

with 10% FBS and no antibiotics. Single cells were sorted for high CD3 expression, an integral component of the T cell receptor complex, before expansion of single-cell clones. Clones were screened for PTPN22, TCPTP, or SHP1 expression by Western blot before sequencing to confirm the disruption of the gene.

6.4.3 Flow cytometry

To evaluate the expression of cell surface receptors in the PTP knockout Jurkats, we collected one million cells by centrifugation, resuspended in flow cytometry buffer (2% FBS in 1X DPBS), and then incubated in fluorescently labelled antibodies for 30 min on ice in the dark. After receptor staining, the cells were washed 3 times in 1 mL, resuspended in 0.1 mL of flow cytometry buffer, then fixed with 2% PFA in the dark for 15 min at room temperature. PFA was washed off the cells 3 times with 1 mL flow cytometry buffer, then resuspended in 0.1 mL flow cytometry buffer before analysis on a Cytex Aurora flow cytometer. The resulting flow cytometry data were analysed and plotted using Floreada.

6.4.4 Inhibitor treatment and TCR stimulation

Inhibition of MEK1/2 was performed using U0126 as previously described (Helou et al., 2013). Briefly, a stock of U0126 (Cell Signaling Technology cat #9903) was resuspended at a concentration of 50 mM in 100% DMSO under sterile conditions and diluted in unsupplemented RPMI 1640 at a concentration of 20 μ M (0.04% DMSO) before use with cells. A control treatment of 0.04% DMSO without U0126 was included for all treatments. For phosphotyrosine mass spectrometry analysis, 1×10^8 cells were used, and for small-scale experiments, 1×10^7 cells were used. For mass spectrometry experiments, a minimum of four biological replicates were used per condition, and for small-scale experiments, a minimum of four biological replicates were used per condition. Cells were harvested by centrifugation before resuspension in U0126 RPMI or control RPMI at a concentration of 1×10^6 cells per millilitre for 2.5 h. After treatment, cells were washed in warm 1 $\times$ DPBS twice before resuspension at 2×10^8 cells per millilitre and a 30-min rest period. T cell receptor stimulation was initiated as previously described by a 1-to-1 addition of α -Jurkat TCR α (Clone C305, a gift from Arthur Weiss) for a final

concentration of 1 $\mu\text{g}/\text{mL}$ and allowed to proceed for 5 min before lysis. Basal state samples were prepared by the addition of urea lysis buffer, vigorous vortexing, and then addition of α -TCR α antibody solution to the lysed samples to maintain cell equivalence amongst samples.

6.4.5 Lysis, reduction, alkylation, and digestion for proteomics

All samples were lysed using urea lysis buffer (8 M urea, 20 mM HEPES pH 7.4, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate) and vigorous vortexing. Samples were incubated on ice for at least 15 min before sonication at 70% amplitude for 30 s, twice. Samples prepared for Western blot analysis were prepared by a 1-to-1 dilution in 2 $\times$ Laemmli sample buffer (2% SDS, 20% Glycerol, 0.01% bromophenol blue, 0.125 M Tris -HCl, 5% β -mercaptoethanol) followed by 5 min at 95 C. Protein concentrations were determined using the Pierce BCA Assay kit (Thermo #23225) using the manufacturer's instructions. The total protein across all samples for phosphotyrosine proteomics was adjusted to 5 mg per replicate, with a total of 5 biological replicates per condition. For global phosphoenrichment, samples were normalised after data acquisition to whole-cell lysate distributions. Urea lysates were reduced using 10 mM dithiothreitol for 30 min at 37 C and subsequently alkylated using 10 mM iodoacetamide for 30 min at room temperature. Lastly, samples were digested using sequencing grade modified trypsin (Promega #V5113) in a 1:50 (w/w) trypsin to protein ratio overnight at 37 C. Tryptic peptides were acidified at a final percentage of 1% (v/v) trifluoroacetic acid (TFA), centrifuged at 1800 $\times g$ at room temperature for 5 min to collect debris, then loaded onto Sep-Pak C18 vacuum cartridges (Waters #WAT020515) for desalting (Nguyen et al., 2009). Samples were desalted in parallel with 12 mL of 0.1% TFA, eluted using 7 mL of 0.1% TFA in 40% acetonitrile, then diluted 1:1 in 0.1% TFA to reduce the acetonitrile concentration to 20%, frozen at - 80 C overnight, and lyophilised for 48 h before phosphoenrichment.

6.4.6 Superbinder SH2 pY enrichment

Selective enrichment of pY-containing peptides was performed using the superbinder SH2 (sSH2) domain as described previously (Callahan et al., 2024; Griffith et al., 2022). Briefly, recombinant sSH2 was purified and conjugated to cyanogen bromide-activated sepharose beads (GE Healthcare, Wauwatosa, Wisconsin) for pY peptide capture. Desalted samples were resuspended in 3 mL IAP buffer (10 mM sodium phosphate monobasic monohydrate, 50 mM sodium chloride, 500 mM MOPS, pH 7.2) before a 2-h incubation with about 1 mg of sSH2-containing beads at 4 C on an end over end rotator. After incubation, the beads were washed with 1 mL of ice-cold IAP buffer thrice, and then 1 mL of ice-cold HPLC-grade water thrice. Peptides were eluted from the sSH2 beads with 0.15% TFA on a 1,150 rpm thermomixer for 10 min at room temperature twice before desalting with Pierce C18 tips (Thermo #87784) according to the manufacturer's instructions. Peptides were then dried by speed-vac and stored at -80°C until LC-MS/MS analysis.

6.4.7 Liquid chromatography-tandem mass spectrometry

Phosphotyrosine-enriched peptides were separated on a lab-packed C18 column (150 mm by $75\text{ }\mu\text{m}$) with XSelect CSH C18 $2\text{ }\mu\text{m}$ resin using an Ultimate 3000 RSLCnano system. Peptides were eluted using a 90-min method starting with a Buffer A (0.1% formic acid in HPLC water) to Buffer B (0.1% formic acid in acetonitrile) ratio of 98:2 and progressing to a final ratio of 70:30 in 69 min, with a 90% buffer B spike at the end of the gradient. Peptides were analysed on an Orbitrap Exploris 480 mass spectrometer in positive ion mode with a spray voltage of 2.2 kV, funnel RF level of 40, and heated capillary temperature of 275°C . Precursor mass range was set to 375-1500 m/z with a resolution of 120,000, normalised AGC target of 300%, IT of 50 ms in profile mode. Fragmentation spectra were collected using a cycle time DDA mode of 2.5 s with a dynamic exclusion time of 20 s, and an intensity threshold of 50,000. Monoisotopic precursor ions with charge states between + 2 and + 6 were fragmented using higher-energy collision-induced dissociation (HCD) using a normalised collision energy of 30%. Fragmentation spectra were acquired using a 0.7 m/z isolation window

at a resolution of 15,000 and a normalised AGC target of 100% in centroid mode with an injection time of 100 ms.

Whole-cell digest peptides were separated on an Aurora C18 column (250 mm by 75 μ m; IonOpticks #AUR3-25075C18) with 1.7 μ m C18 using an Ultimate 3000 nanoRSLC. Peptides were eluted using a 120-min method starting with a Buffer A (0.1% formic acid in HPLC water) to Buffer B (0.1% formic acid in acetonitrile) ratio of 98:2 and progressing to a final ratio of 70:30 in 69 min, with a 90% buffer B spike at the end of the gradient followed by a short wash gradient from 2 to 99%. Peptides were analysed on a QExactive mass spectrometer in positive ion mode with a spray voltage of 2.1 kV, funnel RF level of 40, and heated capillary temperature of 275 C. Precursor mass range was set to 400 -1800 m/z with a resolution of 70,000, AGC target of 3e6, IT of 200 ms in centroid mode. Fragmentation spectra were collected using a Top9 DDA method with a dynamic exclusion time of 30 s, and an intensity threshold of 1e3. Monoisotopic precursor ions with charge states between + 2 and +5 were fragmented using higher-energy collision-induced dissociation (HCD) using a normalised collision energy of 30. Fragmentation spectra were acquired using a 2.5 m/z isolation window at a resolution of 17,500 and an AGC target of 2e4 in centroid mode with a maximum injection time of 200 ms.

6.4.8 Database searching and relative phosphopeptide abundance

For sSH2 enriched samples, Thermo .RAW files were analysed using the high throughput autonomous proteomics pipeline (HTAPP) and PeptideDepot, custom in-house software for the automation of PTM proteomics analysis (Yu and Salomon, 2010; Belmont et al., 2017). MS2 spectra were searched using Mascot against the UniProt complete proteome dataset (93,802 nonredundant forward sequences, downloaded August 2019) to 0.1% FDR using a reverse decoy database. The following parameters were used for the search: trypsin enzyme cleavage (up to 2 missed cleavages), 7 ppm precursor mass tolerance, 20 mmu fragment ion mass tolerance, variable modifications for phosphorylation (S/T/Y; +79.9963 Da), oxidation (M; +15.9949 Da), and static modification of carbamamidomethylation (C;+57.0215 Da). Retention time alignment and integration

of selected ion chromatograms for each peptide spectrum match were used to determine relative peptide abundance as previously described (Belmont et al., 2017; Helou et al., 2015).

Raw files were evaluated for whole-cell digest samples using MaxQuant (Version 2.1.3) with the integrated peptide search engine Andromeda, as described previously (Cox and Mann, 2008; Cox et al., 2011). Briefly, MS2 spectra were searched against the human UniProt database (Homo sapiens, downloaded August 2019) containing 93,802 forward protein sequences. For peptide spectrum matches and proteins, the false discovery rate (FDR) upper threshold was set to 0.01% and was determined using a reverse sequence decoy database approach. The following modifications were set for database searching: carbamidomethylation (cysteine) was fixed, oxidation (methionine) was variable, acetylation (protein N-terminus) was variable, phosphorylation (serine, threonine, tyrosine) was variable. Trypsin specificity was used, and a maximum of two missed cleavages were tolerated. For the main search, the peptide tolerance was set to 7 ppm, whereas the MS/MS tolerance was set to 20 ppm. The search parameter files (mqpar.xml), and the search results Phospho (STY) Sites.txt, proteinGroups, and evidence files are available at the PRIDE (Jones et al., 2006) partner repository under the dataset identifier PXD058624.

6.4.9 Gel electrophoresis and western blotting

Samples were separated on 10% Tris -Glycine polyacrylamide gels cast in-house using the BioRad Mini-Gel apparatus and transferred to a 0.45 μ m polyvinylidene difluoride Immobilon membrane (Millipore Sigma #IPFL00010) before blocking using Intercept TBS Blocking Buffer (LI-COR Biosciences #927-60003). Blocked membranes were incubated with a primary antibody solution containing 5% BSA in 1 $\times$ Tris-buffered saline with Tween 20 (TBST) at 4 C overnight. After primary antibody incubation, membranes were washed three times with 1 $\times$ TBST before incubation with a secondary antibody solution for 1 h at room temperature. After secondary antibody incubation, the membranes were washed three times with 1 $\times$ TBST and then washed twice with 1 $\times$ TBS before visualisation using the Odyssey CLx and Odyssey M Imaging Systems (LI-COR

Biosciences). Western blots were run in 2-replicate pairs for each group for a total of 4 replicates, and quantification was performed by adjusting the signal to the loading control, as previously described (Pillai-Kastoori et al., 2020), normalising to the 0-min group, then combining replicate values between two blots before $\log_2$ transformation and statistical analysis. Due to changes in antibody lots and catalogue numbers for some targets (SFK^{Y416}, Vav1), a direct comparison between cell lines was not performed, and all data presented are relative to their respective 0 min/no treatment samples. Statistical significance between the mean $\log_2$ transformed, basal-state normalised fold changes was performed using a one-way ANOVA with Fisher’s LSD and the Holm Sidak FWER correction (Fisher, 1935; Hayter, 1986; Holm, 1979; Šidák, 1967).

6.4.10 Data analysis and code availability

Post-search analysis of LC -MS/MS data was performed on Ubuntu 22.04 LTS on the Windows Subsystem for Linux (version 2) using Python (version 3.12.5) with the following dependencies: matplotlib (version 3.9.1), scipy (version 1.14.0), numpy (version 2.0.1), scikit-learn (version 1.5.1), pandas (version 2.2.2), MissingPy (modified for updates with scikit-learn), py-venn, and a custom ‘helpers’ module.

The “proteinGroups.txt” file from MaxQuant was used to analyse protein profiling data. The proteinGroups table was filtered to remove “Reverse” and “Potential contaminants” flagged rows, and proteins with $> 10\%$ sequence coverage were kept for further analysis. Missing values were imputed using the Random Forest method using the MissingPy module (Jin et al., 2021; Stekhoven and Bühlmann, 2012). Welch’s T tests (Welch, 1947) with Benjamini & Hochberg’s FDR correction (Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003) were performed to compare the mean log-transformed abundances of each cell line. For plotting, only rows containing > 3 measured values were included. Replicate clustering and reproducibility were evaluated using principal component analysis and multiple linear regression after imputation. KEGG pathway enrichment was performed using ssGSEA2.0 with the KEGG Msig database⁵⁴.

For pY data, the unique pY sites and their abundances were exported from PeptideDepot. Missing values were imputed as described above, and statistical significance

was determined as described above, except comparisons were made as shown in Figures 6.11, 6.13, 6.14, 6.15, and 6.16. Principal component analysis and linear regression were used to show replicate clustering and reproducibility, and ssGSEA2.0 was used to show pathway enrichment using the PTMsig database(Krug et al., 2019).

6.4.11 Data Availability

All mass spectrometry data have been uploaded to the ProteomeXchange Consortium via the PRIDE113 partner repository under the dataset identifier PXD058624. All code used for the analysis and presentation of our mass spectrometry data are publicly available on GitHub (<https://github.com/Aurdeegz/Phosphatase-Project/>) and are available upon corresponding author on reasonable request.

6.5 Acknowledgements

The authors would like to thank the Proteomics Core Facility at the University of Arkansas Medical School for collecting phosphotyrosine proteomics data, and the Proteomics Core Facility at Brown University for collecting whole cell digest proteomics data. We acknowledge the NIH grants R01AI083636, P01AI091580, P20GM121293, S10OD036295, and R24GM137786 for financial support for our research. We also thank Dr. Arthur Weiss and his laboratory for providing α -Jurkat TCR α (clone C305).

Competing Interests

The authors declare no competing interests.

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CHAPTER 7

Phosphoproteomic analysis of successive Jurkat CD19-CAR generations reveals TCR ζ -driven signalling

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Abstract Although chimeric antigen receptor (CAR) T cell therapy has revolutionised individualised cancer therapies for relapsed/refractory lymphomas, low long-term retention due to basal signalling (antigen-independent activation in the absence of cognate antigen) and off-target toxicity limit the broad applicability of CAR-T products. During CAR development, researchers use model systems, like Jurkat T cells (Jurkats), to screen intracellular signalling arrangements based on their ability to activate (e.g., CD69 expression) and withstand repeated antigen encounters. Although Jurkats are standard for CAR screening, the mapping of CAR generations to Jurkat-specific pTyr networks relative to key TCR nodes and CD69 readouts is not well defined, blurring how hierarchical signalling drives activation. Here, we investigated how costimulation influenced tyrosine phosphorylation cascades using LC-MS/MS based phosphotyrosine (pY) proteomics and CD69 expression in the presence of small molecule inhibitors of key TCR signalling regulators. We found that including TCR ζ (CD3 ζ ; gene CD247) in first (ζ -CAR), second (28 ζ -CAR and BB ζ -CAR), and third (28BB ζ -CAR) generation CARs largely determined pY signalling, irrespective of costimulation. Further, we showed that the phosphatase activity of PTPN22 and SHP-1 were largely negligible for activation of CARs, but indiscriminate inhibition of phosphatases using pervanadate (PV) selectively activated BB ζ -CARs without antigen encounter. Finally, we found that selective, partial inhibition of Itk using Soquelitinib reduced basal CD69 expression in CAR-Jurkat cells while maintaining their ability to activate in response to antigen. These data suggest that TCR ζ determines the pY signalling profile and that Itk drives basal activation of

CD19-CAR Jurkats, which may impact evaluation of new CAR designs in CAR-Jurkat screens.

7.1 Introduction

Chimeric antigen receptor (CAR) T cell therapy is a monumental achievement in the advancement of immunotherapies, providing the first potential modular therapeutic for a variety of disease states including autoimmune disorders and cancers (Abbasi et al., 2023; Lindner et al., 2020; da Silva, 2021). CAR designs include an extracellular targeting domain, typically a single chain variable fragment (scFv) of a highly specific antibody, a transmembrane domain, typically from T cell protein CD8 or CD28, and one or more intracellular signalling domains scavenged from the T cell receptor (TCR) itself and/or costimulatory receptors (Lindner et al., 2020; Sadelain et al., 2013). In combination, the structural components of CARs are designed to ligate a disease associated antigen (DAA), promote immune synapse formation (Dong et al., 2020; Davenport et al., 2018; Chockley et al., 2023), initiate T cell activation through canonical TCR signalling networks (Salter et al., 2018, 2021; Griffith et al., 2022), and mount an immune response toward the targeted cell (Qin et al., 2021). Currently, six CARs targeting CD19 (Sadelain et al., 2013) and BCMA (Lindner et al., 2020) are clinically approved for patients with various blood cancers, providing the last line of treatment for relapsed/refractory cancers. These successes are the result of hundreds of clinical trials targeting dozens of cancer-associated cell surface proteins (985 total clinical trials, 159 completed, 88 terminated for ‘Chimeric Antigen Receptor T Cell’ on clinicaltrials.gov as of 9 September 2025). Table 1 displays the results for clinical trials involving CD19-targeted CAR T cells listed “with results” and show 5 active (not recruiting), 10 terminated (only 1 for safety concerns), and 20 completed studies, which have led to the approval of 4 CD19-CAR T cell therapies. High antigen density on tumours and low antigen density on healthy cells is a requirement for the safety and efficacy of CAR T cells (Majzner et al., 2020; Ramakrishna et al., 2019) leading some researchers to propose split-CARs that only promote T cell activation in the presence of two antigens expressed on the target cell (He et al., 2020; Li et al., 2024). Regardless of design, the promise of a treatment adaptable to a specific disease state is

highly desirable and has the potential to reshape personalised medicine.

Chimeric antigen receptors are designed to engage a TCR-like signalling cascade that results in cytokine expression and granzyme/perforin release. Numerous groups have evaluated the signalling capacity of CARs using various low-throughput (Li et al., 2024; Philipson et al., 2020) and high-throughput (Salter et al., 2018, 2021; Griffith et al., 2022; Yao et al., 2024) methods and proposed similar models for CAR-based T cell activation. Work from Salter et al. (2018), Salter et al. (2021), and Griffith et al. (2022) used mass spectrometry based phosphotyrosine (pY) proteomics to show that both primary CAR T cells or model Jurkat CAR T cells (CAR-Jurkats) induce phosphorylation changes similar to those of canonical TCR signalling with some key differences (Salter et al., 2018; Griffith et al., 2022). First, Lck associated with CD28-domain containing CARs phosphorylates TCR ζ ITAMs of high molecular weight (i.e., on the CAR), promoting the binding of Zap70 to the CARs TCR ζ ITAMs and subsequent activation of Zap70 by Lck. Activated Zap70 and Lck engage the LAT-SLP76 signalosome, although LAT itself is dispensable for CAR activation in third generation CARs which include a CD28 and 4-1BB domain (Dong et al., 2020). Studies frequently observe phosphorylation of Itk^{Y512} and Itk substrates PLC γ 1^{Y783} and GADS^{Y45}, which are thought to act as signal integrators for CD28 costimulation (Hallumi et al., 2021). Notably, CD3 ϵ /CD3 γ /CD3 δ ITAMs are seldom observed as significantly induced after CAR engagement in both Jurkat and primary T cells, and further low throughout investigation has shown that CAR- and TCR-based signalling cascades are independent (Salter et al., 2021; Griffith et al., 2022; Barden et al., 2023). Despite the potential similarities in pY signalling, costimulation domains within CARs can have profound effects on constitutive activation, retention, and exhaustion (Cappell and Kochenderfer, 2021; Zhao et al., 2015), suggesting that there exist unexplored activation pathways in CAR T cells with different costimulatory domains.

