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TCR SIGNAL STRENGTH CONTROLS DYNAMIC NFAT ACTIVATION THRESHOLD AND GRADED IRF4 EXPRESSION IN $CD8^+$ T CELLS

A Dissertation Presented By

JAMES M. CONLEY

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

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April 8, 2019

Program of Immunology and Microbiology

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JAMES M. CONLEY

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Leslie J. Berg, Ph. D., Thesis Advisor

Lawrence Stern, Ph. D., Member of Committee

Samuel Behar, M.D. Ph. D., Member of Committee

Michael Brehm, Ph. D., Member of Committee

Stephen Bunnell, Ph. D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Eric Huseby, Ph. D, Chair of Committee

Mary Ellen Lane, Ph. D.,

Dean of the Graduate School of Biomedical Sciences

Program in Immunology and Microbiology

April 8, 2019

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Abstract

TCR signal strength is critical for $CD8^+$ T cell clonal expansion after antigen stimulation. Levels of the transcription factor IRF4 control the magnitude of this process through induction of genes involved in proliferation and glycolytic metabolism. The signaling mechanism connecting graded TCR signaling to the generation of varying amounts of IRF4 is not well understood. Here, using multiple methods to vary TCR signal strength and measure changes in transcriptional activation in single $CD8^+$ T cells, we connect antigen potency to the kinetics of NFAT activation and *Irf4* mRNA expression. T cells that transduce weaker TCR signals exhibit a marked delay in *Irf4* mRNA induction resulting in decreased overall IRF4 expression in individual cells and increased heterogeneity within the clonal population. The activity of the tyrosine kinase ITK acts as a signaling catalyst that accelerates the rate of the cellular response to TCR stimulation, controlling the time to onset of *Irf4* gene transcription. These findings provide insight into the signal transduction pathway accounting for the reduced clonal expansion of low affinity $CD8^+$ T cells following infection. We also describe another context for ITK activity, autoreactive T cell migration. Here, we connect TCR signaling strength to modulation of selectin binding and autoreactive T cell-mediated pathology in an adoptive transfer model system of autoimmune disease. Understanding the signaling mechanisms linking changes in TCR signaling to CD8 T cell function is important in furthering the understanding of vaccine development and T cell adoptive immunotherapy.

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List of Symbols, Abbreviations, or Nomenclature

Ag	Antigen
AP1	Activator Protein 1
APC	Antigen Presenting Cell
APC	Allophycocyanin (Materials and Methods)
APLs	Altered Peptide Ligands
BATF	B-Cell Activating Transcription Factor
Bcl2	B-cell Lymphoma 2
Bcl6	B-cell Lymphoma 6
BCL10	B-cell Lymphoma 10
BCR	B Cell Receptor
Blimp-1	B-lymphocyte Induced Maturation Protein 1
Ca^{2+}	Calcium
CARMA1	CARD-containing membrane-associated guanylate kinase protein-1
CCR5	C-C Chemokine Receptor 5
CCR7	C-C Chemokine Receptor 7
CDK	Cyclin-dependent kinase
CRAC	Calcium-release-activated Calcium Channels
CTLA-4	Cytotoxic T lymphocyte-associated Protein 4
CXCR3	C-X-C Motif Chemokine Receptor 3
CXCR4	C-X-C Motif Chemokine Receptor 4
DAG	Diacylglycerol

EAE	Experimental Autoimmune Encephalomyelitis
Eomes	Eomesodermin
ERK	Extracellular Signal-regulated Kinase
Fc	Fragment Crystallizable
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead Box P3
G4	SIIGFEKL Peptide
hr	Hour
ICAM1	Intercellular Adhesion Molecule 1
IFN	Interferon
IκBα	Nuclear factor of α light polypeptide gene enhancer in B cells inhibitor α
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
i.p.	Intraperitoneal
IP_3	Inositol Triphosphate
IRF4	Interferon Regulatory Factor 4
ITK	(IL-2) Inducible T cell Kinase
i.v.	Intravenous
LAT	Linker of Activated T cells
Lck	lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic Choriomeningitis Virus
LFA1	Lymphocyte function-associated antigen 1

LPS	Lipopolysaccharide
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated Protein Kinase
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
N4	SIINFEKL Peptide
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear factor of κ light polypeptide gene enhancer in B cells
Ova	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed Cell Death Protein 1
PE	Phycoerythrin
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-kinase
PIP_2	Phosphatidylinositol-4,5-Bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-Triphosphate
PKC	Protein Kinase C
PLCγ	Phospholipase C gamma
PHTH	Pleckstrin homology Tec homology
PSGL1	P-selecting binding glycoprotein 1
Ras-GRP	Ras Guanyl-nucleotide releasing protein

RIP	Rat insulin promoter
SEM	Standard Error of the Mean
SH2	Src Homology 2
SH3	Src Homology 3
SLP76	SH2 domain-containing leukocyte protein 76kDa
SOS	Son of Sevenless
T4	SIITFEKL Peptide
T-bet	T-box Transcription Factor TBX21
TCR	T Cell Receptor
TEC	Terminal Effector Cell
TEM	Transendothelial Migration
T_{FH}	Follicular B Helper T Cell
T_H1	Helper T Cell Type 1
T_H2	Helper T Cell Type 2
T_H9	Helper T Cell Type 9
T_H17	Helper T Cell Type 17
TNFα	Tumor Necrosis Factor alpha
T_{REG}	Regulatory T cell
UMMS	University of Massachusetts Medical School
VCAM1	Vascular Intercellular Adhesion Molecule 1
VLA4	Very Late Antigen 4
WT	Wild-Type

ZAP70	Zeta-chain Associated Protein Kinase 70kDa

CHAPTER I: INTRODUCTION

Attributions and Copyright Information

Excerpts in this chapter were taken from a review paper published in *Frontiers in Immunology* in 2016.

T Cells and Gene Regulation: The Switching On and Turning Up of Genes after T Cell Receptor Stimulation in $CD8^+$ T Cells.

James M. Conley, Michael P. Gallagher, and Leslie J. Berg

The adaptive immune system involves highly differentiated cells cooperating for an organism to recognize “self” from “non-self,” thus providing a defense against invading pathogens while maintaining homeostasis within the organism. A central process in adaptive immunity is the generation of memory where cells specific for a particular antigen are able to mount a more effective response upon re-encounter. Here, we focus on one cell type critical in this whole process, the $CD8^+$ T cell. These cells exit the thymus expressing the CD8 co-receptor and are associated with cytotoxic functions, explaining their alterative name, cytotoxic T cells (Butz and Bevan, 1998). However, this name is too general in that $CD4^+$ T cells are also able to perform cytotoxic functions (Marshall and Swain, 2011). We focus on these cells ability to recognize antigen and signal through multiple pathways to change their transcriptome, leading to robust clonal expansion and migration which are critical for an effective T cell-mediated immune response.

T cells bind antigen through their T cell receptor (TCR), activating a signaling cascade that leads to proliferation, differentiation, and pathogen clearance (Weiss, 2005). The affinity of a T cell receptor for its cognate antigen has been directly linked to the regulation of thymic selection and the T cell immune response (Wherry et al., 1999). Upon receptor activation, tyrosine kinases are phosphorylated, creating signaling complexes at the plasma membrane that activate downstream signal transduction pathways. In response to activation, the T cell reorganizes its cytoskeleton, changes its metabolism, and alters its gene expression. This thesis focuses on the signaling pathways that

contribute to changes in gene expression. The three principal pathways activated through the TCR that control transcription are the MAPK, NF-κB, and calcium pathways. These pathways dramatically alter the expression and nuclear localization of various transcription factors that directly regulate genes involved in T cell activation (Brownlie and Zamoyska, 2013).

T cells recognize peptide antigens displayed by the major histocompatibility complex (pMHC) on antigen presenting cells (APC). The interactions between the TCR and pMHC have been extensively characterized in the context of thymic selection and in the immune response to infection (Klein et al., 2014). In response to viral infection, $CD8^+$ T cells recognize antigen from APCs in the lymphoid organs, undergo clonal expansion, migrate to the site of infection, kill infected cells, and then die by apoptosis. Some of the $CD8^+$ T cells will survive this population contraction to form a memory pool (N. Zhang and Bevan, 2011).

Because T cells are polyclonal and contain variable TCRs, they are able to recognize a wide variety of peptide ligands. These ligands can have variable binding affinities for the TCR, creating a range of ligand binding kinetics (Nikolich-Žugich et al., 2004). This variability contributes to the process of graded TCR signaling, where altered peptide ligands can generate different signaling outcomes depending on the binding kinetics of the TCR and pMHC. This introduction chapter highlights recent advances in the field which connect proximal TCR signaling and transcription factor activation to changes in $CD8^+$ T cell function. We then highlight key questions regarding the control of TCR signal

strength on these functions and provide rationale for our studies examining this relationship.

Graded TCR Stimulation and Signal Transduction

The effect of altered peptide ligands (APLs) on the proximal signaling events downstream of the TCR has been extensively examined. In the basal state, the ITAMs of the TCRζ chains are phosphorylated and bound to inactive Zap70. The dwell time of an agonist peptide interaction with the TCR is significantly longer than self-peptide interactions, creating a higher probability that a CD8 co-receptor bound to active Lck can be recruited and bind pMHC. This interaction is highly dependent on peptide sequence, the main factor driving interaction between pMHC and the TCR (Turner et al., 2006). Lck is normally inhibited by an intramolecular interaction between its SH2 domain and a Y505 residue in the C-terminal tail (Sicheri et al., 1997; Xu et al., 1997). This regulatory tyrosine is controlled by CSK phosphorylation and CD45 dephosphorylation (Y. X. Tan et al., 2014). Autophosphorylation of Y394 allows for the open conformation of Lck to interact with the TCR (Hardwick and Sefton, 1995). This interaction increases complex stability and creates an opportunity for Lck to activate Zap70 by first phosphorylating Y319, relieving the autoinhibited conformation. Further activation of other tyrosine residues increases Lck and Zap70 kinase activity and further initiates both kinases to create the downstream LAT signaling complex (W. Zhang et al., 1998) (Chakraborty and Weiss, 2014). These proximal signaling events are absolutely required for downstream TCR signal transduction (Abraham and Weiss, 2004; Au-Yeung et al., 2009). Using an