Recent approaches to improve CAR T cell therapy include screening hundreds of CAR designs in model systems for repeatable T cell activation, high CAR retention, and reduced basal T cell activation. Many researchers use the Jurkat model T cell line (Jurkats hereafter) during CAR screening (Rydzek et al., 2019; Gordon et al., 2025;

ID (clinicaltrials.gov)	Sponsor	Disease(s)	Target(s)	Phase	Outcome
NCT03570892	Noartis Pharmaceuticals	Aggressive B-NHL	CD19	III	Active, not recruiting
NCT03391466	Kite	DLBCL	CD19	III	Completed
NCT02028455	Seattle Children's Hospital	Leukemia	CD19	II	Active, not recruiting
NCT03573700	St. Jude Children's Research Hospital	ALL	CD19	II	Active, not recruiting
NCT01318317	City of Hope Medical Center	B-NHL	CD19	II	Active, not recruiting
NCT03289455	Autolus Limited	B-ALL	CD19/CD22	II	Completed
NCT02614066	Kite	B-precursor ALL	CD19	II	Completed
NCT00924326	NCI	B-cell lymphoma	CD19	II	Completed
NCT01865617	Fred Hutchinson Cancer Center	CLL, NHL, ALL	CD19	II	Completed
NCT01747486	University of Pennsylvania	CLL, SLL	CD19	II	Completed
NCT02601313	Kite	MCL	CD19	II	Completed
NCT02030847	University of Pennsylvania	ALL	CD19	II	Completed
NCT02926833	Kite	DLBCL	CD19	II	Completed
NCT01626495	University of Pennsylvania	Leukemia, Lymphoma	CD19	II	Completed
NCT02030834	University of Pennsylvania	Lymphoma	CD19	II	Completed
NCT03761056	Kite	LBCL	CD19	II	Completed
NCT04002401	Kite	NHL	CD19	II	Completed
NCT02348216	Kite	B-ALL	CD19	II	Completed
NCT03792633	University of Pennsylvania	B-ALL	CD19	II	Completed
NCT02935543	University of Pennsylvania	ALL	CD19	II	Completed
NCT02794246	University of Pennsylvania	MM	CD19	II	Terminated (Administrative reasons)
NCT03743246	Celgene	B-ALL, B-NHL	CD19	II	Terminated (no benefits versus current therapies)
NCT02535364	Juno Therapeutics	B-ALL	CD19	II	Terminated (Safety)
NCT02208362	City of Hope Medical Center	Malignant Glioma	CD19	I	Active, not recruiting
NCT03448393	NCI	B-cell malignancies	CD19/CD22	I	Completed
NCT02659943	NCI	B-cell malignancies	CD19	I	Completed
NCT01593696	NCI	B-cell leukemia, B-cell lymphoma	CD19	I	Completed
NCT03019055	Medical College of Wisconsin	B-cell malignancies	CD20/CD19	I	Completed
NCT01029366	University of Pennsylvania	B-cell lymphoma	CD19	I	Completed
NCT03287817	Autolus Limited	DLBCL	CD19/CD22	I	Terminated (Company's choice)
NCT03624036	Kite	CLL, SLL	CD19	I	Terminated (Development program terminated)
NCT03704298	Kite	LBCL	CD19	I	Terminated (Development program terminated)
NCT04314843	Kite	LBCL	CD19	I	Terminated (Development program terminated)
NCT02706405	Fred Hutchinson Cancer Center	B-NHL	CD19	I	Terminated (slow accrual)

Table 7.1: Clinical trials involving CD19-CARs listed as ‘with results’ at clinicaltrials.gov (as of 9 September 2025). B-NHL, B-cell Non Hodgkin’s Lymphoma. DLBCL, Diffuse Large B-cell Lymphoma. ALL, Acute Lymphoblastic Leukemia. B-ALL, B-cell Acute Lymphoblastic Leukemia. CLL, Chronic Lymphocytic Leukemia. SLL, Small Lymphocytic Leukemia. MCL, Mantle Cell Lymphoma. LBCL, Large B-cell Lymphoma. MM, Multiple Myeloma.

Bloemberg et al., 2020; Gordon et al., 2022) or for basic research on CAR-T cell activation due to its ease of modification, heavily studied T cell activation mechanisms, and speed with which CAR function can be assessed (Abraham and Weiss, 2004). Recently, Jahan et al. (2023) used a CAR-Jurkat model to evaluate the role of spacer length preceding the scFv region of CARs and found that CAR-Jurkats recapitulate many phenotypes of primary cells, including cytotoxicity, NF- κ B/NFAT engagement, and intracellular phosphosignalling (Jahan et al., 2023). Similarly, Wan et al. (2025) used a CAR-Jurkat model to show that SMAD4 is involved in regulating Lck expression and, therefore, basal expression of CD69 and CAR T cell proliferation (Wan et al., 2025). Finally, CAR-Jurkats were recently used by Gordon et al. (2022) to develop the CARPOOL library, which aims to determine an optimal set of intracellular signalling domains by barcoding hundreds of ICD combinations, expressing them in Jurkats, and assessing their activation potential (Gordon et al., 2022). Despite the many drawbacks of clonal cell lines for biomedical research (Sarntivijai et al., 2014), the use of CAR-Jurkats has provided a platform for evaluating new CARs and the mechanism of CAR T cell activation that is demonstrably useful for CAR engineering.

To define the Jurkat-specific signalling signature that underlies CAR screening readouts, we applied our stable isotopic labelling of amino acids in cell culture (SILAC) co-culture pY mass spectrometry workflow (Griffith et al., 2022) together with CD69 flow cytometry and selective perturbation of TCR regulators. Using variable minimised first, second, and third generation CAR constructs mimicking the therapeutic Yescarta (Locke et al., 2019), we found that Jurkat-CAR-induced pY signalling is dominated by inclusion of TCR ζ . Further, we showed that all CARs show higher CD69+ populations compared to non-transfected Jurkats, but costimulation domains are required for a large-scale induction of CD69 expression. We also observed that CD19-CAR Jurkats differ from primary CAR T cell studies in that SHP-1 phosphatase activity showed no influence on CAR activation, but phosphatase activity globally was crucial for thresholding T cell activation. Finally, we found that pharmacological inhibition of Itk reduced baseline CD69 expression while maintaining co-culture induced activation. Jurkat cells are PTEN-deficient and differ from primary T cells in phenotype. Therefore, our results should be interpreted as model-specific and not assumed to generalise to primary CAR

T cells without dedicated validation. Overall, we provide a thorough evaluation of CAR signalling in Jurkats and highlighted the importance of Itk for basal activation in TCR ζ -containing CD19-CAR Jurkats.

7.2 Materials and Methods

7.2.1 Cell culture

Jurkats and CD19-CAR expressing Jurkats, Raji B cells, J.muOT1.hCD8 (J^{OT1}), and T2-K^b cells were maintained in RPMI 1640 supplemented with 10 % FBS (Peak Serum #PS-FB3 Lot 01Z2233), 100 U/mL penicillin, 2 mM L-glutamine, 100 μ g/mL streptomycin (100 $\times$ PSQ, Cytiva #SV30082.01), and 2.5 μ g/mL Plasmocin (InvivoGen #ant-mpp) in a humidified incubator at 37C and 5 % CO₂. Chimeric antigen receptor-expressing Jurkats were transduced as previously described(Callahan et al., 2025). All four CARs used in this study contained the same scFv domain (FMC63-based CD19 targeting), transmembrane domain (CD8 TM), the primary TCR-based signalling domain the intracellular domain of TCR ζ (CD3 ζ ; gene CD247), and a terminal GFP tag (superfold GFP). Each construct differed only in the number of costimulatory domains, which were placed between the CD8 TM domain and the intracellular TCR ζ domain. ζ -CAR contained no costimulatory domains, 28 ζ -CAR contained the intracellular domain of CD28, BB ζ -CAR contained the intracellular domain of 4-1BB, and 28BB ζ -CAR contained both the intracellular domain of CD28 and 4-1BB in sequence (Figure 7.1A, 7.2). For SILAC co-culture experiments, Jurkats were maintained in SILAC RPMI 1640 (Thermo #A33823) supplemented with 10 % dialysed FBS, 100 U/mL penicillin, 2 mM L-glutamine, 100 μ g/mL streptomycin, 2.5 μ g/mL Plasmocin, 0.28 mM ¹³C₆ ¹⁵N₄ Arginine (Sigma Aldrich #608003–4.00G) and 0.22 mM ¹³C₆ ¹⁵N₂ Lysine (Cambridge Isotope Laboratories #CNLM-291-H-1) for seven days.

7.2.2 Inhibitor treatment and stimulation

For inhibitor treatment experiments, Jurkats were collected, washed once in plain RPMI 1640, then resuspended in the inhibitor solutions – 0.2 % DMSO, 5 μ M PP1 (Med-

ChemExpress #HY-13804-5 mg), 2 μ M Soquelitinib (MedChemExpress #HY-150298), 5 μ M U0126 (Cell Signalling Technologies #9903), 4 μ M TPI-1 (MedChemExpress #HY-100463-5 mg), 2 μ M PTPN22-IN-1 (MedChemExpress #HY-139693-5 mg), 10 μ M pervanadate - at a concentration of 3×10^7 cells/mL for 3 h at 37C, 5 % CO₂ in a humidified incubator. After pretreatment, cells were washed once in 1 $\times$ DPBS (with inhibitor), resuspended in inhibitor DPBS at a concentration of 2×10^8 cells per millilitre, before stimulation was induced by crosslinking 2 μ g/mL α -CD3 ϵ (clone OKT3, Invitrogen #16-0037-85) and 2 μ g/mL α -CD28 (clone CD28.6, Invitrogen #16-0288-85) with 22 μ g/mL α -Mouse IgG (Jackson ImmunoResearch #115-005-062) secondary antibody. Cells were lysed after 5 min of stimulation by 1-to-1 addition of Urea Lysis Buffer (8 M Urea, 1 μ M sodium orthovanadate, 1 μ M β -glycerophosphate, 1 μ M sodium pyrophosphate) before being briefly sonicated at 70 % amplitude for 15 s (Q55 Sonicator, Qsonica, USA) and diluted in 2 $\times$ Laemmli Buffer (4 % SDS, 125 mM TRIS-HCl pH 6.8, 20 % glycerol, 5 % β -mercaptoethanol, 0.01 % bromophenol blue). Samples were stored at -20C until use.

7.2.3 Flow cytometry

To evaluate CAR expression, one million CAR-expressing Jurkats were collected and washed twice in FACS buffer (2 % FBS in 1 $\times$ DPBS), then fixed with 2 % paraformaldehyde (PFA) at RT in the dark. After fixation, the cells were washed thrice with FACS buffer and stored before analysis on a Cytex Aurora flow cytometer. Analysis of expression was performed using Floreada and raw counts were visualised using Python 3.

For evaluating CD69 expression before and after co-culture, Jurkats and CD19-CARs were pretreated with inhibitor for 1 h as described in Chapter 7.2.2. For co-cultures, Raji B cells were fixed by 1:1 addition of PFA at room temperature for 15 min. After pretreatment with inhibitor, Jurkat cells were washed once in complete RPMI 1640 with inhibitor, then incubated at a concentration of 5×10^6 T cells/mL in complete RPMI 1640 with inhibitor. For baseline signalling assays, Jurkats were maintained alone and for co-culture activation assays, Jurkats were maintained with fixed Raji B cells

(pipetting up and down to mix) for 24 h at 37C, 5 % CO₂ in a humidified incubator. After 24 h of incubation, cells were collected and resuspended in FACS buffer before staining with α -CD3 ϵ PE (BD Biosciences #555333, for Jurkats) and/or α -CD69 APC (BD Biosciences #560967, for all samples). Samples were washed 3 times with 1 mL of FACS buffer, then fixed by 1-to-1 addition of 4 % PFA for 15 min at room temperature, washed 3 times in 1 mL FACS buffer, then stored in FACS buffer until analysis on a Cytex Aurora flow cytometer. Analysis of percent CD69+ cells was performed using Floreada and statistical analysis was performed using Python3.

7.2.4 Co-culture stimulation

Co-culture sample preparation for Western blotting or mass spectrometry sample preparation was performed as previously described(Callahan et al., 2025). Briefly, T cells (Jurkats, J^{OT1} cells, or CD19-CARs) and live Raji B cells were washed once in 1 $\times$ DPBS, then resuspended at a concentration of 2×10^8 (T cells) or 1×10^8 (target cells). T2-K^b cells were loaded with OVA_{257–264} peptide by washing once in plain RPMI 1640, then incubated in plain RPMI 1640 supplemented with 1 μ M OVA^{257–264} for 3 h at 37C, 5 % CO₂ in a humidified incubator. After incubation, the peptide-loaded T2-K^b cells (pT2-K^b) were collected and washed once in 1 $\times$ DPBS, then resuspended at a concentration of 1×10^8 cells/mL. Cells were co-cultured by 1:1 (v/v, 2:1 E:T) addition of T cells to target cells, briefly centrifuged for 30 s at 500 $\times$ g, incubated at 37C for the indicated times, then lysed with Urea Lysis Buffer, and cooled on ice for at least 15 min. Samples were then sonicated using a microtip sonicator (Q55 Sonicator, Qsonica, USA) at 70 % amplitude for 15 s once (Western blotting samples) or 30 s once (mass spectrometry samples). Samples for Western blotting were diluted 1:1 in 2 $\times$ Lamelli buffer (4 % SDS, 125 mM TRIS-HCl pH 6.8, 20 % glycerol, 5 % β -mercaptoethanol, 0.01 % bromophenol blue), boiled at 95C for 5 min, then stored at -20C until use.

7.2.5 Western blotting

Western blotting was performed as previously described(Callahan et al., 2025). Ten percent Tris-Glycine gels were poured in-house, and samples were separated at 100 V for 130 min before being transferred to a PVDF membrane (EMD Millipore #IPFL00010)

at 100 V for 100 min in an ice-cold wet transfer apparatus. Membranes were blocked with Intercept TBS blocking buffer (LI-COR #927–60,001) that was diluted 1 to 1 in 1× TBS, then incubated with primary antibody solution (antibody in 5 % BSA dissolved in 1× TBST) overnight with gentle shaking. Membranes were washed 3 times in 1× TBST before being incubated with secondary antibody solution for 1 h at room temperature on a rocker. Finally, membranes were washed 3× in 1× TBST, 2× in 1× TBS, then imaged on a LI-COR Odyssey CLx or LI-COR Odyssey M scanner. Western blot quantification was performed as previously described(Callahan et al., 2025; Pillai-Kastoori et al., 2020).

Primary Antibodies: PLC γ 1 (1:10000, Millipore Sigma #05–163), PLC γ 1^{Y783} (1:2000, Cell Signalling Technologies #2821), Erk1/2 (Cell Signalling Technologies #9107), ErkT202Y204 (1:2000, Cell Signalling Technologies #9101), Lck (1:4000, Cell Signalling Technologies #2657), Src family kinase activation site (SrcY416; 1:2000, Cell Signalling Technologies #2101).

Secondary Antibodies: IRDye 680RD Donkey anti Rabbit IgG (LI-COR #926–68,073), IRDye 800CW Donkey anti Rabbit IgG (LI-COR #926–32,213).

7.2.6 Sample preparation for LC-MS/MS

Mass spectrometry samples were prepared as previously described(Callahan et al., 2025). Briefly, Urea-lysed samples were protein-input normalised (15 mg per sample for Jurkat, ζ -CAR, 28 ζ -CAR, BB ζ -CAR, 28BB ζ -CAR and 10 mg per sample for J^{OT1} pT2-K^b) by BCA (Thermo #23225), adjusted to 2 mL with 4 M Urea Lysis Buffer, then diluted with 2.45 mL of 20 mM HEPES. Proteins were reduced with 50 μ L of 1 M dithiothreitol (final concentration 10 mM) then alkylated with 500 μ L of 100 mM iodoacetamide (final concentration 10 mM) before being digested overnight with trypsin (Promega #V5113) at a 1:100 trypsin:protein ratio. The following day, samples were acidified to 1 % trifluoroacetic acid and desalted using Waters Sep-Pak C18 vacuum cartridges (Waters #WAT020515) as previously described(Griffith et al., 2022). For pY peptide enrichment, the Src SH2 superbinder (sSH2) method was used as previously described(Griffith et al., 2022; Callahan et al., 2025, 2024).

7.2.7 LC-MS/MS, database searching, and analysis

Enriched pY peptides were separated on a Vanquish Neo ultra-high-performance liquid chromatography (UHPLC) using a trap and elute setup. Peptides were first loaded onto a 7 cm Acclaim PepMap C18 trap column (2 cm bed length, 3 mm particle size, 100 Å; Thermo #164946) before elution and separation using an Aurora Ultimate C18 column (25 cm × 75 mm ID, 1.7 mm particle size; IonOpticks #AUR3-25075C18) using a 60 min gradient at a constant 300 nL/min starting at 5 % Solvent B (80 % acetonitrile, 0.1 % formic acid) and 95 % Solvent A (0.1 % formic acid) and ending at 30 % Solvent B and 70 % Solvent A over a course of 50 min, then spiked to 95 % Solvent B for 5 min and 99 % Solvent B for the remaining 5 min. After each sample, the trap column was washed using a dedicated 60 min method consisting of 12 zebra washes, and the analytical column was washed with 3 aqueous/organic cycles before equilibrating for the next sample. In between each set of 5 samples, 20 µL of flush solution was injected, and 1 µg of BSA was run to assess instrument sensitivity.

For MS settings, the spray voltage was set to 2050 V, the ion transfer tube was set to 275°C, and a lock mass of 445.12003 was used. The orbitrap detector was used for MS1 scans with wide quad isolation, a resolution of 120,000, a mass range of 300–1500 m/z, a maximum injection time of 50 ms, 250 % normalised AGC target, and RF lens at 30 % in centroid mode. The following filters were included: monoisotopic peak and peptide mode, charge state 2–7, 375–1500 m/z precursor selection range, dynamic exclusion (exclude after 1 time, 15 s exclusion time, 10 ppm low and high mass tolerances, excluding isotopes), intensity range of 20,000 (min) - 1E20 (max) with relative intensity threshold of 20 %. MS2 were collected in data-dependent acquisition mode using a 3 s cycle time, 30 % normalised higher-energy collisional dissociation (HCD), and a quadrupole isolation width of 0.7 m/z. Fragment ions were collected in centroid mode and detected using the ion trap in Turbo Mode with a scan range of 150–2000 m/z with a maximum injection time of 35 ms, 100 % normalised AGC, and the dynamic mode was used for maximum injection time.

Thermo .RAW files containing MS spectral data were analysed using the high-throughput autonomous proteomics pipeline (HTAPP) and PeptideDepot as previously

described (Yu and Salomon, 2009, 2010). Briefly, MS2 spectra were searched against the UniProt complete proteome dataset (downloaded August 2019 with 98,300 unique forward sequences) using the Mascot search engine to 0.1 % FDR using a reverse decoy database strategy. For peptide spectrum matching, the following parameters were used: trypsin enzyme cleavage with up to 2 missed cleavages, 7 ppm precursor mass tolerance, 500 mmu fragment ion mass tolerance, variable modifications for phosphorylation (S/T/Y + 79.9963 Da) and oxidation (M + 15.9949 Da), with static modifications of carbamamidomethylation (C + 57.0215 Da). Retention time alignment and integration of selected ion chromatograms were used to determine the relative peptide abundance for each PSM as previously described 36. The resulting peak areas were further processed in PeptideDepot to normalise peak areas to a spike in pY peptide (LIEDpYTAK; samples #44 and #75 were dropped due to no sequencing of this peptide), choose unique pY sites (fewest missing values and highest median intensity). Then statistical significance between $\log_2$ mean abundance for the time points was determined using Welch's t-test and FDR was estimated using the method of Storey (Storey and Tibshirani, 2003). Replicate reproducibility was determined with multiple linear regression and principal component analysis. Post-translational modification signature enrichment analysis was performed as previously described (Callahan et al., 2025; Krug et al., 2019) using the R-script ssGSEA2.0.R with the PTM-signature database version 2.0.0.