engineered mutant allele of Zap70, which is sensitive to a small molecule kinase inhibitor, a sharp threshold of signaling was observed downstream of the kinase. Using Nur77-GFP as a readout for TCR signaling, Zap70 was determined to be required for signaling and cell division. This requirement was independent of both the strength of the pMHC-TCR interaction and IL-2 (Au-Yeung et al., 2014).

Not all pathways downstream of the TCR are similarly affected by antigen avidity. The calcium signaling pathway has been shown to be disproportionately affected by weak antigen stimulation (J. L. Chen et al., 2010; Miller et al., 2004; Wülfing et al., 1997). The MAPK and NF-κB pathways, while activated by the TCR, have a threshold level of activation that is less affected by weak stimuli. The MAPK pathway starts with Ras activation and subsequently leads to downstream Erk activation and the formation of the transcription factor dimer AP-1. In T cells, Ras is activated downstream of the TCR by RasGRP or SOS. Initially, RasGRP is activated by binding diacylglycerol (DAG), a byproduct of PtdIns(4,5)P_2 (PIP_2) cleavage, followed by phosphorylation by protein kinase c (PKC) at the plasma membrane. This process is dependent on TCR signal transduction and ultimately, on activation of phospholipase c (PLCγ). PLCγ activation gradually increases the local concentration of RasGRP at the LAT signaling complex. While this initial signaling event is graded in nature, it rapidly triggers a positive feedback loop. SOS acts as the integration point in this feedback loop; not only is it activated like RasGRP and recruited to the signaling complex, it has an allosteric binding site for RasGTP which substantially increases its GTPase activity (Das et al., 2009). This feedback loop ensures that

low affinity TCR ligation can trigger robust Ras activation. Many signaling outputs are often described as “digital” or “analog,” with a non-linear relationship observed between signaling input and output in the context of digital signaling and a linear response observed in the context of analog signaling. Indeed, a digital signal response is observed with p-Erk, with different affinity antigens triggering maximal pathway activation. CD69 was also observed to have this digital response and its expression was highly dependent on the MAPK pathway (Altan-Bonnet and Germain, 2005; Balyan et al., 2017; Das et al., 2009).

A similar signaling effect was observed in the NF-κB pathway. Jurkat cells stimulated with different concentrations of αCD3 antibody were able to activate T cells in a graded manner. However, looking at IκBα degradation, the pathway was digital in nature with cells forming a bimodal population (Kingeter et al., 2010). Using a mutant form of the OT-I TCR (bTMD) having intact antigen binding capacity but dampened signaling, a defect in PKC recruitment to the immunosynapse was observed shortly after stimulation. This defect lead to impaired $CD8^+$ T cell memory formation. However, only a signaling defect in the NF-κB pathway was observed downstream of the TCR suggesting that a small signaling defect in this pathway could impair $CD8^+$ T cell differentiation (Teixeiro et al., 2009). The diverse functions of NF-κB signaling downstream of the TCR have been extensively reviewed (Paul and Schaefer, 2013).

The quality of the calcium signaling response is highly sensitive to TCR antigen affinity. Specifically, the influx oscillation patterns: time of onset, amplitude of influx, and frequency of influx of this cation are highly mutable (J. L.

Chen et al., 2010; Christo et al., 2015; Rosette et al., 2001; Wülfing et al., 1997). Using calcium indicator dyes to measure changes in intracellular ion concentration, changes in both the amplitude and duration of calcium influx have been observed upon stimulation with antigens of different affinities (J. L. Chen et al., 2010; Rosette et al., 2001). Low affinity pMHC does not trigger robust calcium influx in multiple in vitro T cell experiments. This has been linked to the regulation of calcium egress from the endoplasmic reticulum, a process that is mediated by the inositol trisphosphate receptor (IP3R) (J. L. Chen et al., 2010). In $CD4^+$ T cells, the time of onset for calcium flux was directly proportional to the affinity of the antigen. Weaker pMHC stimulation also reduced the magnitude of the influx peak and the duration of the influx (Wülfing et al., 1997). Calcium oscillation patterns have been directly linked to nuclear signaling in T cells with a nonlinear relationship between $[Ca^{2+}]$ and transcription factor activation observed (Dolmetsch et al., 1998). Specifically, high frequency oscillation drove both NFAT and NF-κB activation, while low frequency oscillation drove only NF-κB activation. This, combined with differences in nuclear dwell time (>15m for NF-kB and <10m for NFAT), accounts for variable pathway activation in response to changes in calcium influx (Dolmetsch et al., 1997).

The calcium-sensitive transcription factor NFAT is well known for its contribution to T cell activation, specifically through the production of IL-2 and pro-survival factors. NFAT's binding partners are also critical for T cell activation. Recently, the genetic targets of NFAT were further characterized, comparing constructs that allowed or ablated binding with its most common partner, AP-1. A

subset of genes critical for the promotion of $CD8^+$ T cell exhaustion were highly dependent on NFAT, with transcription occurring in the absence of AP-1 (Martinez et al., 2015a). The amount of time that NFAT spends inside the nucleus is critical for specific transcriptional targets. Using multiphoton intravital microscopy, the half-lives of NFAT nuclear import and export were calculated to be ~1min for import and ~20min for export. The tolerance gene *Egr2* was sensitive to short low affinity antigen stimulation, while the effector gene *Ifng* needed prolonged high affinity stimulation (Marangoni et al., 2013).

ITK and Graded TCR Signal Strength

Downstream of Lck, the LAT signaling complex is critical for subsequent signaling pathway activation. Inducible T cell kinase (ITK) is a Tec family tyrosine kinase, which is activated by Lck and phosphorylates PLCγ. Unlike its sister kinase Bruton's tyrosine kinase (BTK), ITK's expression is largely limited to T cells and its catalytic activity is in the weak micromolar range. In resting T cells, ITK resides in the cytosol. Upon activation, the kinase is recruited to the plasma membrane through its pleckstrin homology (PH) domain, which binds PtdIns(3,4,5)P_3 (PIP_3). ITK also associates with the LAT-SLP76 complex through its SH2 and SH3 domains, creating a signaling complex that is directly dependent on upstream Lck and Zap70 signaling. ITK is directly phosphorylated by Lck and subsequently undergoes cis-autophosphorylation. Then, ITK is able to phosphorylate the lipase PLCγ that cleaves PIP_2 in the plasma membrane, generating the secondary messengers IP_3 and DAG (Andreotti et al., 2018). These secondary messengers primarily activate the downstream MAPK, NF-κB,

and calcium signaling pathways (Berg, 2007; Berg et al., 2005; Schwartzberg et al., 2005).

Unlike the upstream Src kinases, ITK is not required for T cell activation. Indeed, *Itk*-deficient T cells are capable of proliferation and cytokine production in response to antigen stimulation (Liao and Littman, 1995; Liu et al., 1998). Mice lacking ITK are also able to clear pathogens in multiple infection models (Bachmann et al., 1997; Fowell et al., 1999; Schaeffer et al., 1999). However, *Itk*-deficient T cells are not normal, and a diminished response is observed rather than an ablated response. While the MAPK pathway is only modestly impaired in *Itk*-deficient T cells, the magnitude of calcium flux is greatly reduced in response to antigen (Miller et al., 2004). This reduction in signaling correlates with a reduction in $CD8^+$ T cell clonal expansion in response to infection (Atherly et al., 2006a). A key set of experiments directly compared kinase dead mutants of Lck and ITK overexpressed in activated T cells (Donnadieu et al., 2001). While both mutants inhibited calcium flux in the T cells, the mutants had very different effects on the whole population. The Lck mutants ablated calcium signaling in a proportion of the T cells, only cells with lower expression of the mutant were able to flux calcium. Conversely, ITK mutants dampened the calcium response of each cell expressing the mutant in the entire population, both in amplitude and duration. This suggests a digital regulation of calcium signaling through Lck and an analog regulation through ITK. A simplified model of TCR and ITK signaling that drives changes in T cell transcription is shown in Figure 1.1.

Figure 1.1: Representative model of TCR signaling and regulation of transcription in activated $CD8^+$ T cells.