7.2.8 Data availability

All plotting was performed using Python3.12.5 in a JupyterLab environment with the dependencies matplotlib (version 3.9.1), scipy (version 1.14.0), numpy (version 2.0.1), scikit-learn (version 1.5.1), pandas (version 2.2.2), and a custom 'helpers' module used for beautifying matplotlib graphs. Statistical analysis of Western blots and flow cytometry data was performed using a self-coded version of the Holm-Sidak FWER correction based on the algorithm provided by GraphPad Prism. All Image Studio files (Westerns), post-gating flow cytometry .csv files, and code used in this project are available in the supporting materials and on GitHub (<https://github.com/Aurdeegz/CAR-Generations>). Mass spectrometry .RAW files are available from the ProteomeXchange Consortium via the PRIDE repository (Dataset ID: PXD065669).

7.3 Results

7.3.1 CD19-CAR expression and mass spectrometry experimental design

Because of the large clinical variation in CAR function between CARs with different costimulatory domains, we sought to evaluate the pY signalling and co-culture-induced CD69 expression in CD19-CAR Jurkats with clinically relevant costimulatory domains modelled after the therapeutic Yescarta (Axicabtagene ciloleucel)(Locke et al., 2019). We engineered Jurkats to express a first generation (TCR ζ ; ζ -CAR hereafter), two second generation (CD28co-TCR ζ ; 28 ζ -CAR hereafter, 41BBco-TCR ζ ; BB ζ -CAR hereafter), and one third generation (CD28co-41BBco-TCR ζ ; 28BB ζ -CAR hereafter) with an FMC63-based scFv that recognizes CD19, CD8 transmembrane domain and hinge region, and an intracellular superfold GFP tag (Figure 7.1A, 7.2). All CD19-CARs were highly expressed compared with a Jurkat control, with little variation in the GFP+ population between CD19-CAR constructs (Figure 7.1B). Co-culture between CD19-CARs and Raji B cells showed significant induction of Erk1^{T202Y204}/Erk2^{T185Y187} and PLC γ 1^{Y783} phosphorylation at 5 and 15 min after co-culture, and 28BB ζ -CAR showed significantly lower levels of basal Erk1^{T202Y204}/Erk2^{T185Y187} and PLC γ 1^{Y783} phosphorylation than WT Jurkats (Figure 7.1C-E, 7.3). It is important to note that Raji B cells also expressed Erk1/2 and PLC γ 1, and thus the basal levels of Erk1^{T202Y204}/Erk2^{T185Y187} and PLC γ 1^{Y783} may have been influenced by Raji phosphorylation levels. However, we have previously shown that Raji B cells displayed little to no pY changes after co-culture(Griffith et al., 2022; Callahan et al., 2025); thus the induction we observed was most likely due to the presence of CD19-CARs. To gain precise information about the pY signalling potential, we adopted a co-culture Western blot and phosphoproteomics workflow as described previously(Griffith et al., 2022; Callahan et al., 2025). Briefly, CD19-CAR T cells and Raji B cells were cultured to confluence in standard RPMI and ¹³C₆ ¹⁵N₄ Arginine/¹³C₆ ¹⁵N₂ Lysine RPMI, respectively, before mixing at a 2:1 effector-to-target (E:T) ratio, were briefly centrifuged to promote contact, and were allowed to incubate for 0 min, 5 min, or 15 min before lysis in 8 M Urea. A portion of the lysate was saved for phosphosite specific Western blotting, and the remaining lysate (15 mg protein per sample)

was processed through a standard bottom-up proteomics workflow(Griffith et al., 2022; Callahan et al., 2025; Belmont et al., 2017). After, pY peptides were enriched using the Src SH2 superbinder domain (Griffith et al., 2022; Callahan et al., 2025, 2024) and were analysed by LC-MS/MS on an Orbitrap Ascend mass spectrometer. These methods provide precise information regarding the pY signalling cascades initiated by each CAR during co-culture.

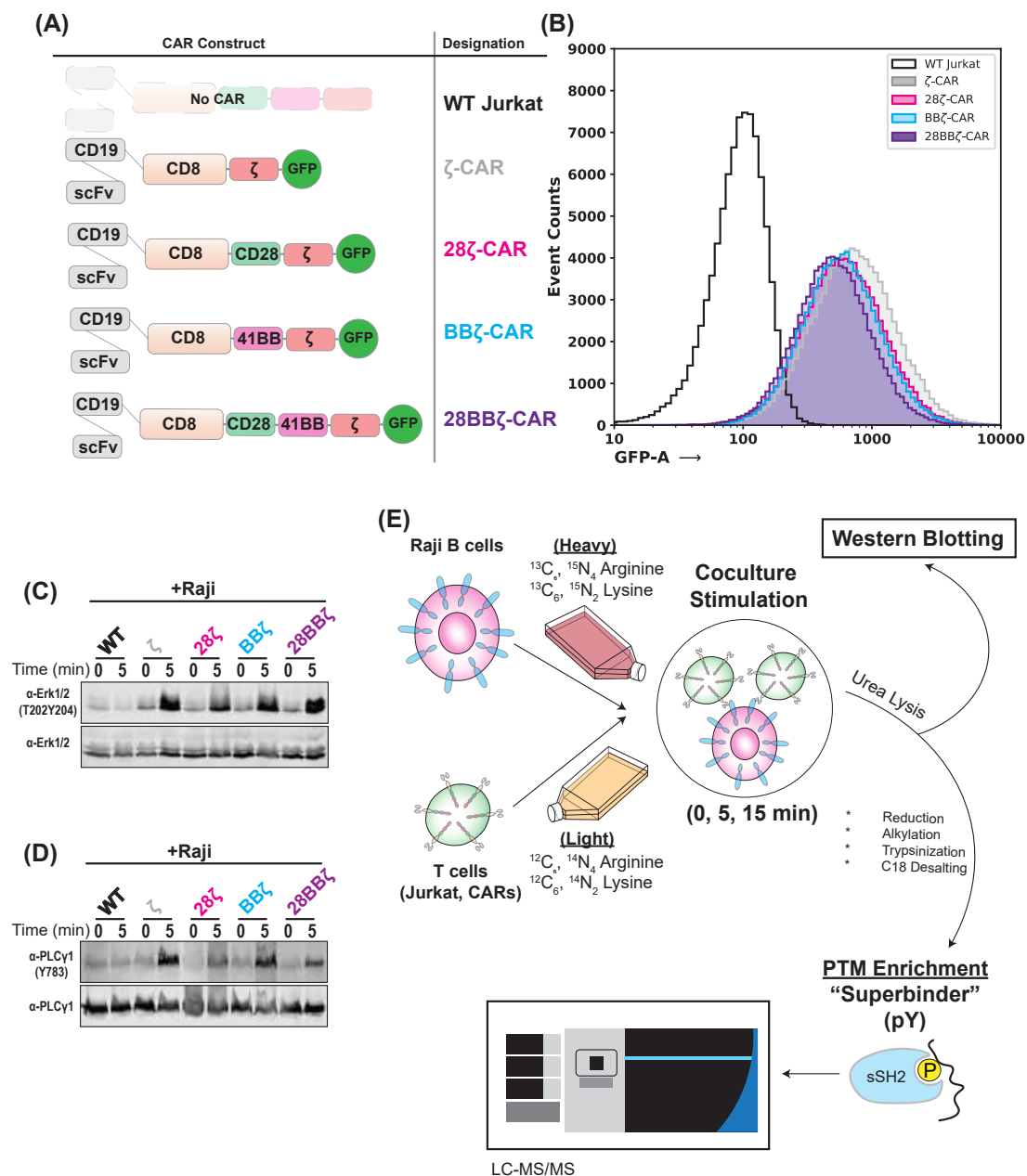


Figure 7.1: Expression of CD19-CARs and phosphoproteomic workflow. (A) Schematic representation of the cell lines used in this study. (B) Flow cytometry analysis showing total GFP expression in CD19-CAR Jurkats. (C-D) Western blot analysis of co-culture samples showing phosphorylation of Erk1/2 and PLCγ1, respectively. (E) Flow chart depicting the co-culture stimulation phosphoproteomics workflow used in this study. Details are in the Methods section.

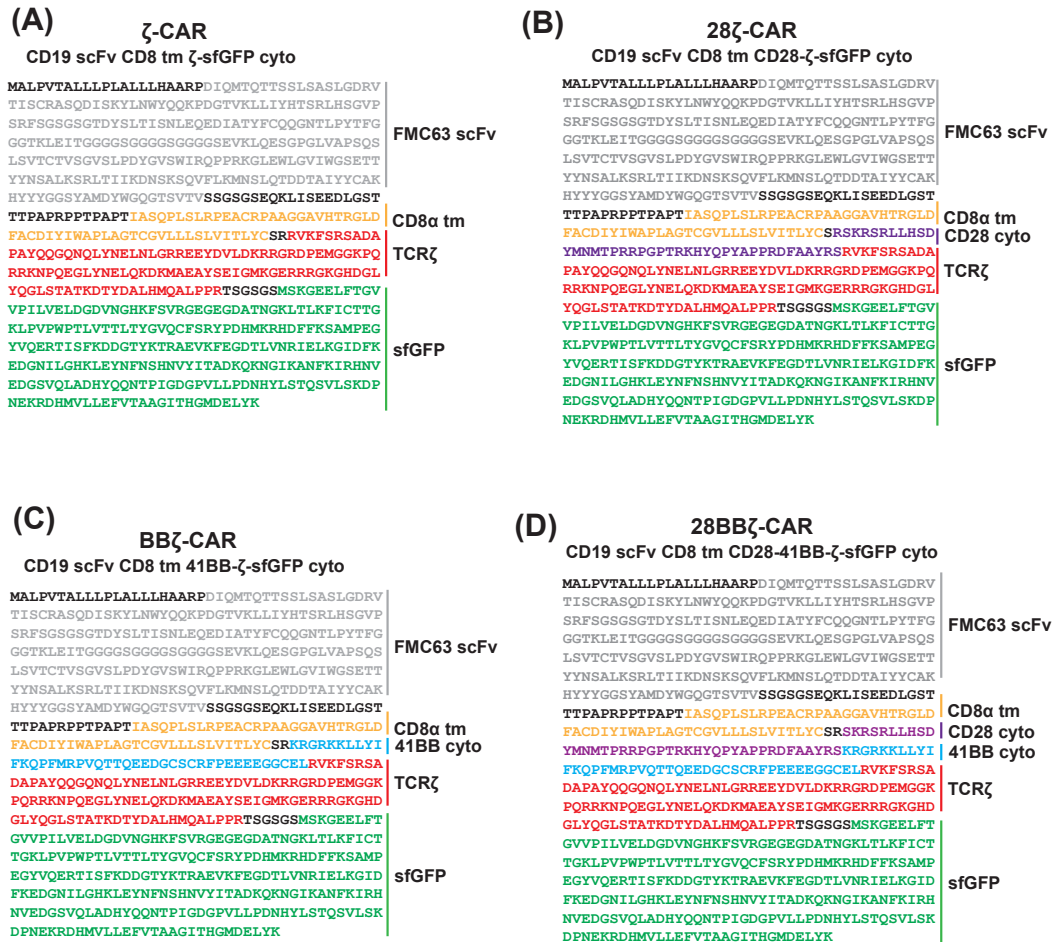


Figure 7.2: Amino acid sequences for (A) ζ-CAR, (B) 28ζ-CAR, (C) BBζ-CAR, and (D) 28BBζ-CAR.

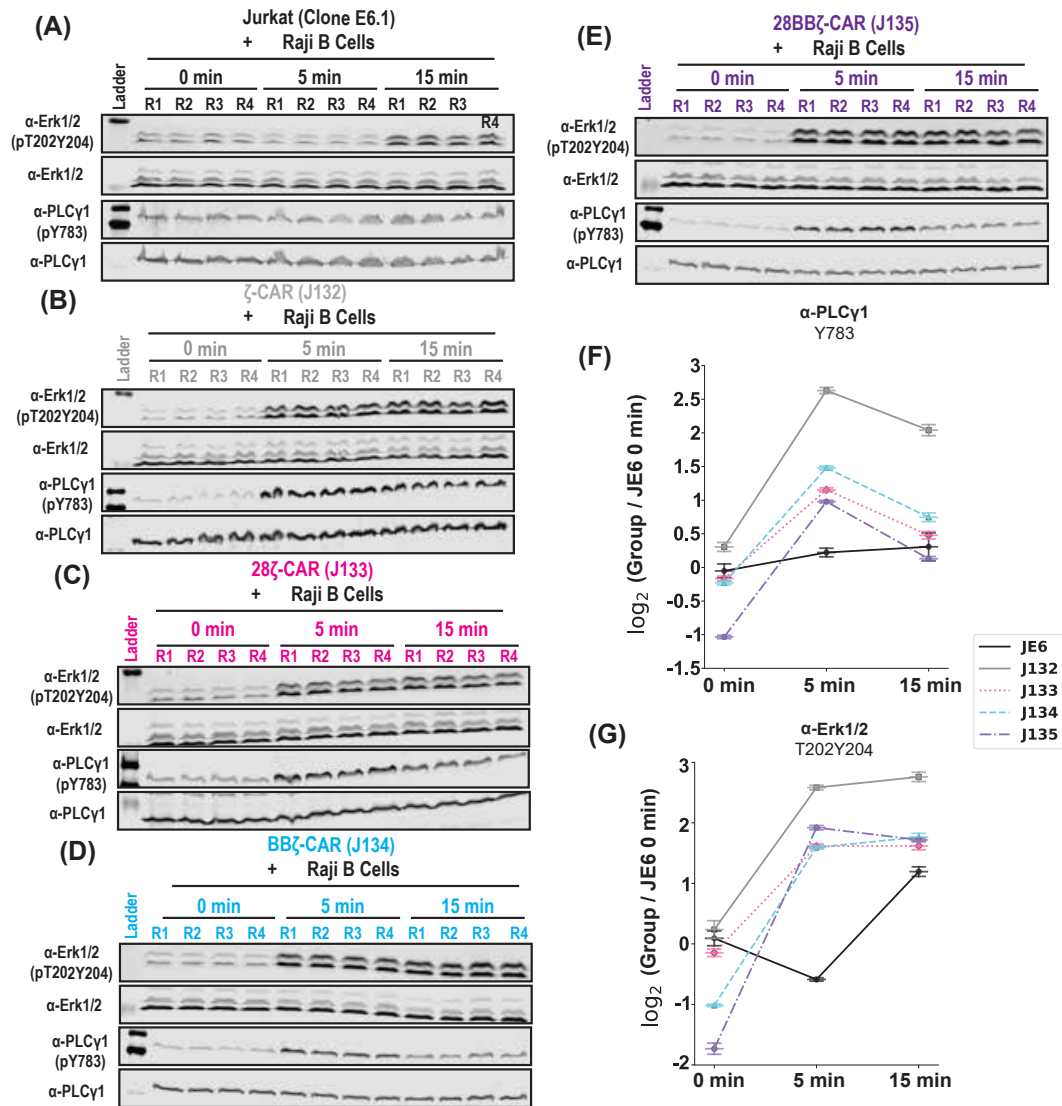


Figure 7.3: Co-culture stimulation experiments show similar induction of pY sites on TCR signalling proteins. (A) Western blot analysis of Erk1^{T202Y204}/Erk2^{T185Y187}, and PLC γ 1^{Y783} in co-cultures of Jurkat T cells and Raji B cells. (B-E) As in A, except for co-cultures of Raji B cells and ζ -CAR, 28 ζ -CAR, BB ζ -CAR, and 28BB ζ -CAR, respectively. (F, G) Quantification of PLC1Y783 and Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation, respectively. All groups are relative to the JE6 0m group.

7.3.2 Inclusion of TCR ζ in CD19-CARs dominates pY signalling cascades

Using SILAC co-cultures with pY enrichment mass spectrometry (Figure 7.1C), we sequenced a total of 496 pY sites on 340 unique proteins across all samples with high reproducibility ($R > 0.8$) and replicate clustering by principal component analysis (Figure 7.5). In line with our own previous work (Griffith et al., 2022; Callahan et al., 2025), we observed few pY sites significantly elevated in co-cultures of Jurkat and Raji B cells, and many pY sites significantly increased in abundance after co-culture of CD19-CARs with Raji B cells (Figure 7.4A-E). Notably, the pattern of significantly changing pY sites after 5 min of co-culture closely matched co-cultures of Jurkat cells expressing the murine OT1 transgenic TCR and CD8 (J^{OT1}) co-cultured with T2-K^b cells expressing a class I MHC loaded with OVA²⁵⁷⁻²⁶⁴ (pT2-K^b; Figure 7.6C-D, 7.7A-B), which is a well-established model for studying T cell signalling and T cell activation (Clarke et al., 2000; Chua and Salomon, 2021; Lo et al., 2019). Across all CD19-CARs, many of the significantly induced pY sites are involved in the forward progression of T cell signalling, including the immunotyrosine based activation motifs (ITAMs) TCR ζ^{Y123} and TCR ζ^{Y142} , CD28^{Y191}, GADS^{Y45}, LAT^{Y220}, Erk1^{Y204} and Erk2^{Y187}, Nck^{Y105}, PLC γ 1^{Y771}, PLC γ 1^{Y775}, PLC γ 1^{Y783}, PKC δ^{Y313} , SHC1^{Y427}, WASL^{Y256}, Zap70^{Y292}, Zap70^{Y492}, and Zap70^{Y493} (Figure 7.4F-J). In contrast, co-cultures of J^{OT1} and pT2-K^b cells showed significant phosphorylation of ITAMs on other components of the TCR, such as CD3 δ^{Y149} , CD3 δ^{Y160} , CD3 ϵ^{Y188} , and CD3 ϵ^{Y190} , and regulatory sites on Zap70 such as Zap70^{Y315} and Zap70^{Y319} (Figure 7.7C), suggesting that in this system CD19-CAR T cells engage a subset of the TCR signalling pathway that is independent of the TCR itself. Post-translational modification signature analysis (PTM-SEA), a computational tool that discerns patterns between measured PTM proteomics data and annotated databases, showed downregulation of both kinase substrate signatures and pathways in co-cultures of WT Jurkats with Raji B cells (Figure 7.4K, 7.8A). In contrast, all CD19-CARs showed upregulation of kinase substrate signatures, particularly the Src family kinase Lck and the Syk family kinase Zap70, which are both necessary for the forward progression of T cell activation through CARs and the TCR (Figure 7.4L-O) (Gaud et al., 2018). Similarly, all

CD19-CARs showed upregulation of signalling pathways, generally, with ζ -CAR showing the weakest and 28BB ζ -CAR showing the strongest engagement with signalling cascades (Figure 7.8B-E). Together, our data suggest that the inclusion of TCR ζ is the primary determinant of pY signalling in CD19-CAR Jurkats and that TCR ζ -containing CARs engage only a subset of canonical TCR signalling.