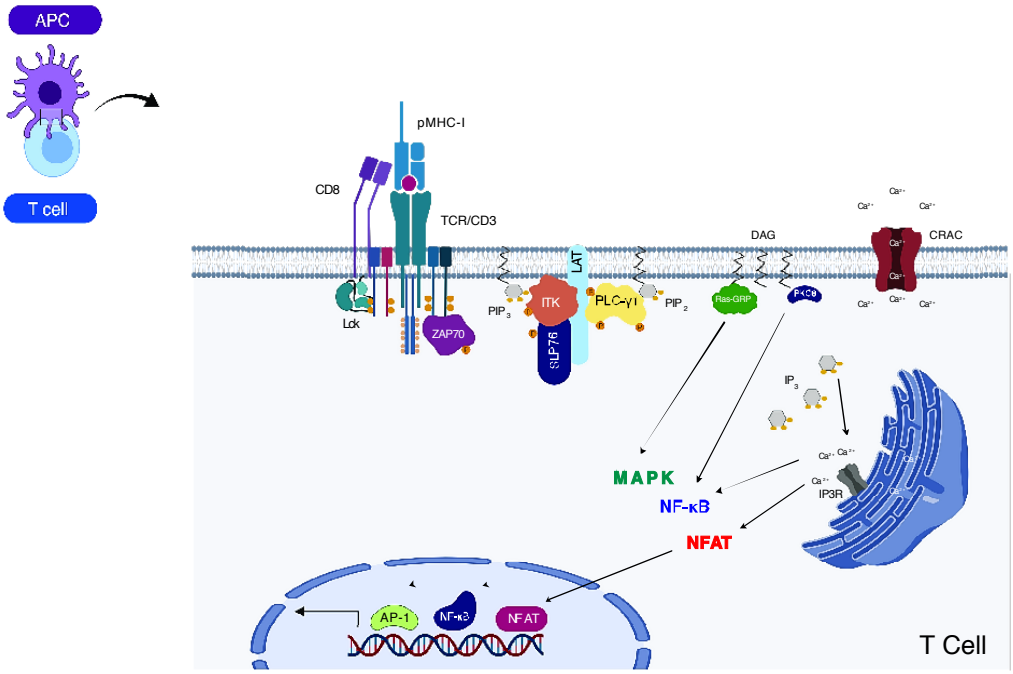


Figure 1.1: Representative model of TCR signaling and regulation of transcription in activated $CD8^+$ T cells.

This is a simplified model of TCR signaling in $CD8^+$ T cells, focusing on the downstream pathways that alter transcription in early activated cells: MAPK, NF-κB, and Calcium. This model also highlights the LAT signaling complex and the Tec kinase ITK, which phosphorylates PLCγ leading to generation of the secondary messengers DAG and IP_3 acting as the anchor points for cytoplasmic signaling cascades.

Graded TCR Signaling and Transcription Factors

In addition to the canonical transcription factors AP-1, NF-κB, and NFAT, which are activated by the TCR, $CD8^+$ T cells rely on a diverse set of transcription factors that drive different functions. Recently, the balance of Blimp-1, T-Bet, Bcl-6, and Eomesodermin have been linked to changes in $CD8^+$ T cell differentiation with high TCR signal strength driving T-Bet and Blimp-1 expression, leading to terminal effector differentiation (Daniels and Teixeiro, 2015; Joshi et al., 2007; Kaech and Cui, 2012). Changes in transcription have also been linked to TCR affinity, specifically the transcription factor Interferon Regulatory Factor 4 (IRF4) (Huber and Lohoff, 2014). While it shares homology with other family members like IRF3, Type 1 IFN does not regulate IRF4 (Honda and Taniguchi, 2006). Rather, its expression is directly regulated by antigen receptors in both T cells and B cells. Undetectable in naïve lymphocytes, IRF4 is rapidly upregulated by antigen stimulation and the magnitude of expression is proportional to the strength of stimulus (Nayar et al., 2012).The NF-κB family transcription factor c-Rel directly regulates *Irf4* at the promoter locus (Grumont and Gerondakis, 2000). In $CD8^+$ T cells, IRF4 expression directly impacts clonal expansion and viral clearance. Specifically, the amount of IRF4 expressed in the $CD8^+$ T cell contributed to the expansion of terminal effector cells (TECs) (Man et al., 2013; Nayar et al., 2014; Yao et al., 2013). These terminally differentiated T cells are critical for pathogen control and require robust proliferation before the population contracts through apoptosis. IRF4 contributes to TEC functionality by regulating multiple genes associated with glycolysis (Man et al., 2013; Man and Kallies, 2015).

$CD8^+$ T cells rapidly shift their metabolism in response to antigen and like cancer cells, use glycolytic pathways to increase glucose import providing energy and macromolecules for biomass expansion and division (Pearce et al., 2013; 2009). IRF4 can also act as a repressor and binds sites in genes associated with apoptosis and cell cycle inhibition, supporting its important role in T cell survival and expansion (Yao et al., 2013). Directly comparing *Irf4*$^{+/fl}$ with *Irf4*$^{fl/fl}$ (using CD4-Cre) and WT mice, viral clearance and TEC expansion was directly proportional to the maximum level of IRF4 expression. The haploinsufficient mice had approximately 2-5 fold less IRF4 production, a reduction that was mirrored in their ability to clear infection (Nayar et al., 2014). Reduction in TCR signaling is also linked to impaired protein translation and ribosome biogenesis in OT-I T cells stimulated with low affinity ligand with and without Lck (T. C. J. Tan et al., 2017).