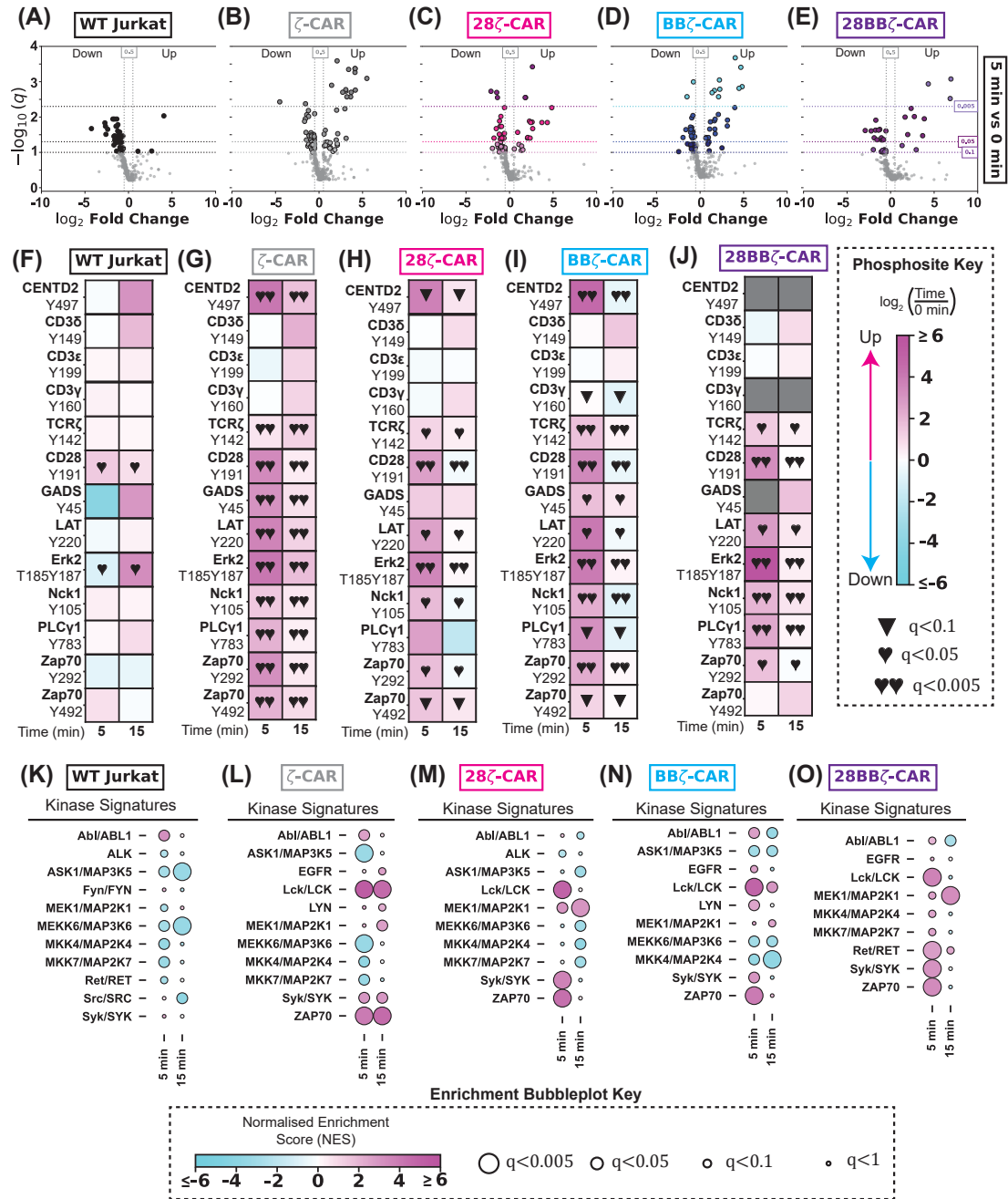


Figure 7.4: Inclusion of TCR ζ in CD19-CARs dominates pY signalling. (A-E) Volcano plot comparing unique pY sites observed from 5 min and 0 min of co-culture between Raji cells and WT Jurkats, ζ -CAR, 28 ζ -CAR, BB ζ -CAR, and 28BB ζ -CAR, respectively (n = 4 or 5). (F-J) Heat maps comparing 5 min or 15 min with 0 min of co-culture between Raji cells and WT Jurkats, ζ -CAR, 28 ζ -CAR, BB ζ -CAR, and 28BB ζ -CAR, respectively. Sites presented are a selection of known TCR-responsive pY sites. ▽ indicates $q < 0.1$ (trending toward significance), ♥ indicates $q < 0.05$, and ♥♥ indicates $q < 0.005$. (K-O) Post translational modification signature enrichment analysis using pY site data showing enrichment of PhosphoSitePlus kinase substrate signatures for WT Jurkats, ζ -CAR, 28 ζ -CAR, BB ζ -CAR, and 28BB ζ -CAR, respectively.

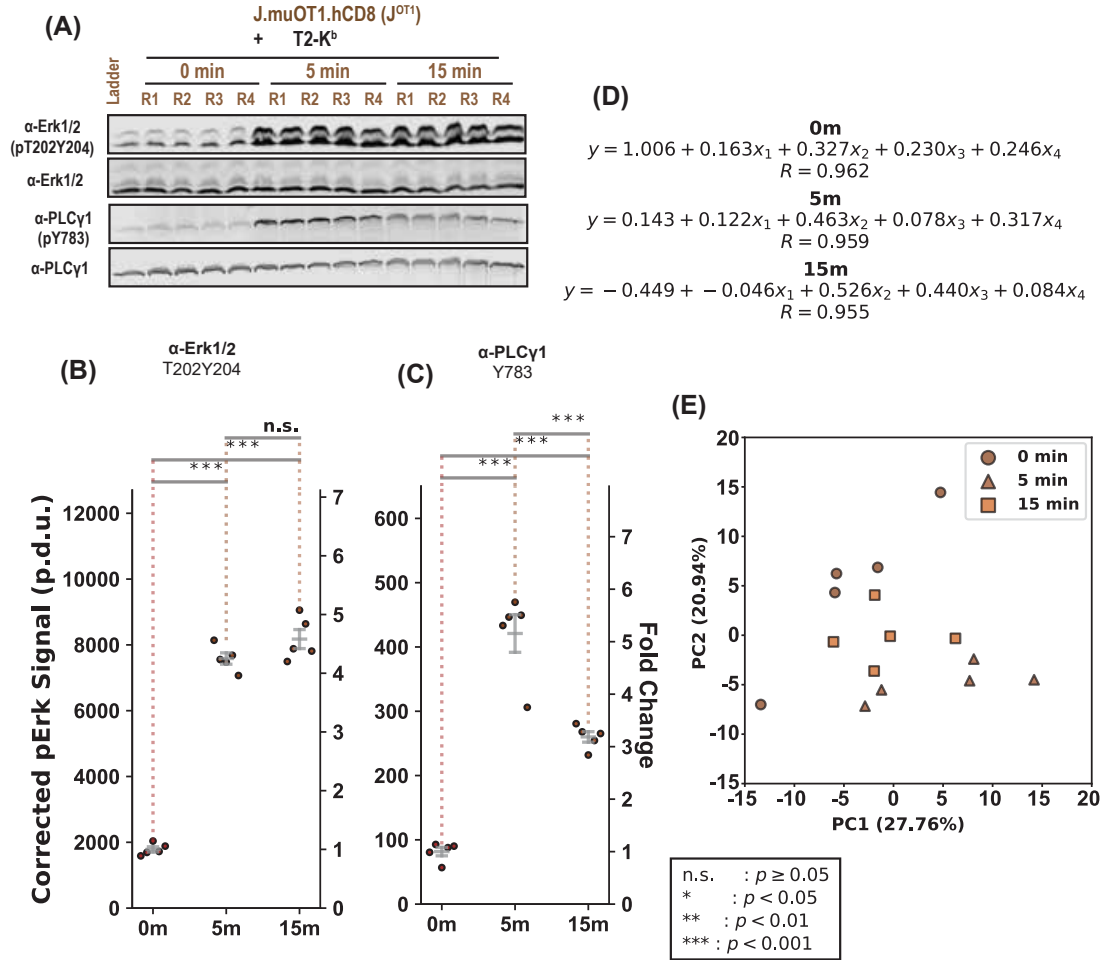


Figure 7.5: Co-culture of Jurkat cells expressing the murine OT1 transgenic TCR and human CD8 with OVA²⁵⁷⁻²⁶⁴ loaded T2-K^b cells induces highly reproducible TCR stimulation. (A) Western blot analysis of Erk1^{T202Y204}/Erk2^{T185Y187} and PLC γ 1^{Y783} in co-cultures of J^{OT1} cells and pT2-K^b cells. (B, C) Quantification of phosphorylation of Erk1^{T202Y204}/Erk2^{T185Y187} and PLC γ 1^{Y783}, respectively, in J^{OT1} cell and pT2-K^b cell co-cultures. (D) Multiple linear regression on pY sites measured in all replicates during co-culture of J^{OT1} and pT2-K^b. (E) Principal component analysis of pY sites measured in all replicates of J^{OT1} cells during co-culture with pT2-K^b cells.

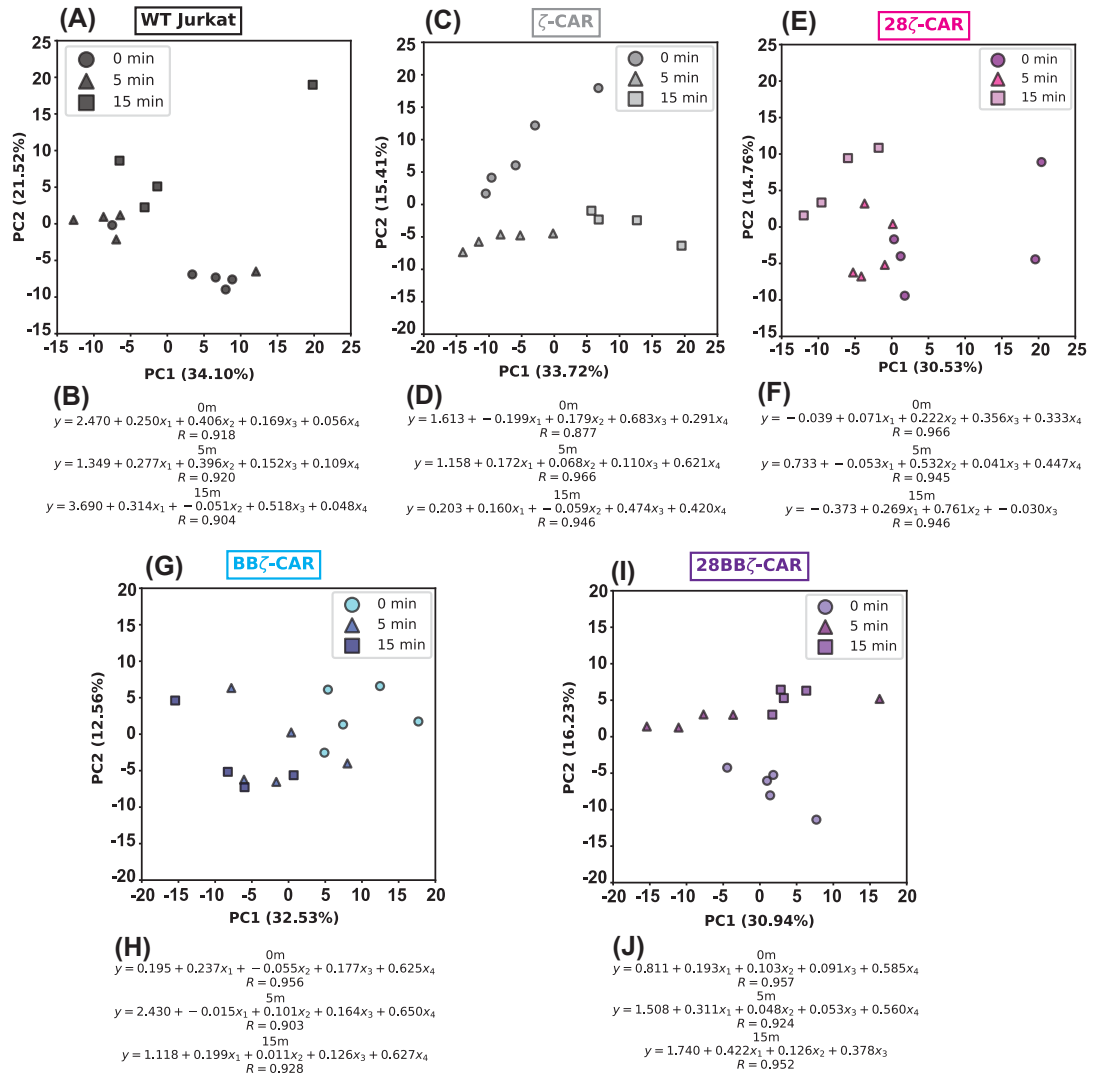


Figure 7.6: pY proteomics data for CD19-CAR T cells is highly reproducible. (A) Principal component analysis of pY sites measured in all replicates of WT Jurkat cells during co-culture with Raji B cells. (B) Multiple linear regression on pY sites measured in all replicates during co-culture of WT Jurkat cells with Raji B cells. (C-D) As in A & B, except for ζ-CAR. (E-F) As in A & B, except for 28ζ-CAR. (G-H) As in A & B, except for BBζ-CAR. (I-J) As in A & B, except for 28BBζ-CAR.

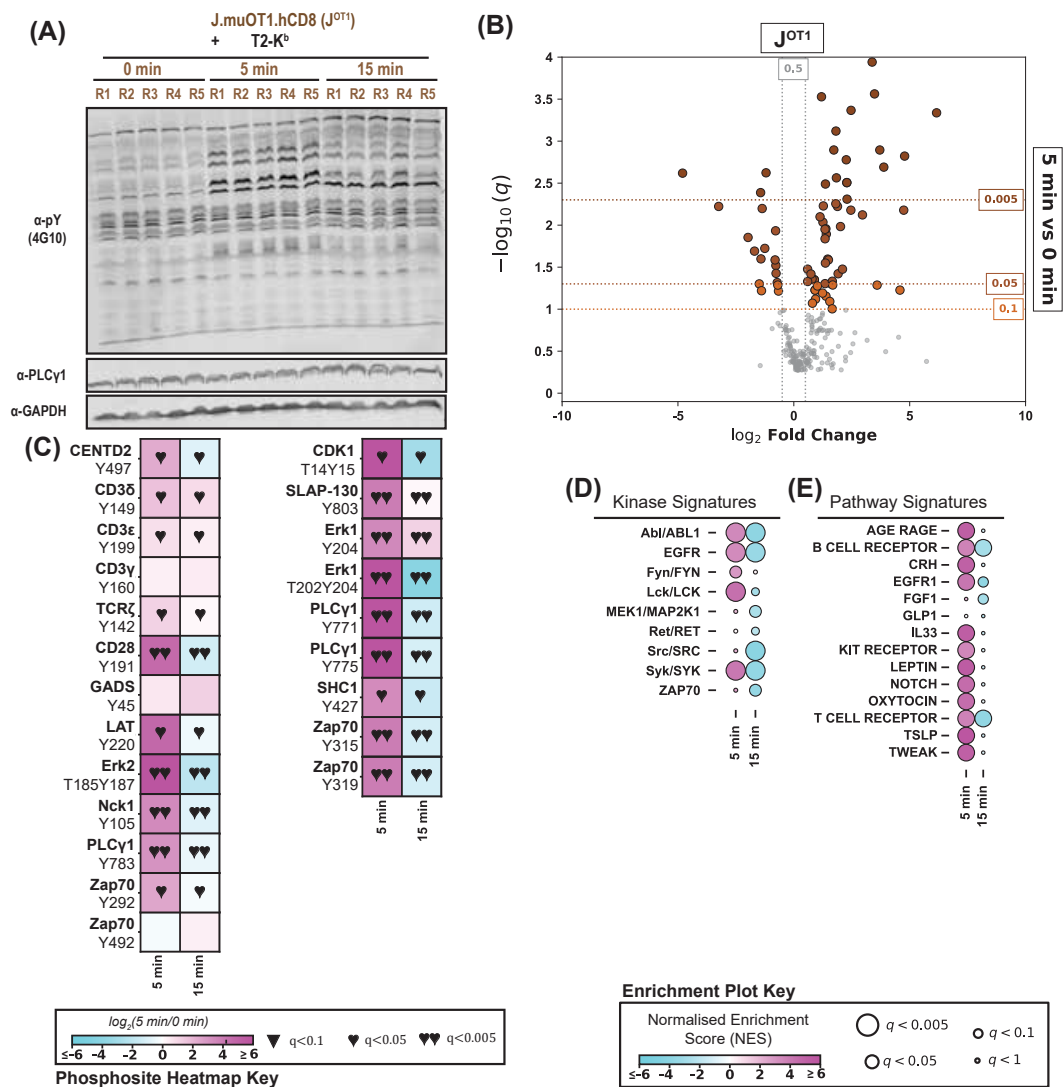


Figure 7.7: pT2-K^b cells induce strong TCR pY signalling in J^{OT1} cells. (A) Western blot analysis of global tyrosine phosphorylation in pY proteomics samples during a time course co-culture of J^{OT1} cells and pT2-K^b cells. (B) Volcano plot analysis showing significance and fold-change of pY sites after 5 minutes of J^{OT1} and pT2-K^b co-culture. (C) Heat maps comparing 5 minutes or 15 minutes with 0 minutes of co-culture between pT2-K^b cells and J^{OT1} cells. Sites presented are a selection of known TCR-responsive pY sites. ▽ indicates $q < 0.1$ (trending towards significance), ♥ indicates $q < 0.05$, and ♥♥ indicates $q < 0.005$. (D-E) Post translational modification signature enrichment analysis using pY site data showing enrichment of PhosphoSitePlus kinase substrate signatures and NetPath pathway signatures, respectively, for co-culture between pT2-K^b cells and J^{OT1} cells.

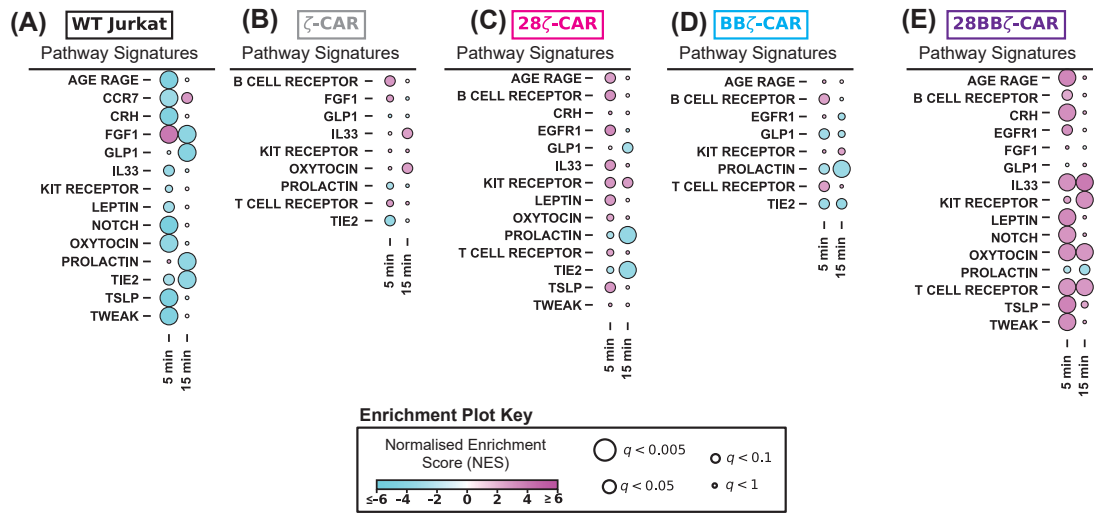


Figure 7.8: PTM-SEA reveals high engagement of signalling cascades in the third generation 28BB ζ -CAR. (A-E) Post translational modification signature enrichment analysis using pY site data showing enrichment of NetPath pathway signatures for WT Jurkats, ζ -CAR, 28 ζ -CAR, BB ζ -CAR, and 28BB ζ -CAR, respectively.

7.3.3 Inclusion of TCR ζ in CD19-CARs increases basal CD69 expression but is not sufficient for activation after antigen encounter

Although CD19-CAR Jurkats all effectively engage pY sites in the known T cell receptor signalling pathway, previous literature suggests that costimulation is necessary for primary T cell activation (Srivastava and Riddell, 2015). To establish whether CAR-specific costimulation domains are necessary for CAR Jurkat activation, we performed 24 h co-culture stimulation experiments using PFA fixed Raji B cells and assess CD69 expression (Figure 7.9A), a known marker of T cell activation used in CAR-Jurkat screens (Bloemberg et al., 2020; Gordon et al., 2022), by flow cytometry. We assessed baseline T cell activation by gating the main cell population, then gating on high signal in the GFP (CD19-CARs) or PE (α -CD3 ϵ PE for WT Jurkats), then high signal for APC (α -CD69 APC) (Figure 7.9B). We observed a significant increase in percent CD69+ cells in ζ -CAR and 28 ζ -CAR, a trend toward significance in BB ζ -CAR, and no significant increase in 28BB ζ -CAR (Figure 7.9C) - with no significant change in mean fluorescence intensity (MFI) between CD69+ WT Jurkats and any CD19-CAR (Figure 7.9D). To assess T cell activation during co-culture with PFA fixed Raji B cells, a similar gating strategy was used considering the large Raji B cell cluster that appears in GFP/PE around 10^4 (Figure 7.9E). We again observed a significant increase in percent CD69+ cells in ζ -CAR compared to WT Jurkats; however all CD19-CARs with costimulation domains showed significantly higher percent CD69+ cell populations than WT Jurkats or ζ -CAR (Figure 7.9F). In co-cultured samples, the MFI of CD69+ cells was significantly increased in all CD19-CARs (Figure 7.9G). Our data show that percent CD69+ cells is a metric that reproduces known and consistently observed phenotypes for CD19-CAR T cells, particularly baseline CD69 expression.