$CD4^+$ T cells, or "helper T cells" are often characterized by their ability to produce cytokines in response to TCR stimulation. Various subsets have been defined: T_H1, T_H2, T_H9, T_H17, and T_{REG} are the most commonly studied subsets. T_H1 T cells are mainly defined by their ability to secrete IFNγ with T_H2, T_H9, and T_H17 cells producing IL-4, IL-9, and IL-17 respectively. T_{REG}s are a subset of T cells that produce the immunosuppressor IL-10 in response to auto-antigen stimulation (Kaplan et al., 2015; Murphy and Reiner, 2002; Walker and McKenzie, 2018). IRF4 expression levels controlled T_H9 and T_H17 differentiation and IL-9/IL-17 production (Gomez-Rodriguez et al., 2016; 2014). In the absence of ITK, differentiation was inhibited and was rescued by exogenous IRF4

transduction. T_R1 cells, a $IL10^{hi}Foxp3^{lo}$ population of Tregs, are also dependent on ITK activity, driven by Ras pathway-mediated induction of IRF4 (Huang et al., 2017). In humans, *IRF4* haploinsufficiency was linked to a rare inactivating mutation at R98W leading to impaired transcription factor activity. This led carriers to be more susceptible to *Tropheryma whipplei* infection, a bacterium normally controlled in the majority of the population (Guérin et al., 2018).

The frequent binding partner of IRF4, BATF, has also been linked to $CD8^+$ T cell expansion. However, unlike IRF4, the levels of expression are not as critical. The two transcription factors are regulators of T-bet and Blimp1 expression, the two transcription factors that drive effector differentiation (Kurachi et al., 2014). Interestingly, BATF was determined to act as a "persistence detector" of antigen signaling. Maximum BATF-mediated effector differentiation was only triggered by durable antigen signaling. Late in the T cell response, BATF could also repress critical genes like Perforin and Granzyme B, dampening the late effector response (Kurachi et al., 2014). Further research led to identification of a DNA binding motif that was occupied by BATF-IRF4 only in T_H2 cells stimulated with high dose antigen (Iwata et al., 2017).

Another factor associated with TCR affinity is Nur77, a transcription factor primarily associated with apoptosis (Woronicz et al., 1994). Nur77 is often used as a reporter for TCR activation with its expression proportional to antigen avidity. The amount of Nur77 expression correlates with thymic selection, with negatively selected thymocytes having higher levels than their positively selected counterparts (Baldwin and Hogquist, 2007; Moran et al., 2011). Nur77 is rapidly

upregulated after antigen stimulation and its expression is dependent solely on TCR signaling. Using a Nur77-GFP reporter mouse, the levels of GFP were proportional to the affinity of TCR ligand in the OT-I model system, showing a graded expression similar to IRF4 (Moran et al., 2011). However, a binary response was observed in activated $CD8^+$ T cells stimulated with different doses of antigen. The threshold of Nur77-GFP reporter activation was then connected to IL-2, with exogenous IL-2 leading to a lower threshold of reporter expression (Au-Yeung et al., 2017).

The signaling data suggests that certain factors like CD69 and IL-2 are digital in nature, while others like IRF4 are analog in nature. The strength of TCR stimulus, whether through increased dose or affinity of antigen, is not proportional to the output of the MAPK and NF-κB signaling pathways. However, the activation marker IRF4 is proportional to the strength of stimulus.

Graded TCR Signaling and $CD8^+$ Effector Function

$CD8^+$ T cell development and function have been studied using the OT-I transgenic mouse model. The OT-I TCR recognizes the SIINFEKL peptide (N4), an antigen derived from chicken ovalbumin (Hogquist et al., 1994). These OT-I T cells are MHC class I restricted, creating a large population of naïve $CD8^+$ T cells that recognize the peptide antigen. The OT-I model provides an excellent system to study TCR signal strength because of the exquisitely high affinty N4 peptide ligand. Altered peptide ligands (APLs) with single amino acid substitutions greatly reduce TCR binding affinity while maintaining equivalent MHC-I binding capacity. Scientists have also engineered a variety of pathogens to express these APLs,

providing excellent infection models to study TCR signal strength in vivo. In the context of $CD8^+$ T cell development in the thymus, graded TCR signal strength drives the balance between positive and negative selection, a process that can directly impact autoimmune disease (Daniels et al., 2006; Enouz et al., 2012; Koehli et al., 2014). By designing a DNA-based chimeric antigen receptor, researchers were recently able to show that a single hydrogen bond, added through nucleotide substitution, could increase both antigen binding and downstream receptor signaling, emphasizing the importance of the affinity of the antigen for the TCR on signaling dynamics (Taylor et al., 2017).