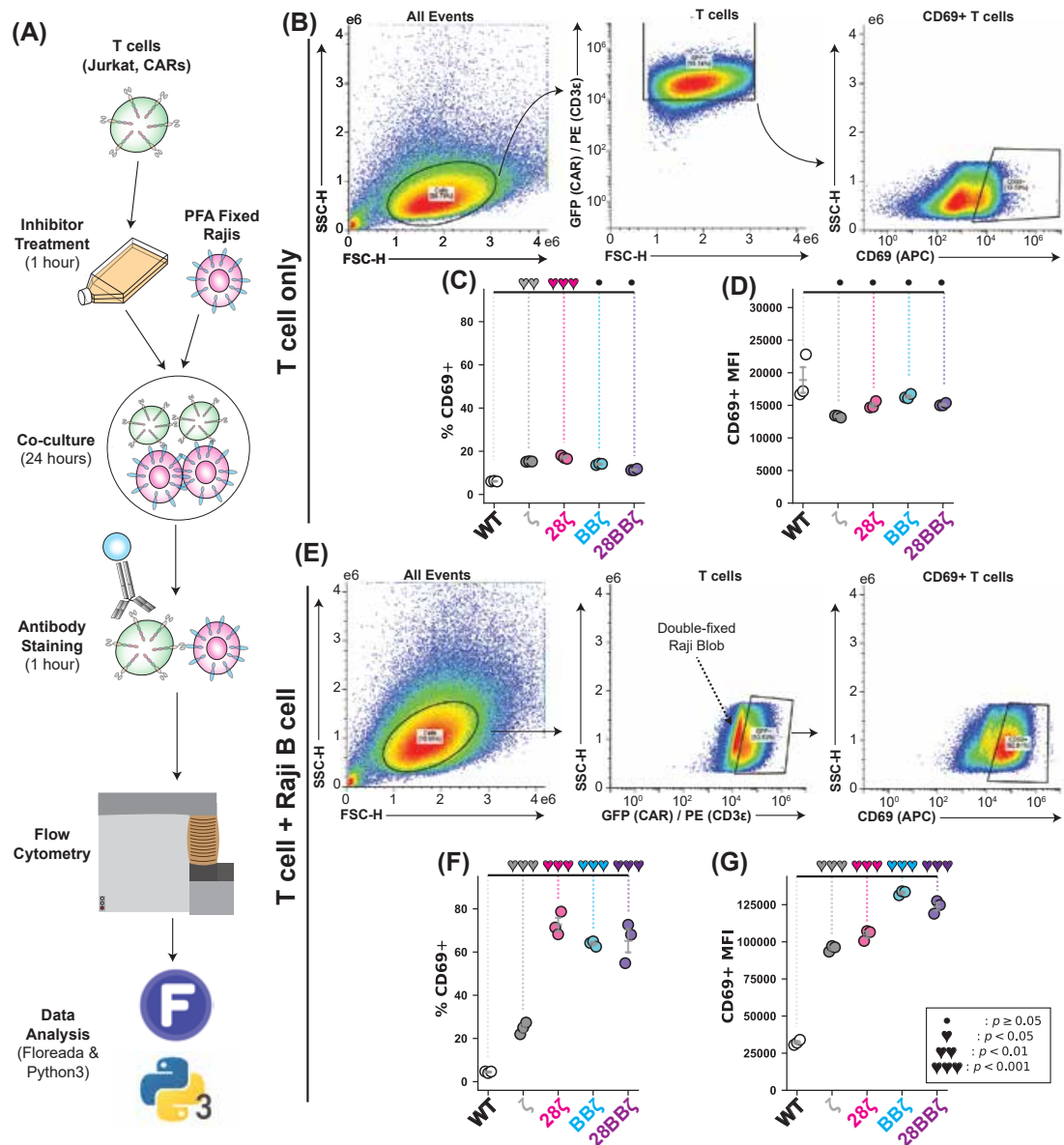


Figure 7.9: Costimulation is required for elevated CD69 expression after co-culture; however, ζ -CAR Jurkats show baseline CD69 expression. (A) Flow chart showing the experimental design of CD69 activation assays. (B) Gating strategy for non co-culture CD69 flow cytometry data analysis. (C-D) Evaluation of percent CD69+ cells and the mean fluorescence intensity of CD69+ cells, respectively, for non co-cultured CAR T cells. (E) Gating strategy for co-culture CD69 flow cytometry data analysis. (F-G) As in C and D, except for co-culture samples. Three biological replicates are shown for each condition.

7.3.4 Global PTP activity, but not PTPN22 or SHP-1, threshold CAR T cell activation

Phosphatases are quickly activated to coordinate the deactivation of T cells after T cell receptor ligation (Gaud et al., 2018); however, phenotypic differences in CAR T cell activation (e.g., exhaustion, baseline activation) suggest the potential dysregulation of these feedback mechanisms. To evaluate the role of PTPs in CAR T cell activation, we leveraged small molecule inhibitors of the T cell PTPs PTPN22 (PTPN22-IN-1) and SHP-1 (TPI-1), as well as the pan-PTP inhibitor pervanadate (PV). First, we validated the activity of 2 μ M PTPN22-IN-1 (Bosch et al., 2024), 4 μ M TPI-1 (Kundu et al., 2010), and 10 μ M PV (Hehner et al., 1999) in Jurkats using CD3 ϵ and CD28 crosslinking to stimulate the TCR. While PV treatment alone significantly elevated basal and stimulated Src family kinase activation site (SFK^{Y416}), PLC γ 1^{Y783}, and Erk1^{T202Y204}/Erk2^{T185Y187} abundance, TPI-1 and PTPN22-IN-1 treatment significantly elevated the SHP-1 substrate (Matalon et al., 2016) PLC γ 1^{Y783} and the PTPN22 pathway target (Duan et al., 2017; Zhang et al., 2020) Erk1^{T202Y204}/Erk2^{T185Y187}, respectively (Figure 7.10B-D, 7.11), showing that these inhibitors functioned at the given concentrations. To investigate whether PTP activity influences CAR T cell activation in the Jurkat model system, we treated CD19-CARs with each PTP inhibitor and assessed CD69 expression after 24 h of co-culture. In WT Jurkats and ζ -CAR, inhibition of PTPN22 or SHP-1 did not significantly alter percent CD69+ cells in either basal or co-culture conditions; however, treatment with 10 μ M PV significantly increased percent CD69+ cells in both basal and co-culture conditions (Figure 7.10E-H). Similarly, inhibition of PTPN22 or SHP-1 did not alter basal percent CD69+ cells in 28 ζ -CAR, BB ζ -CAR, or 28BB ζ -CAR and did not prevent co-culture-induced increases in percent CD69+ cells, while PV treatment uniformly elevated basal percent CD69+ cells (Figure 4I-N). Notably, BB ζ -CAR alone showed a significant reduction in percent CD69+ cells when treated with the SHP-1 inhibitor TPI-1 during co-culture, and greater than 90 % CD69+ cells when treated with PV in the basal state (Figure 7.10K-L), suggesting CD19-CARs without CD28 domains are thresholded more by PTPs than those that include CD28 domains. In general, the MFI of CD69+ CD19-CARs in the context of inhibitor treatment trended similarly to what we observed

with CD69+ cell populations (Figure 7.12). Together, our results demonstrate that PTP activity globally in the Jurkat model system is critical for regulating CD19-CAR activation, but the TCR-specific phosphatases PTPN22 and SHP-1 are not involved in thresholding CD19-CAR activation.

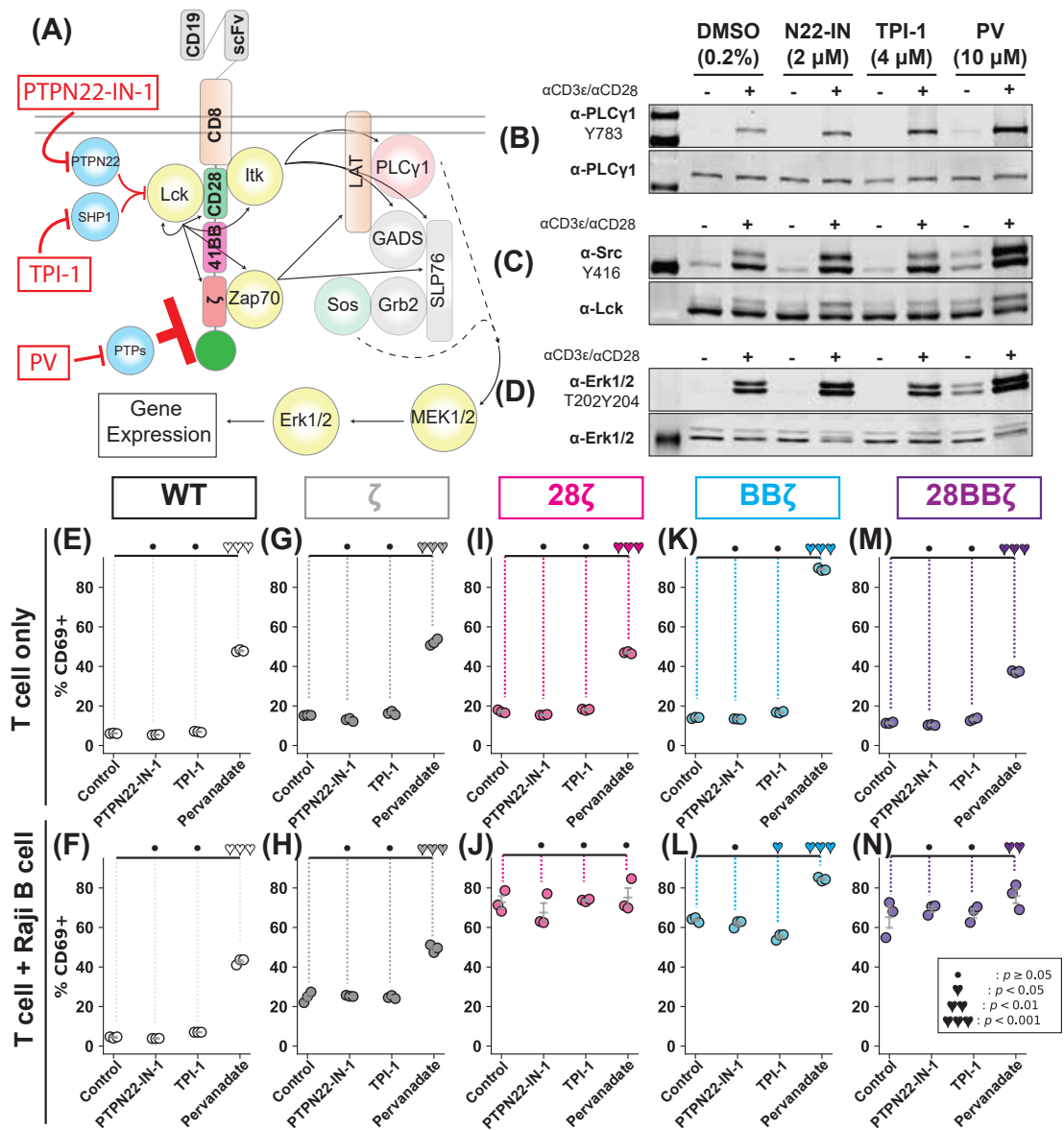


Figure 7.10: PTP activity globally, but not TCR-specific regulatory PTPs, restrict CD69 expression in a costimulation-dependent manner. (A) Simplified schematic representation of CAR T cell signalling with inhibitors and their targets noted. (B-D) Western blot analysis of PLCγ1^{Y783}, Lck^{Y394}, and Erk1^{T202Y204}/Erk2^{T185Y187}, respectively, in samples treated with PTPN22-IN-1, TPI-1, or PV. One sample is shown here for display. Three replicates and quantification are shown in Figure 7.11. (E-F) Dotplot showing the effect of PTPN22-IN-1, TPI-1, and PV on the percentage of CD69+ WT Jurkats in non co-culture and co-culture samples, respectively. (G-H) As in E and F, except for ζ-CAR Jurkats. (I-J) As in E and F, except for 28ζ-CAR Jurkats. (K-L) As in E and F, except for BBζ-CAR Jurkats. (M-N) As in E and F, except for 28BBζ-CAR Jurkats. Three biological replicates are shown for each flow cytometry experiment.

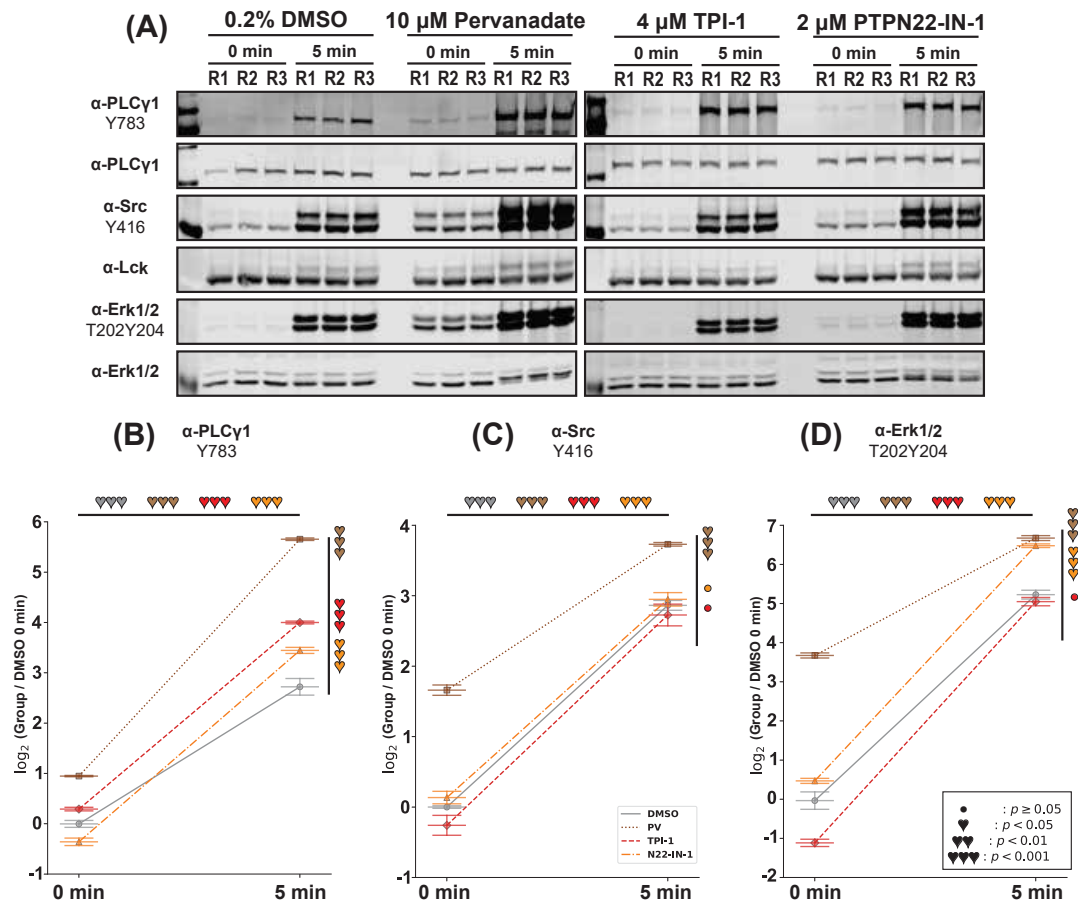


Figure 7.11: Effects of phosphatase inhibitors Pervanadate (global), TPI-1 (SHP-1), and PTPN22-IN-1 (PTPN22) on pY induction of T cell signalling proteins after CD3 ϵ /CD28 antibody crosslinking. (A) Western blot analysis of PLC γ 1^{Y783}, Lck^{Y394}, and Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation in the basal and stimulated states of untreated or phosphatase inhibitor treated Jurkat T cells. (B-D) Quantification of PLC γ 1^{Y783}, Lck^{Y394}, and Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation, respectively.

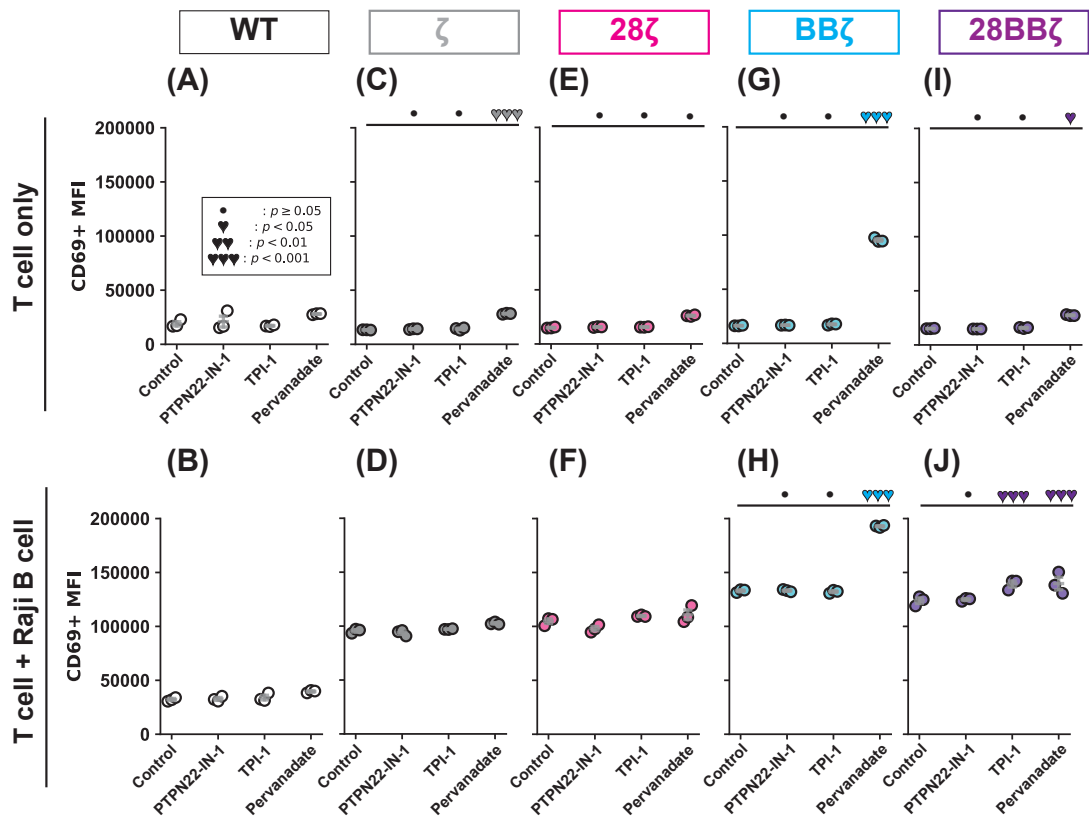


Figure 7.12: 4-1BB containing CD19-CAR Jurkats show elevated CD69 mean fluorescence intensity after PV treatment in both co-culture and non co-culture conditions. (A-B) Dotplot showing the effect of PTPN22-IN-1, TPI-1, and PV on the mean fluorescence intensity of CD69 positive WT Jurkats in non co-culture and co-culture samples, respectively. (C-D) As in A and B, except for ζ -CAR Jurkats. (E-F) As in A and B, except for 28 ζ -CAR Jurkats. (G-H) As in A and B, except for BB ζ -CAR Jurkats. (I-J) As in E and F, except for 28BB ζ -CAR Jurkats. Three biological replicates are shown for each flow cytometry experiment.

7.3.5 Inhibition of Itk reduces baseline CD69 expression while maintaining CD19-CAR T cell activation in the Jurkat model system

The kinases Lck and Zap70, which are required for T cell activation through the TCR(Gaud et al., 2018), play a major role in CAR T cell activation(Ajina and Maher, 2018); however, the role of other proximal and distal kinases, like Itk and MEK1/2, in CAR T cell activation in the Jurkat model system remains unclear. To understand the role of kinases in CAR Jurkat activation, we first showed that small molecule inhibitors of Lck (5 μ M PP1)(Hanke et al., 1996), Itk (2 μ M Soquelitinib)(Hsu et al., 2024), and MEK1/2 (5 μ M U0126)(Favata et al., 1998) functioned appropriately in Jurkats. As expected, 5 μ M PP1 and 5 μ M U0126 significantly dampened TCR-induced phosphorylation of PLC γ 1^{Y783}, Lck^{Y394}, and Erk1^{T202Y204}/Erk2^{T185Y187}(Hanke et al., 1996; Helou et al., 2013), while treatment with 2 μ M Soquelitinib significantly reduced phosphorylation of the Itk substrate PLC γ 1^{Y783} and pathway target Erk1^{T202Y204}/Erk2^{T185Y187}(Hsu et al., 2024)(Figure 7.13B-D, Figure 7.14). To determine whether these kinases perturb CAR Jurkat activation, we treated CD19-CARs with each kinase inhibitor and assessed CD69 expression after 24 h of co-culture. In WT Jurkats, we found that PP1, Soquelitinib, and U0126 reduced the percent CD69+ cells in the basal state but not during co-culture (Figure 7.13E-F). In all CD19-CARs, PP1 treatment reduced the percent CD69+ cells without changing the MFI of CD69+ cells and PP1 or U0126 treatment significantly reduced the percent CD69+ cells after co-culture (Figure 7.13I-N, Figure 7.15). In agreement, Soquelitinib treatment significantly reduced percent CD69+ cells in the basal state of 28 ζ -CARs and 28BB ζ -CARs while BB ζ -CARs were trending toward significance ($p = 0.06$; Figure 7.13I, K, M). No change was observed in the MFI of CD69+ cells after treatment with any inhibitor in the basal state (Figure 7.15 A, 7.15C, 7.15E, 7.15G, 7.15I), suggesting that the remaining CD69+ cells were on the lower end of the intensity threshold to be considered CD69+. However, Soquelitinib-treated CD19-CARs could maintain the co-culture-induced increase in percent CD69+ cells and increases in MFI of CD69+ cells at levels similar to untreated CD19-CAR Jurkats (Figure 7.13J, L, N, Figure 7.15B, D, F, H, J). Together, these data suggest that Itk acts to elevate basal

levels of CD69 in CD19-CAR Jurkats but is not required for activation-induced CD69 expression.

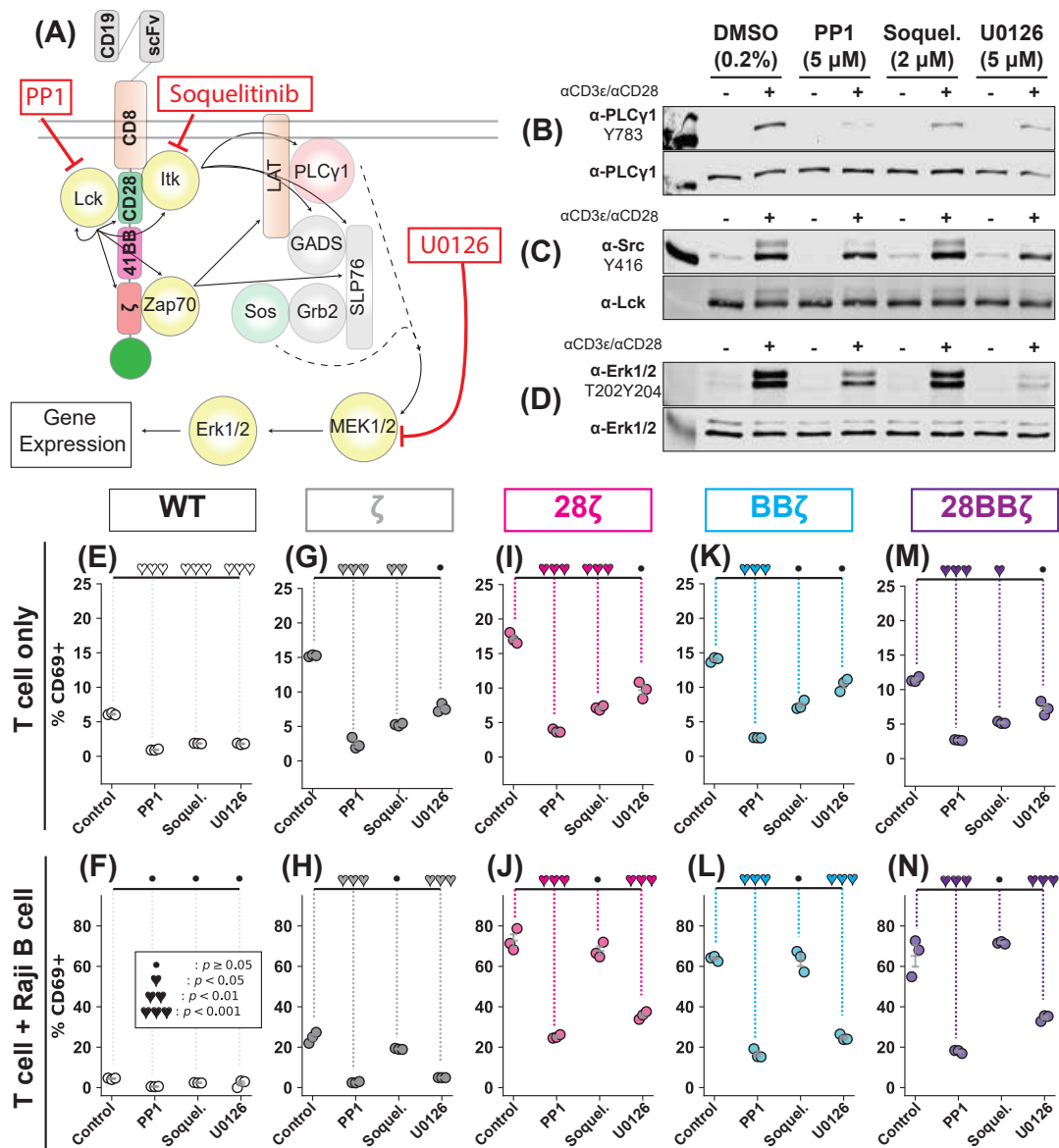


Figure 7.13: Pharmacological inhibition of Itk with Soquelitinib reduces baseline CD69 expression without impairing CAR T cell activation. (A) Simplified schematic representation of CAR T cell signalling with inhibitors and their targets noted. (B-D) Western blot analysis of Lck^{Y394}, PLCγ1^{Y783}, and Erk1^{T202Y204}/Erk2^{T185Y187}, respectively, in samples treated with PP1, Soquelitinib, or U0126. One sample is shown here for display. Three replicates and quantification are shown in Figure 7.14. (E-F) Dotplot showing the effect of PP1, Soquelitinib, and U0126 on the percentage of CD69+ WT Jurkats in non co-culture and co-culture samples, respectively. (G-H) As in E and F, except for ζ-CAR Jurkats. (I-J) As in E and F, except for 28ζ-CAR Jurkats. (K-L) As in E and F, except for BBζ-CAR Jurkats. (M-N) As in E and F, except for 28BBζ-CAR Jurkats. Three biological replicates are shown for each flow cytometry experiment.

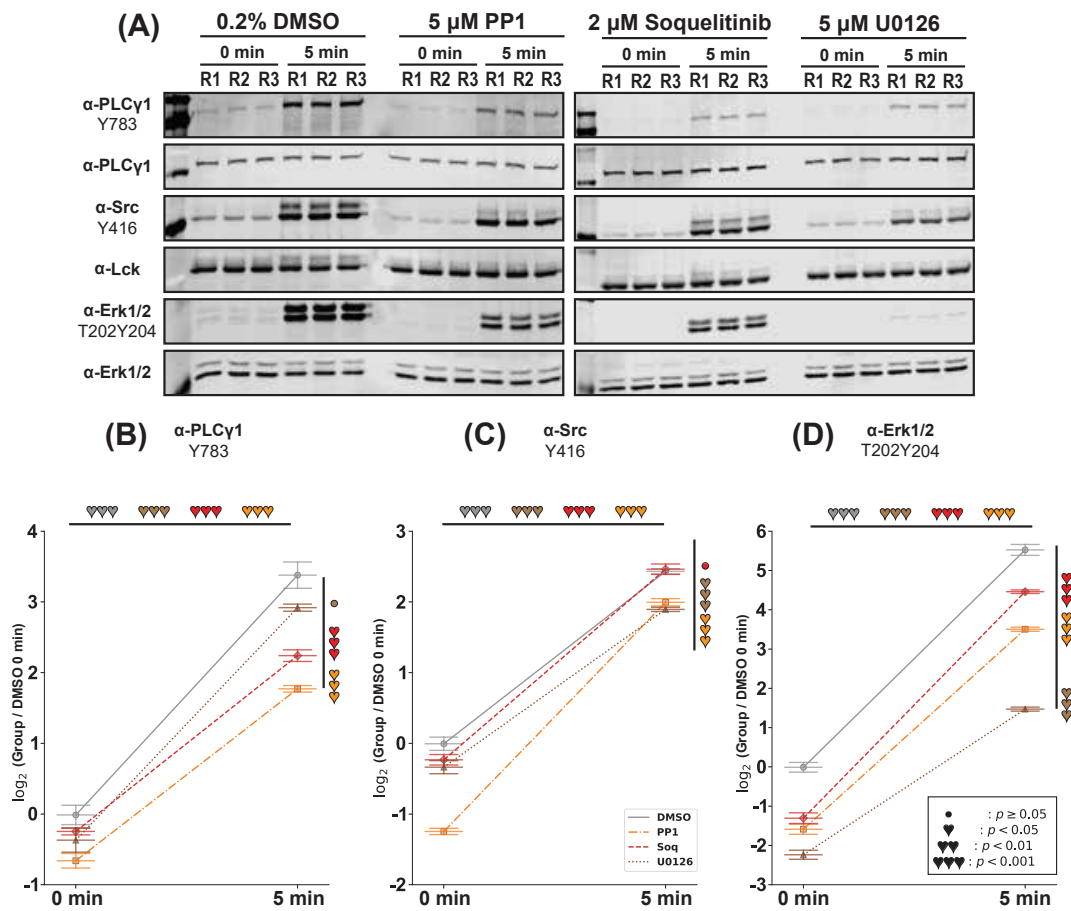


Figure 7.14: Effects of kinase inhibitors PP1 (Lck), Soquelitinib (Itk), and U0126 (MEK1/2) on pY induction of T cell signalling proteins after CD3 ϵ /CD28 antibody crosslinking. (A) Western blot analysis of PLC γ 1^{Y783}, Lck^{Y394}, and Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation in the basal and stimulated states of untreated or kinase inhibitor treated Jurkat T cells. (B-D) Quantification of PLC γ 1^{Y783}, Lck^{Y394}, and Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation, respectively.

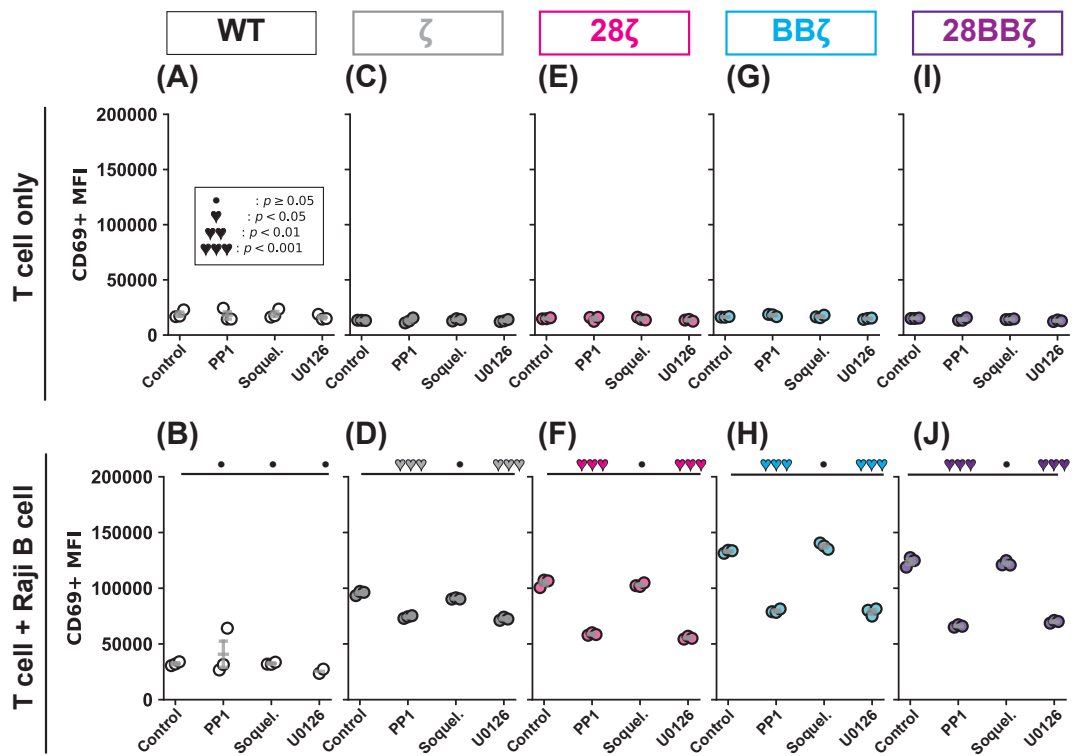


Figure 7.15: Soquelitinib treatment does not effect MFI for CD69+ CD19-CAR Jurkats after co-culture. (A-B) Dotplot showing the effect of PP1, Soquelitinib, and U0126 on the mean fluorescence intensity of CD69 positive WT Jurkats in non co-culture and co-culture samples, respectively. (C-D) As in A and B, except for ζ -CAR Jurkats. (E-F) As in A and B, except for 28 ζ -CAR Jurkats. (G-H) As in A and B, except for BB ζ -CAR Jurkats. (I-J) As in E and F, except for 28BB ζ -CAR Jurkats. Three biological replicates are shown for each flow cytometry experiment.

7.4 Discussion

The development of novel chimeric antigen receptors with increased antigen sensitivity, specificity, and target clearance is necessary to improve the range of CAR T cell applications. Current methods for the identification of functional chimeric antigen receptors require high-throughput screening of CAR constructs expressed in model systems (Rydzek et al., 2019; Gordon et al., 2025; Bloemberg et al., 2020; Gordon et al., 2022; Jahan et al., 2023), such as Jurkats, or low-throughput, logic driven construction (Salter et al., 2021; Tousley et al., 2023). Using these systems, researchers have identified and verified novel CARs that are highly efficacious, outperforming the current clinically approved constructs in mouse models (Gordon et al., 2025). However, Jurkats are PTEN deficient, resulting in mislocalisation of Itk (Shan et al., 2000), which is a well-known limitation of the Jurkat model system. Additionally, Bartelt et al. (2009) report that Jurkats display hyperactivation of early and proximal TCR signalling proteins, including PLC γ 1, Vav1, and Erk1/2 in response to TCR stimulation when compared to another immortalised line (HuT78) and primary human T cells (Bartelt et al., 2009). Jurkats and other T-leukemic cell models, which often display aberrant activity in the PI3K pathway, are thought to underrepresent the importance of PI3K signalling to Akt/mTOR in T cell activation (Kim and Suresh, 2013; Astoul et al., 2001). Despite these limitations, Jurkats remain a cornerstone of innovation in immunology generally (Abraham and Weiss, 2004) and with regards to high-throughput screening platforms for CAR designs. To date, various luciferase Jurkat reporter cells are available for purchase from several companies (Promega, Creative Biogene, and BPS Bioscience), highlighting the desire the biological community has for model systems like Jurkats for biological development and discovery. Despite many studies successfully using CAR Jurkats to screen for new CARs, the molecular effect of Jurkat lines on CAR activation in the context of costimulation domains remains unclear.

Here, we evaluate pY-based signalling in the CAR Jurkat model system using pY-based LC-MS/MS, activation assays, and small molecule inhibition of key TCR regulators. We use four CAR constructs containing an FMC63 scFv, CD8 transmembrane domain, varying costimulatory domains (none ζ -CAR, CD28 intracellular 28 ζ -CAR, 4-

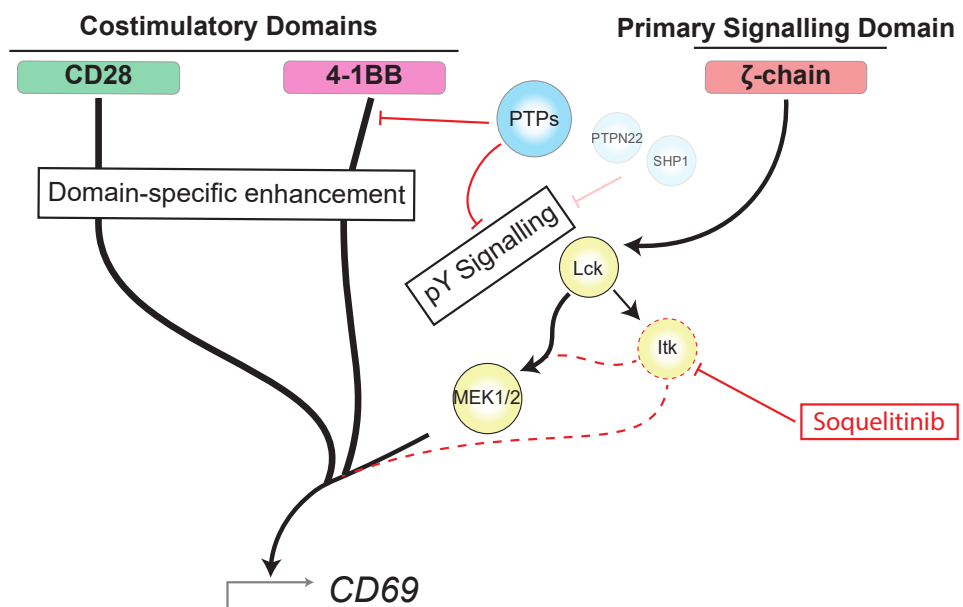


Figure 7.16: Inclusion of TCR ζ drives pY signalling and Itk is dispensable for pY signalling and CD60 production in CD19-CAR Jurkats. CD28 and 4-1BB costimulation provide domain-specific, non-pY based signals to enhance CD69 production, while the TCR ζ is primarily responsible for pY signal induction. Inhibiting protein tyrosine phosphatases (PTPs) globally, which restrict pY signalling, enhances basal CD69 production, however neither the known TCR regulators SHP-1 nor PTPN22 alone drive this phenotype. Inhibiting either Lck or MEK1/2 ablates co-culture induced CD69 production, whereas inhibiting Itk reduces CD69+ cell population while maintaining the ability to induce CD69 production.