A series of studies have looked at $CD8^+$ T cell activation in vivo in response to infection and have teased apart contributions between antigen interactions and environmental inflammatory signals. While T cell expansion was directly proportional to antigen avidity, cytolytic function was more dependent on inflammatory signals (Zehn et al., 2009; 2013). Using peptide-pulsed dendritic cells with or without *Listeria monocytogenes* infection, Granzyme B and IFN-γ production were only seen in the context of infection. However, IL-2 and the chemokine receptor CCR7 were regulated in the absence of inflammation and their expression was directly proportional to the avidity of the antigen. The high-affinity IL-2 receptor (CD25) was driven by both signals with its expression proportional to both dose and affinity of the stimulus (King et al., 2012; Zehn et al., 2009). These *Listeria* experiments show that certain aspects of T cell function are driven by the affinity of the pMHC-TCR interaction, while others are driven by cytokine activation. Low affinity antigen stimulation downregulated CCR7 earlier

than high affinity stimulation and the T cells were able to migrate out of the periarteriolar lymphocyte sheaths (PALS) at an earlier time, creating a larger proportion of T cells in the blood and the red pulp of the spleen. The high affinity stimulated T cells retained CCR7 expression out to 4d and did not migrate out of the PALS. Further confirmation of this phenotype followed with recent studies using intravital imaging of lymph nodes in OT-I mice stimulated with transferred peptide-pulsed dendritic cells. TCR affinity did not control early APC:T cell contact, however, sustained contact past 24h was only observed with high affinity N4 antigen. These high affinity clones then migrated to interfollicular regions, a process mediated by CXCR3 (Ozga et al., 2016).

Effects on T cell migration and tissue infiltration were also observed in the context of autoimmunity. In a model where membrane-bound ovalbumin (mOVA) is under the control of the rat insulin promoter (RIP), OT-I T cells were transferred into mice after immunization with high and low affinity peptide antigen. This model is useful in that antigen is expressed specifically in the pancreas and OT-I T cells can recognize the antigen even in the absence of infection. Only T cells stimulated with high affinity peptide were able to infiltrate the pancreas and migrate to the site of antigen, a process that was dependent on the integrin VLA-4 (King et al., 2012).

The altered expression in both magnitude and kinetics of CD25, CD69, and CCR7 show that antigen affinity directly affects IL-2 signaling and lymph node egress. This suggests that T cells stimulated with high-affinity antigen are able to sequester more IL-2 from the environment and remain in the lymph node,

thereby allowing them a longer time to receive stimulation. This delay in egress contributes to a more robust clonal expansion, a process that is directly proportional to antigen avidity. Signaling through the IL-2 receptor is required for $CD8^+$ T cell memory formation (Malek, 2008). Mice lacking IL-2 receptors in mature $CD8^+$ T cells, mixed chimeras that retain a normal population of T regulatory cells, were able to mount a normal primary response to LCMV infection. However, the secondary response was impaired. Thus IL-2 signaling is required during the primary response to produce a functional memory pool (Williams et al., 2006). IL-2 can also promote proliferation of T cells stimulated by low-affinity antigen. Using an in vitro co-culture system of P14 and OT-I CD8 T cells, P14 T cells stimulated with high-affinity GP33 peptide could produce IL-2 and trigger increased proliferation in neighboring OT-I T cells that were stimulated with the low-affinity G4 peptide. The IL-2 is required within 20-30h of antigen stimulation and PI3K signaling through the IL-2 receptor cooperates with TCR signaling to promote cell cycle entry (Voisinne et al., 2015). IL-2 is not the only secondary signal that cooperates with TCR activation, a wide variety of co-stimulatory pathways interplay with graded TCR signaling to promote diverse $CD8^+$ T cell functions (L. Chen and Flies, 2013). The regulation of T cell memory has been extensively linked to a combination of graded TCR signaling and co-stimulation using multiple transgenic and pathogen models (Daniels and Teixeiro, 2015).

Affinity of the antigen has also been linked to regulation of asymmetric cell division with proximal daughter T cells becoming TECs (Chang et al., 2007; King

et al., 2012). High affinity interactions led to a distinct progeny after the first division with proximal daughters containing the immunosynapse and increased glycolytic capacity. In contrast, low affinity TCR interactions did not lead to this phenotype. Single-cell genomic studies revealed that changes in TCR affinity did not alter the T cells cytotoxic function, rather changes in transcriptional upregulation were delayed (Richard et al., 2018). Graded TCR signaling controls many aspects of $CD8^+$ T cell differentiation. The signaling mechanisms that connect graded TCR stimulation with these changes in differentiation while not completely understood have been extensively examined in the recent years (Kaech and Cui, 2012; Zikherman and Au-Yeung, 2015).

CD28 Costimulation and Autoreactive T Cell Migration

Since early work discovering that TCR ligation by itself induced T cell anergy, constimulatory signals presented by APCs were known to be critical for T cell activation (Jenkins et al., 1988; Mueller et al., 1989). Discovery of the CD28 receptor and its cognate ligands of B7-1 (CD80) and B7-2 (CD86) on APCs soon followed (Jenkins et al., 1991). Dosing mice with CD28 antagonists induced tolerance in organ engraftment experiments, suggesting a role in controlling autoreactive T cells (Lenschow et al., 1992). This then lead to the characterization of CTLA-4, a competitive receptor for B7, which inhibited T cell activation. CTLA-4 binds more strongly to B7 ligands, contributing to its dominant role as a T cell suppressor, now considered an immune checkpoint similar to PD-1 (Leach et al., 1996).