1BB intracellular BB ζ -CAR, or both CD28 and 4-1BB intracellular 28BB ζ -CAR), the TCR ζ intracellular domain, and a superfold GFP tag. Our results demonstrate CAR signalling is highly similar, independent of costimulation domains, and very similar to pMHC/TCR ligation (Figure 7.1C-E, 7.4, 7.9, 7.10, 7.13, 7.16). We observe increases in TCR ζ -ITAMs in CAR co-cultures but only TCR ligation induces phosphorylation of ITAMs on CD3 ϵ , CD3 γ , and CD3 δ (Figure 7.4, 7.7), supporting the literature precedent that CAR- and TCR-induced signalling have non-overlapping origins (Barden et al., 2023). Our results are in line with our own previous work, where we have demonstrated that CD19-28 ζ -CAR Jurkats (Callahan et al., 2025) and CD19-28BB ζ -CAR Jurkats (Griffith et al., 2022; Callahan et al., 2025) very strongly engage a TCR-like signalling cascade including robust induction of activating phosphorylation on Itk, PLC γ 1, and Erk. While there does not exist a variable minimised comparison with primary CAR T cells, studies evaluating CAR signalling in primary cells have demonstrated similar but notably different results. Salter et al. (2018) compare phosphorylation after activation of CD19-BB ζ -CARs and CD19-28 ζ -CARs in primary human T cells and find that 28 ζ -CARs more strongly engage the phosphoproteome than BB ζ -CARs; however, both engage pY sites in the TCR signalling pathway including phosphorylation of CD28Y209 (Salter et al., 2018). In our work, we observe little difference in the strength of phosphoproteome engagement between 28 ζ -CAR Jurkats and BB ζ -CAR Jurkats; however, BB ζ -CAR Jurkats do engage CD28^{Y191} similarly to ζ -CAR, 28 ζ -CAR, and 28BB ζ -CAR (Figure 7.4). In a follow-up study, Salter et al. (2021) directly compare TCR stimulation with MHC tetramer and 28 ζ -CAR stimulation with activating antibodies in primary T cells, showing that TCR signalling is stronger than CAR signalling in a simplified stimulation model (Salter et al., 2021). Our results are in agreement, as OVA^{257–264} loaded T2-K^b cells strongly induce tyrosine phosphorylation along the TCR signalling pathway (Figure 7.7). Although our data and those of Salter et al. (2018) and Salter et al. (2021) have different signalling kinetics and fold-changes, the different methods of stimulation (antigen/antibody coated beads versus centrifugal cell-to-cell contact) likely account for these differences. Overall, our results agree with the literature in that TCR ζ containing CARs engage a similar TCR-like pathway; however, the similar induction levels we observe may be an artefact of the Jurkat model system which must be considered in Jurkat-based screens for CAR

function.

It is well established that inclusion of costimulatory domains can greatly affect the strength and timing of CAR T cell activation and whether CAR T cells exhaust or persist in primary cell systems(Lindner et al., 2020), which is likely independent of early, forward progressing phosphorylation-based signalling. Our data resolve the relative roles of early forward phosphorylation versus phosphatase feedback in CAR-Jurkat activation. This clarifies how hierarchical pY nodes connect to CD69 output and provides a practical framework for interpreting Jurkat-based CAR screens and informing construct design. Using a CD69 flow cytometry activation assay, we show that CAR Jurkats require costimulation domains to increase the CD69+ population, and that ζ -CAR and 28 ζ -CAR show high basal CD69 populations (Figure 7.9). Using the CD69 activation assay with small molecule inhibitors of SHP-1 (TPI-1), PTPN22 (PTPN22-IN-1), and PTPs globally (PV), we find that the phosphatase activity of SHP-1 and PTPN22 is not important for regulating the activation of CD19-CAR Jurkats; however, global PTP activity thresholds CD69 expression particularly in BB ζ -CARs (Figure 7.10, 7.16). Previous literature has shown that PTPN22 deletion does not affect CAR clearance of solid tumours(Du et al., 2022); however, some literature suggests SHP-1 knockout in primary T cells enhances the killing ability of some CAR T cells(Petersen et al., 2019), SHP-1 negatively regulates CD19-BB ζ -CAR activation(Sun et al., 2020), and SHP-2 constitutively associates with BB ζ -CARs and dissociates after stimulation(Ritmeester-Loy et al., 2025). Our finding that PV treatment elevates CD69 expression in BB ζ -CAR Jurkats is likely due to complete inhibition of all 4-1BB-associated phosphatases, and suggests that phosphatases downregulating BB ζ -CARs may be responsible for the low basal activation associated with BB ζ -CARs(Long et al., 2015). Further, T cell protein tyrosine phosphatase (TCPTP; gene name PTPN2) deletion has also been implicated in thresholding HER2-28 ζ -CAR cytotoxicity(Wiede et al., 2020). Our results demonstrating that SHP-1 phosphatase activity does not influence CD19-CAR Jurkat activation, but global phosphatase inhibition does, suggest that negative regulation of CD19-CARs in Jurkats differs from CAR primary T cells and should be taken into account when considering candidates produced during CAR-Jurkat screens.

Finally, we examine downstream signal propagation through the Lck-Itk-PLC γ 1-MEK-ERK pathway in CD19-CAR Jurkats using small molecule inhibitors of Lck (PP1), Itk (Soquelitinib), and MEK1/2 (U0126). In our study, treatment of CD19-CAR Jurkats with these inhibitors after TCR stimulation recapitulated the canonical Itk-dependent TCR signalling pathway reported in primary T cells, despite PTEN loss, constitutive PI3K–Akt activity, and constitutive Itk membrane localisation in Jurkats (Figure 7.13B-D, 7.14). As expected, PP1 treatment ablates CD19-CAR-induced baseline CD69 expression and stunts antigen-induced activation, and inhibition of MEK1/2 acts similarly, although to a lesser extent (Figure 7.13E-N). Interestingly, inhibition of Itk reduces the CD69+ population to the level of untreated WT Jurkats in all CD19-CAR Jurkats, with significant reductions in CD69+ CAR Jurkats in ζ -CAR and 28 ζ -CAR compared to untreated ζ -CAR and 28 ζ -CAR (trending toward significance in BB ζ -CAR; Figure 7.13E-N, 7.16). Soquelitinib (also known as CPI-818) is a recently described selective, irreversible Itk inhibitor that has been recently used to reduce exhaustion markers and restore effector function to previously exhausted T cells (Hsu et al., 2024) and has been recently given an FDA Orphan Drug Designation for the treatment of T cell lymphomas. Soquelitinib was recently used to show that the kinase activity determines the differentiation of naive CD4+ T cells into T helper 17 cells in a calcium dependent, MAPK independent manner (Anannya et al., 2025), further demonstrating the unique role of Itk in T cell maintenance. The trend toward decreased basal CD69 expression in all CD19-CAR Jurkats likely stems from mislocalisation and hyperactivity of Itk in Jurkats; however, point mutations to the Itk binding sites of CARs within CD28 domains have been shown to reduce exhaustion without compromising cytotoxicity (Boucher et al., 2021). Our results, showing that Itk inhibition can reduce baseline CD69 expression without impairing antigen-induced CAR Jurkat activation, suggest that Itk is a promising target for reducing antigen-independent effects of CAR T cell therapies.

7.5 Conclusion

The rush to develop new chimeric antigen receptors with high specificity and low off-target effects leads to the use of classical T cell model systems for their reliability

and well characterised activation pathways. Our results suggest that activation in CAR Jurkats is dominated by TCR ζ on the CARs at the pY signalling level, but full activation occurs only with costimulation domains, which replicate in vivo studies. Further, we find that global PTP activity thresholds CAR Jurkat activation, but PTPN22 and SHP-1 phosphatase activity are not important for CAR Jurkat regulation. Finally, we provide evidence that Itk inhibition can reduce baseline activation of CAR Jurkats irrespective of inclusion of costimulation domains, without compromising antigen-induced activation. Our findings show that the CAR Jurkat model displays a strong phosphotyrosine signalling phenotype independent of inclusion of costimulation domains and that perturbation of the CAR Jurkat signalling pathway (e.g., Lck or Itk inhibition) replicates expected pathway dependencies in primary T cells.

7.6 Acknowledgments

We would like to thank Dr. Nicholas DaSilva at the Brown University Proteomics Core facility for helping us operate the mass spectrometer, Dr. Kevin Carlson at the Brown University Flow Cytometry and Cell Sorting Facility for running the flow samples, and Dr. Christoph Schorl at the Brown University Genomics Core Facility for allowing us to use the LI-COR scanner for Western blotting.

Funding Sources

NIH grant P01AI091580 (ARS), 1S10OD036295 (ARS), R35 GM138299(XS), R21 CA286364(XS), R21 CA294038(XS), NIH Director's Transformative Research Award EB037112(XS), American Cancer Society Research Scholar Grant 135926(XS), Gabrielle's Angel Foundation Medical Research Award(XS), Pershing Square Sohn Prize for Young Investigators in Cancer research(XS), Human Frontier Science Program Early-Career Research Grant RGY0088/2021(XS).

Competing Interest Statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Arthur Salomon reports financial support was provided by National Institute of Allergy and Infectious Diseases. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 8

Discussion

The use of modern mass spectrometers to assess global protein and PTM abundance has revolutionised our understanding of the immune system. Improvements in the engineering of mass spectrometers has reduced the sample input requirements for confident identifications, while novel sample preparation techniques have improved overall identifications in complex samples. Mass spectrometry-based proteomics has historically and recently pushed T cell biology forward; from the initial mapping of T cell receptor signalling events to recent studies evaluating the interactome of integral TCR signalling proteins. Further, mass spectrometry-based proteomics has been instrumental in understanding the effects of chimeric antigen receptor (CAR) T cells and allowed researchers to design more effective CARs. The aim of the research presented here is threefold: (1) to evaluate current methods for mass spectrometry sample preparation and how they impact phosphotyrosine (pY) site identification, (2) to use mass spectrometry based pY proteomics to understand regulatory mechanisms of T cell signalling, and (3) to use mass spectrometry-based pY proteomics to understand signalling in CAR T cells and their targeted cancers.

8.1 Developing methods for reproducible, deep identification of tyrosine phosphorylation in T cells

Modern mass spectrometers are highly sensitive and fast, allowing for the rapid identification of many peptides from complex samples. Improvements in instrumentation and methodologies has allowed for highly reproducible identification of low abundance PTM peptides, particularly those containing pY, which are instrumental in maintaining homeostasis in human cells(He et al., 2023; Lancaster et al., 2024). Despite these advancements, many research groups are dedicated to pushing the limitations of mass spectrometers for PTM identification through optimisation of sample preparation and leveraging the speed of newer instruments(Xie et al., 2022; Gluth et al., 2024; Yang and Qiao, 2023). In Chapters 2 and 3, I present research on two methodological approaches for improving pY site identification. First, I provide a rigorous evaluation of high protein input sample preparation products, and second I demonstrate the feasibility of using sample multiplexing with carrier channels for primary T cell pY proteomics.

A necessary step in the preparation of proteomics samples is desalting, as non-peptide ions from samples compete for space during the first ion acquisition step (MS1) and reduce peptide identifications. Historically, desalting has been performed using affinity purification on reverse phase (C18) columns, where peptides bind with high affinity, are washed with acidic solvent, and eluted using an organic solvent (e.g. 40% acetonitrile). Suspension trapping is an adaptation of this technique where whole proteins are trapped in a matrix, washed, digested using trypsin while trapped in the matrix, and free peptides are then eluted from the matrix. While many papers have evaluated these technologies for small protein input ($<100\text{ }\mu\text{g}$) samples, little if any work has evaluated these products for high protein input ($> 3\text{ mg}$) samples or downstream enrichments. In Chapter 2, I provided an evaluation of four products for sample preparation of high protein input samples with unique protocols: Waters Sep-Pak C18 vacuum columns, Pierce C18 microcentrifuge spin columns, TECAN Narrow Bore Extraction (NBE) columns (compatible with the TECAN Resolvex A200 liquid handling system), and ProtiFi s-Trap

spin columns. While sample preparation using TECAN NBE columns was greatly aided by using the Resolvex A200, ProtiFi s-Trap provided superior peptide recovery ($\sim 80\%$), protein identification (4800 with $3 \leq n \leq 4$), and pY site identifications (848 with $n \geq 1$, 300-400 with $3 \leq n \leq 4$). My data are in agreement with previous work, as Uslan *et al.* 2025 compared low protein input s-Trap and C18 desalting columns and found that s-Trap had superior recovery compared to traditional Urea/C18 sample preparation techniques (Uslan *et al.*, 2025). The recovery percentage for C18 desalting columns in my work is lower than what has been reported previously (Uslan *et al.*, 2025), however low protein input preparation methods use lower volumes at every step (lysis, washes, elution) and different matrix geometries, which can impact peptide recovery (Nie *et al.*, 2022; Kassem *et al.*, 2021; Martin *et al.*, 2020). While ProtiFi s-Trap was superior by most analytical metrics, the spin-column format of s-Trap was the most time consuming to use, especially considering that high protein input samples can clog columns and require repeated centrifugation during wash steps. In summary, these data show that ProtiFi s-Trap provides a reproducible, although optimisable, workflow for pY proteomics sample preparation.

While desalting plays a necessary function in pY proteomics sample preparation, researchers have found ways to exploit the design of mass spectrometers themselves to improve identification of low abundance peptide ions. In the blooming field of single cell proteomics, isobaric tags are chemically attached to peptides in very low input (< 1 ng) samples from single cells and an overloaded sample with $\sim 50\times$ the sample input of any other sample. When all the samples are pooled and peptide ions are acquired in MS1 and MS2 on the mass spectrometer, the overloaded sample ‘carries’ the low-abundance ions from samples along with it, thus tricking the instrument into identifying ions well below the limit of sensitivity of the instrument. In Chapter 3, I demonstrated the feasibility of broad spectrum optimisation of selective triggering (BOOST), which uses a pY overloaded sample similar to those used in single-cell proteomics, in primary murine T cells. Developed by Chua *et al.* (2020) in the Jurkat T cell model system, Chua *et al.* (2020) was able to identify ~ 2000 pY PSMs using 1 mg of receptor stimulated Jurkat T cells when using BOOST, whereas only 150 pY PSMs were identified without BOOST. I found similar results using primary cells, where I identified > 2000 pY

PSMs with quantified reporter ions per sample using BOOST, and only ~ 300 pY PSMs were identified without BOOST. These results demonstrate a remarkable jump in pY site characterisation in primary murine T cells, as a recent study reported using ~ 20 mg of primary murine T cell protein for pY proteomics, only characterising < 300 pY sites total (Locard-Paulet et al., 2020). The improvements in pY identification led directly to uncharacterised, TCR-responsive pY sites, as I uniquely identified Cbl-b^{Y763}, SLP76^{Y483}, and Csk^{Y184} as significantly TCR-responsive when using BOOST in addition to many previously characterised TCR signalling sites like TCR ζ and CD3 ϵ ITAMs and Erk1^{Y185}/Erk2^{Y205} observed in BOOST and control experiments. While the BOOST method improves identification of pY sites, it is limited by the number of isobaric tags available and their price (the 11-plex kit used in this work is now 3000–6000), which limits the scope of pY proteomics studies using BOOST. Regardless, a 7-fold improvement in pY identifications in primary cells is a monumental step forward in characterising the function of uncharacterised pY sites.

The current era of mass spectrometers are highly sensitive, fast instruments that have the potential to identify more PTMs than ever before. Improving reproducible identification of low abundance PTM species like tyrosine phosphorylation, however, remains a large challenge in the proteomics field. While the data I provide herein provide an evaluation of sample preparation techniques to improve pY site identifications, considerable work remains to be done to expand the potential of these techniques. In particular, semi-automation of sample desalting by marrying ProtiFi s-Trap columns with an instrument like the Resolvex A200 will reduce technical variability between samples. Manual washing steps during bead-based enrichment techniques like Src SH2 Superbinder pY enrichment leads to unnecessary sample loss due to accidentally removing and discarding beads¹. Semi-automation of this process through packing beads into Resolvex A200-compatible columns will remove the technical variation in pY enrichments, reduce the overall time required for sample preparation, and would be compatible with commercially available enrichment products (e.g. Cell Signaling Technologies PTMScan kits). The BOOST method can, in theory, be expanded to global phosphorylation

¹It's actually insane how many beads you lose during the wash steps. It doesn't even seem to matter how careful you are, beads will ALWAYS be lost.

(pS/pT/pY) or other low abundance PTMs like O-GlcNacylation, S-nitrosylation, or arginine methylation. Further, newer TMT kits are available that can accommodate more samples (e.g. a 35-plex set is available as 3 stand alone products from Thermo Fisher), which will allow higher replicate numbers and conditions than were previously available. Together, my data provide a basis with which to improve and expand the applicability of PTM enrichment proteomics.

8.2 Novel roles for protein tyrosine phosphatases and kinases in the TCR signalling pathway

Coordinated activation and deactivation of the immune response is essential to maintaining human health. T cell activation through the T cell receptor is rather well described, with known initiators (Lck/Fyn), propagators (Zap70/Itk/Rel/Tec/PLC γ 1), aggregators (LAT/SLP76), and deactivators (SHP1/SHP2/TCPTP/PTPN22/JKAP) which each play vital roles in maintaining homeostasis before, during, and after binding of the TCR to a peptide-bound MHC. Mutations in T cell signalling proteins that influence the activity or timing of T cell activation are a well known cause of immune disorders (Ashouri et al., 2022; Keller et al., 2024; Lanz et al., 2024; Lui et al., 2023; Hauck et al., 2012; Wallace et al., 2021; Ghosh et al., 2014; Croker et al., 2008; Shultz et al., 1997; Tizaoui et al., 2022; Fousteri et al., 2013; Zikherman and Weiss, 2011; Wiede et al., 2011; Bacchelli et al., 2017; Lev et al., 2020). While we have come a long way to understanding the intricacies and balance in T cells, many T cell signalling proteins remain incompletely described outside of their canonical role in the TCR signalling pathway. In Chapters 5 and 6, I use pY proteomics workflows to evaluate the roles of kinases and phosphatases in the context of global pY maintenance and signalling mechanisms in the Jurkat T cell model.

In T cell signalling, the kinases Zap70 and Itk play primary roles in signal transduction and T cell activation. After ITAM phosphorylation by Lck/Fyn, Zap70 binds phosphorylated ITAMs through its dual SH3 domains before Zap70^{Y492Y493} phosphorylation by Lck. After activation, Lck bridges Zap70 to LAT, providing Zap70 with access to multiple

substrates (including LAT^{Y132}, LAT^{Y220}, SLP76^{Y145}) that regulate distal TCR signalling and activation. Similarly, activation of Itk by Lck-mediated phosphorylation of Itk^{Y512} during CD28 costimulation promotes Itk-mediated phosphorylation of PLC γ 1^{Y783} and GADS^{Y45} post LAT/SLP76 signalosome formation, integrating CD28 costimulation with the mainline TCR signalling cascade. Outside of these canonical roles for Zap70 and Itk, our understanding of their broader roles in pY maintenance and branch pathways is incomplete. In Chapter 5, I coupled pY proteomics with novel, covalent inhibitors of Zap70 (RDN2150) and Itk (Soquelitinib) and multiple T cell activation methods (soluble antibodies, APC-pMHC/TCR, and CD19-CAR/Raji) to better understand their roles in T cell signalling and activation. I showed that the strength of TCR pY site induction and expression of the activation markers CD69 and IL-2 do not correlate, and that Zap70 uniquely controls of activation-induced CD69 expression whereas the kinase activity of Zap70 and Itk is required for IL-2 production. These results are in agreement with previous literature, as soluble antibodies do not provide sufficient immune synapse formation or force generation for T cell activation(Manger et al., 1987; Trickett and Kwan, 2003) and Itk is a primary driver of IL-2 production downstream of Zap70 in T cells(von Bonin et al., 2011). Using pY proteomics, I show that pY induction occurs in the presence of 10 μ M RDN2150 or 10 μ M Soquelitinib, however the number of significantly induced pY sites drastically varies by stimulation method. Regardless, RDN2150 has the most profound impact on pY abundance overall, significantly reducing the abundance of pY sites compared to DMSO treatment irrespective of stimulation method. Soquelitinib treatment significantly reduces pY abundance in soluble antibody treated samples however no clear trend is observed in APC-pMHC/TCR or CD19-CAR/Raji stimulation samples, which may be due to differences in the strength of stimulation or differences between TCR and CAR-induced signalling pathways(Salter et al., 2021). Notably, many pY sites on heterogeneous nuclear ribonucleoproteins (hnRNPs) were significantly reduced after treatment with RDN2150, however treatment with Soquelitinib tended to increase pY abundance on hnRNP pY sites. Currently, only hnRNPK has been linked directly to TCR signalling(Chang et al., 2009), however hnRNPs have generally been shown to be impactful for T cell physiology(Meininger et al., 2016; White et al., 2025; Shankarling et al., 2014). Further, the phosphorylation state of tyrosine residues on

hnRNPs have been implicated in the phase separation properties and therefore function of hnRNPs (Ryan et al., 2021). Together, my data suggests that the kinase activity of Zap70 and Itk control distinct branches of T cell activation, particularly with respect to hnRNP phosphorylation and RNA processing. While feedforward activation of T cells is dominated by tyrosine kinases, negative feedback pathways use protein tyrosine phosphatases (PTPs) to reel T cells back in.

After successful activation, PTPs are activated and promote deactivation of many components of the TCR signalling pathway. Particular attention has been paid to deactivation of Lck through dephosphorylation of Lck^{Y394}. Phosphorylated Lck^{Y394} has been independently shown to be substrates of four PTPs, T cell protein tyrosine phosphatase (TCPTP), PTP non-receptor type 22 (PTPN22), SH2 domain containing tyrosine phosphatase 1 (SHP1), and JNK-associated phosphatase (JKAP), although the cellular context in which each phosphatase acts to dephosphorylate Lck^{Y394} is not well understood for each phosphatase. Further, outside of their role in Lck^{Y394} dephosphorylation, the roles of PTPs in feedback regulation of the TCR and pY maintenance in T cells is not well characterised. In Chapter 6, I used Jurkat T cells with CRISPR/Cas9 knockouts of *PTPN2* (TCPTP), *PTPN22*, and *PTPN6* (SHP1) with pY proteomics to understand the specific roles of TCPTP, PTPN22, and SHP1 in TCR signalling. After clone selection, I used shotgun proteomics to evaluate changes to the proteome in each PTP KO cell line. I found that all PTP KOs maintained expression of TCR signalling proteins, but each PTP KO showed unique changes in expression of non-TCR proteins. Using pY proteomics, I found that each PTP played unique roles in pY maintenance in resting and TCR activated T cells, with PTPN22 KO cell line showing elevated pY site abundance, SHP1 showing reduced pY site abundance in resting and TCR activated T cells, and TCPTP KO showing slightly elevated pY site abundance in resting T cells but even upregulated/downregulated pY sites in TCR activated T cells. Thus, it appears that each phosphatase, either directly or indirectly, has a unique impact on the pY proteome of T cells outside of their canonical role as ‘negative regulators’ of TCR signalling (Chiang and Sefton, 2001; Gjörloff-Wingren et al., 1999; Wiede et al., 2011). Previous work from the Salomon Laboratory showed the inhibition of MEK1/2 using the small molecule U0126 led to a global decrease in tyrosine phosphorylation, which

was thought to be a consequence of the controversial positive feedback loop between Erk1/2, SHP1, and Lck (Helou et al., 2013). To determine whether the pY reduction caused by MEK1/2 inhibition involves TCPTP, PTPN22, or SHP1, I treated PTP KO cell lines with U0126 and performed pY proteomics. Wild-type Jurkat T cells, PTPN22 KO Jurkats, and SHP1 KO Jurkats all show a universal decrease in pY abundance after treatment with U0126 in the resting and TCR activated states, however the TCPTP KO Jurkats showed a mild decrease in the rested state with no appreciable trend in the TCR activated state. Importantly uncharacterised pY sites on critical TCR signalling proteins were identified as significantly upregulated in the TCPTP KO cells treated with U0126, including Lck^{Y414}, Lck^{Y209}, Zap70^{Y248}, and Itk^{Y273}, which may be MEK1/2 and/or TCPTP-regulated pY sites involved in feedback regulation of the TCR. These data are in contrast to the established model of Erk positive feedback, where Erk1/2 mediated phosphorylation of Lck^{S59}, which disrupts SHP1 docking to Lck (Štefanová et al., 2003). Instead, my data suggest the existence of a feedback pathway involving TCPTP and MEK1/2 which helps maintain proper pY abundance during TCR signalling.

Regulatory mechanisms governing T cell receptor signalling are complex, with many seemingly redundant proteins and proteins that are necessary yet not well understood. In Chapters 5 and 6, I used mass spectrometry based proteomics to show that Itk controls a distinct subset of forward TCR signalling and that the phosphatases TCPTP, PTPN22, and SHP1 play unique roles in T cell phosphotyrosine maintenance, respectively. Recently, reducing Itk activity through inhibition or induced deficiency has been shown to improve TCR and CAR T cell function against solid tumours, reduce antigen-independent activation, and preserve CAR T cell stemness (Fan et al., 2021; Song et al., 2025; Jiang et al., 2023; Fu et al., 2025), presumably by modulating activity of the CD28 costimulatory domain (Guedan et al., 2020; Hsieh et al., 2020; Boucher et al., 2021). However, contradictory evidence suggests that Itk overexpression can improve TCR-dependent CD8⁺ T cell cytotoxicity towards dual large B cell lymphomas (Liu et al., 2025), suggesting that Itk may play more nuanced, context-dependent roles in regulation of TCR activation. Given my results that Itk inhibition elevates tyrosine phosphorylation of hnRNP proteins, some of which are known to be involved in late-stage TCR-based activation and T cell differentiation (Chang, 2016; Shankarling et al., 2014;

Ozay et al., 2020; Mallory et al., 2020; White et al., 2025), future work may focus on discovering the function of hnRNPs in T and CAR T cells and the connection between Itk function and hnRNP tyrosine phosphorylation. Further, given the immunotherapeutic potential of Itk(Weeks et al., 2021), the beneficial effects of Itk-disrupting mutations on CD28 costimulated CARs(Guedan et al., 2020; Hsieh et al., 2020; Boucher et al., 2021), and reducing T cell exhaustion in patients with T cell lymphomas(Miller et al., 2025), Soquelitinib should be evaluated as a combination therapy to improve function of currently available CAR treatments. Additionally, PTPs in T cells have long been thought to play redundant roles in the regulation of TCR signalling, namely through dephosphorylation of Lck^{Y394}(Chiang and Sefton, 2001; Gjörloff-Wingren et al., 1999; Wiede et al., 2011). TCPTP, PTPN22, and SHP1 have all been implicated in various immune disorders(Zikherman and Weiss, 2011; Bottini et al., 2006; Croker et al., 2008), however each has ambiguous yet unique biological contexts that inform how effective the immunotherapies are. For example, PTPN22 deficiency does not improve CAR T cell function, however SHP1 acts as a context-dependent negative regulator of CAR T cell function(Sun et al., 2020) and loss of TCPTP enhances *in vitro* and *in vivo* efficacy of CAR T cells(Wiede et al., 2020; Du et al., 2025; Miao et al., 2023). My results suggest that TCPTP, PTPN22, and SHP1 play unique yet critical roles in feedback regulation of the TCR and pY maintenance in T cells, and understanding the specific pathways each PTP governs is instrumental for understanding the origins of PTP-based immune disorders and immunotherapies. Specifically, using interactomic approaches to determine the positive feedback network between Erk/MEK, TCPTP, and the TCR may provide new druggable targets for TCPTP-dependent autoimmune disorders. While the substrates of PTPN22 and TCPTP have been mapped in T cells(Wu et al., 2006) and podocytes(LeBleu et al., 2026), respectively, mapping of TCPTP and SHP1 substrates in T cells is necessary understand their roles in pY maintenance, as my pY proteomics study does not account for indirect effects of PTP knockouts (e.g., hyperactivity of kinases due to loss of a negative regulator). Regardless, my data provide a global characterisation of how T cell kinases (Zap70 and Itk) and phosphatases (TCPTP, PTPN22, and SHP1) influence the pY proteome in human T cells, providing a basis for future research regulation of T cell signalling and homeostasis.

8.3 Ubiquity of CAR signalling in models and target-dependent effects of CARs

Chimeric antigen receptor T cell therapy relies on condensing the minimum necessary components for T cell activation into a single-pass transmembrane protein. While successful for specific cancers(Thirumalaisamy et al., 2025), the differential construction of CAR T cells has led to divergence in long-term phenotypes and clinical outcomes(Abbasi et al., 2023). Rather than understanding the basic biology of a chimeric antigen receptor, how it differs from its natural counterparts, and whether interactions with the targeted tumour impact efficacy, current research has hyperfocused on optimisation of CAR function through logical design and high-throughput screening(Roex et al., 2025). Despite their efforts and hundreds of proposed CAR designs our current efforts have only resulted in a handful of clinically successful CAR therapies, highlighting the need for a more thorough understanding of CAR biology. In Chapters 4 and 7, I evaluated the impact of CAR design on physiological responses in the targeted cells and whether costimulation on CARs impacts pY signalling or feedback to the CARs in a Jurkat T cell model.

The current design of CARs are modelled after Gross and Eshhar (1992), who showed that single chain variable fragments (scFvs) provided T cells with antibody-like specificity toward a targeted antigen and the ability to engage TCR-like signalling. All clinically available CARs use scFv-based targeting moieties, however researchers have recently begun investigating native ligand-based CARs. Ideally, native ligands provide high, well characterised specificity toward antigens overexpressed on tumours, and targets can be chosen with a high tumour/non-tumour expression to minimise off-target effects(Ramírez-Chacón et al., 2022). However, this design choice neglects the fact that many (if not all) of these ligands naturally induce signalling cascades after binding to their target receptors, potentially altering the target cell's physiology(Uings and Farrow, 2000). In Chapter 4, I used a co-culture based pY proteomics approach to show that a CSF1R-based CAR (CSF1R-CAR) induces CSF1R signalling in THP1 cells expressing CSF1R, which corresponds to ratio-dependent THP1 growth. In particular, phosphorylation sites associated with cell growth and differentiation on CSF1R increase in abundance shortly (<5 min) after co-culture with CSF1R-CAR Jurkat cells. CSF1R signalling is

tissue-dependent and incompletely characterised in macrophage-lineage cells(Han et al., 2022; Otero et al., 2009; McVicar and Trinchieri, 2009; Hu et al., 2021; Chaintreuil et al., 2023), however I observed phosphorylation of CSF1R pathway components like Erk1^{T202Y204}/Erk2^{T185Y187}, Pyk2^{Y579Y580}, and PLC γ 2^{Y759}. These results contrast a clinically approved, scFv-based CD19-CAR design, where targeted Raji cells show little-to-no significant changes in pY abundance after co-culture. Disruption of the kinase activity of CSF1R, but not T cell activation or T cell cytoskeletal reorganisation, prevents CSF1R-CAR induced CSF1R^{Y723} and Erk1^{T202Y204}/Erk2^{T185Y187} phosphorylation. Thus, it appears that only the ligand-receptor interaction is necessary for the ligand-based CSF1R-CAR to induce CSF1R signalling in THP1 cells. Considering that CSF1R signalling is involved in growth and differentiation of macrophage lineage cells(Rojo et al., 2019), I then demonstrated that high expression of CSF1R in THP1 cells and low effector-to-target (E:T) ratios during co-culture can induce proliferation in THP1 cells, rather than killing. At high E:T ratios, however, primary T cells expressing CSF1R-CAR are able to induce lysis of THP1 cells, suggesting that CSF1R-CAR induced growth signals compete with CSF1R-CAR induced lysis signals in targeted cells. These results again contrast the scFv-based CD19-CAR design, where Raji cell killing is induced at all E:T ratios tested. Previous studies evaluating ligand-based CARs, particularly those targeting CSF1R and EGFR, use a static, relatively high E:T ratio ($\geq 1:1$)(Chinsuwan et al., 2023; Shinagawa et al., 2025; Achkova et al., 2022), which may obscure any CAR-induced responses in the targeted cells. Few studies have evaluated target cell responses to CAR co-culture, however a recent report by Zhong et al. (2023) showed that scFv-based Mesothelin targeted CAR T cells induced pancreatic ductal adenocarcinomas to release PD-L1 filled small extracellular vesicles to impede CAR T cell function, which can be attenuated via inhibition of exocytosis(Zhong et al., 2023). My results, in combination with those of Zhong et al. (2023), suggest that targeted cells play a more active role in CAR T cell therapies than previously thought.

While investigating target-dependent responses to CAR T cell therapy is understudied, much research has been dedicated to phenotypic characterisation of current therapeutic CARs and discovery of novel, superior CAR designs. All therapeutically used CAR designs are second generation, use either a CD19 or BCMA targeting scFv, and either

CD28 or 4-1BB costimulation, and display markedly different phenotypes *in vivo*. CD28 costimulated CARs are known to be aggressive, quickly activating and lysing targeted cells before exhausting and being cleared. In contrast, 4-1BB costimulated CARs often display a prolonged activation period before differentiation into memory T cells for long-term surveillance(Cappell and Kochenderfer, 2021). Recently, researchers have relied on high throughput screens in cell lines, namely Jurkat T cells, to identify candidate CARs for further evaluation using *in vitro* lysis assays and *in vivo* tumour clearance models(Roex et al., 2025). In Chapter 7, I used pY proteomics to evaluate multiple generations of short term (≤ 15 min) CAR signalling before assessing mid-term (24 hour) activation during perturbation of known TCR signalling pathway components. I found that the primary determinant of pY signalling was the inclusion of TCR ζ in the CAR design, as a first generation CAR without costimulation displayed similar pY signalling patterns to all costimulated CARs. These results largely agree with the literature, although previous studies in primary T cells have demonstrated that some BB ζ -CAR T cells engage the TCR signalling pathway to a lesser extent than 28 ζ -CAR T cells(Sun et al., 2020; Salter et al., 2018; Yao et al., 2024), contrary to my results in CAR Jurkats. In agreement with clinical literature(Kershaw et al., 2006), I found that costimulation is required for CAR-based activation, which I measured using CD69 surface expression(Rydzek et al., 2019; Gordon et al., 2025, 2022; Bloemberg et al., 2020). Considering that previous reports point to differences in negative regulation of CD28 and 4-1BB costimulated CAR T cells(Sun et al., 2020; Zolov et al., 2018), I next evaluated whether negative regulators of T cell signalling impact CAR Jurkat physiology. In line with work in primary T cells, pharmacological inhibition of PTPN22 did not impact basal or antigen-induced CD69 expression in CAR Jurkats. Pharmacological inhibition of SHP1 also did not impact CD69 expression in CAR Jurkats, although SHP1 has been identified as a negative regulator of 4-1BB costimulated CAR T cells(Sun et al., 2020). Global PTP inhibition using 10 μ M Pervanadate, however, significantly increases basal CD69 expression in CAR and WT Jurkats, confirming that PTP activity is important for maintaining low basal T cell activation. Importantly, 100 μ M Pervanadate treatment was recently shown to induce oxidation of Src, relieving autoinhibition and increasing Src activity(Mulholland et al., 2026), indicating that the increased T cell activation I observed

in 10 μ M Pervanadate-treated Jurkats may be, in part, due to off-target activation of the Src family kinases Lck/Fyn. Finally, I assessed the role of the canonical TCR signalling proteins Lck, Itk, and MEK1/2 in CD19-CAR Jurkat activation. In agreement with prior literature(Wan et al., 2025), I found that the kinase activity of Lck was necessary for CAR-based Jurkat activation, as treatment with 5 μ M PP1 completely ablated CD69 induction. However, treatment with the Itk inhibitor Soquelitinib significantly reduced the CD69 positive population in the basal state of almost all CARs while maintaining antigen-induced activation. These results are in agreement with studies demonstrating that point mutations to the Itk binding domain of CD28 costimulated CARs can reduce exhaustion and activation in the absence of antigen(Guedan et al., 2020; Hsieh et al., 2020; Boucher et al., 2021). I observed a similar trend in 4-1BB costimulated CARs which does not have literature precedence, which may be due to PTEN deficiency causing Itk mislocalisation and hyperactivation in the Jurkat cell line(Shan et al., 2000) and impact candidates identified in screens using Jurkats. Together, my results demonstrate that CAR-based Jurkat activation largely resembles what has been observed in primary T cells, however the strength of signalling and activation in CAR Jurkats may be stronger in CAR Jurkats than in primary cells.

Chimeric antigen receptor T cell therapy has revolutionised immunotherapies, providing a modular platform for creating new therapies towards difficult to address cancers. Although successful in particular blood cancers, our understanding of CAR-based T cell activation and the impact on targeted tumours is incomplete. In Chapters 4 and 7, I used pY proteomics to uncover signalling pathways in both CAR T cells and their target cells before evaluating target cell responses and CAR-based T cell activation. Recent reports indicating that tumours may not be “bystanders” during CAR therapy and may instead play an active role in downregulating CAR T cells(Zhong et al., 2023; Callahan et al., 2025) has forced researchers to reevaluate what a ‘good’ target really is. I found that CAR T cells targeted using CSF1, a natural ligand to the RTK CSF1R, induce CSF1R signalling and proliferation in THP1 monocytes. Signalling depended entirely on CSF1R kinase activity, not CAR T cell signalling/activation, and proliferation depended on the expression level of CSF1R and the E:T ratio during co-culture. While my work focuses on CSF1-based CARs, ligand-targeted CARs are a recent innovation

in the CAR field with controversy(Ramírez-Chacón et al., 2022; Branella and Spencer, 2022). Researchers have consistently shown that ligand-based CARs are effective for use targeting EGFR, CSF1R, BAFF, and others(Chinsuwan et al., 2023; Shinagawa et al., 2025; Achkova et al., 2022), however a recent clinical trial evaluating ligand-based CARs for BCMA and TACI described the clinical responses as “disappointing”, noting reduced overall activation and off-target quenching by soluble receptor(Lee et al., 2023). For many targets that have failed clinical trials previously, like EGFR and HER2, the impact of CAR therapy on intracellular activation remains unknown. Thus, future work should evaluate whether other ligand-CARs induce similar signalling responses in targeted tumours, and whether those responses provide a mechanistic/physiological basis for CAR T cell failure. Given the potential for signalling responses in RTK targets, CARs could be administered alongside low doses of well established inhibitors of RTK activity (e.g. Erlotinib for EGFR, Pexidartinib for CSF1R) to prevent CAR-induced activation while providing the desired specificity. In contrast to target-based effects of CAR T cell therapy, much research has been dedicated to the optimisation of CAR activity and discovery of novel, superior CAR constructs(Minguet et al., 2025). High throughput screening tools rely on the use of cell lines, namely Jurkats, due to their ease of genetic manipulation and well-characterised T cell activation patterns(Roex et al., 2025; Rydzek et al., 2019; Gordon et al., 2025, 2022; Bloemberg et al., 2020), however CAR-based Jurkat activation is not as well characterised. I showed that TCR ζ dominates pY signalling in CD19-CAR Jurkats and regulation by known TCR signalling proteins is identical between CD19-CARs with different costimulation, partially contradicting literature on primary CAR T cells. Recently, He *et al.* engineered immortalised primary human T cells through inserting human telomerase reverse transcriptase(He et al., 2025) similar to the hTERT-immortalised Cells available from ATCC, which provides a mechanism for long-term culture of primary-like cells. Importantly, Jurkat cells do not express exhaustion markers like PD-1(Liu et al., 2023), which severely limit their utility for identifying persistent CAR constructs in screens. Transitioning screens towards a less artefactual cell system for CAR screening will reduce artefactual positives during CAR screens and allow researchers to screen both for activation and exhaustion, which is currently not possible. Together, my data suggest that, while identification of new

CARs is important, better model systems for identifying new CARs is required and the notion that tumours are static during CAR T cell therapy should be reconsidered.

8.4 Conclusion

Mass spectrometry based proteomics has played a vital role in the development of the T and CAR T cell fields, namely in identifying the signalling pathways underlying T cell activation. The goal of my dissertation was threefold: to identify methods to improve mass spectrometry-based proteomics for studying T cells, to understand feedback regulation of the T cell receptor, and to understand the construction and effects of chimeric antigen receptors. I have shown that mass spectrometry based phosphotyrosine proteomics can be improved to reduce protein input while maintaining sequencing depth and pitfalls in current sample preparation methods that lead to sample loss. Further, I have shown that proteins involved in feedforward and feedback regulation of T cell signalling have previously unappreciated roles in T cell signalling and global phosphotyrosine maintenance. Lastly, I have shown that the construction of chimeric antigen receptors can unintentionally induce signalling proliferation in target cells and that current screening tools to identify novel CARs lack the nuance of primary cells required for accurate identification of novel CAR candidates. Together, through the marriage of technology and discovery, my work provides the basis for thoroughly characterising T cell signalling and the informed improvement of T cell immunotherapies.

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