CD28 resides on the surface of the plasma membrane as a 44 kDa homodimer, with important cytoplasmic tail domains that are required for downstream signaling (Carreno and Collins, 2002). These motifs include 3 tyrosine residues that are phosphorylated after TCR stimulation and bind multiple signaling proteins critical for signal transduction, GRB2, VAV3, PI3K, SLP-76, and CSK to name a few. Proline-rich sequences can also bind SH3 domains of other signaling proteins. These signaling domains are critical for different T cell functions, one of which is IL-2 production, a process dependent on CD28 (Fraser et al., 1991; June et al., 1987; Thompson et al., 1989). Importantly, Src kinase Lck can also interact with CD28 through its SH3 domain, creating a codependent signaling mechanism uniting TCR and CD28 pathways (Holdorf et al., 1999). Lck can phosphorylate the tyrosines in the CD28 tail. The main function of CD28 signaling in CD8 T cells seems to be the regulation of the cells glycolytic rate through Akt and mTORC1 activation, a process that is also highly regulated by IRF4 (Frauwirth et al., 2002; Man et al., 2013).

The complexity of CD28 biology, and co-stimulation in general, is beyond the scope of this thesis. We were interested in exploring one key function of T cells, transendothelial migration, a process that required both CD28 and ITK signaling in an autoimmune pathology model (Jain et al., 2013). Briefly, transendothelial migration is a multi-step process by which T cells (and generally other cell types) bind to integrins on the surface of the endothelium. The “stop” binding step is then followed by a “rolling” step which halts the T cell in place before it can migrate through the tight junctions between the endothelial cells and pass

through to other tissues. This complex process is largely driven by the selectins (P- and E-selectin) and integrin ligands ICAM-1 and VCAM-1 (Vestweber, 2015).

Summary of Key Research Questions

The connection between ITK activity and IRF4 was first identified in a microarray experiment comparing CD8SP thymocytes from WT and *Itk*$^{-/-}$ mice. IRF4 expression was reduced in the KO cells (Nayar et al., 2012). Nayar et. al. then went on to further explore the relationship between the kinase and the transcription factor in T cells. Comparing *Itk*$^{-/-}$ and *Irf4*$^{fl/fl}$ (under control of CD4-Cre) mice, their peripheral T cells showed an innate/memory phenotype with CD44^{hi}CD62L^{hi} expression. They both also had very high levels of Eomes, a transcription factor associated with T cell memory. Stimulation of T cells with high and low doses of CD3 and CD28 antibodies along with treatment with a small molecule inhibitor or ITK, 10n, resulted in a graded inhibition of IRF4 expression that was dose-responsive. These experiments gave us the first evidence that ITK activity could fine-tune IRF4 expression, however the mechanism was unknown (Nayar et al., 2012).

Shortly thereafter, a series of papers went on to describe the importance of IRF4 function in CD8^{+} T cells, specifically in the context of clonal expansion in response to infection. *Irf4*$^{-/-}$ CD8^{+} T cells were unable to clear *L. monocytogenes* infection due to their impaired proliferation, cytotoxicity, and cytokine production. The T cells also expressed lower levels of T-bet, Blimp-1, and Id2, transcription factors critical for TEC function (Raczkowski et al., 2013). A similar defect in

clonal expansion was observed in the context of LCMV and Influenza A infection (Man et al., 2013; Nayar et al., 2014) (Yao et al., 2013).

Nayar et al went a step further and compared *Irf4*$^{fl/fl}$ and *Irf4*$^{+/fl}$ T cells. Haplosufficient T cells had about 2-fold less IRF4 expression and also had defective clonal expansion post infection. Importantly, these haplosufficient mice are able to control the LCMV infection and viral load is undetectable at d8, unlike knockout mice who still have active infection. This allows for a better comparison of *Irf4*$^{+/+}$ and *Irf4*$^{+/fl}$ CD8^{+} T cells during their expansion and contraction phases, eliminating any confounding inflammatory factors present in mice with viral burden. Adoptive transfer experiments with P14 T cells, the TCR specific for the GP33 epitope of LCMV, confirmed an intrinsic CD8^{+} T cell defect in clonal expansion caused by a 2-fold reduction in IRF4 expression (Nayar et al., 2014). Man et. al. went on to link IRF4 to aerobic glycolysis with RNA-seq experiments comparing *Irf4*$^{+/+}$ and *Irf4*$^{-/-}$ T cells. Yao et. al. connected IRF4 function to cell cycle progression and survival with defects in CDK inhibitors and Bim expression (Man et al., 2013; Yao et al., 2013). These papers provided a rationale for looking at IRF4 regulation in CD8^{+} T cells, because of the defect in clonal expansion seen with a modest decrease in expression. There were also striking similarities between the anti-viral responses of *Itk*$^{-/-}$ and *Irf4*$^{+/fl}$ mice, with both CD8^{+} T cell populations able to clear LCMV infection with delayed kinetics of clonal expansion (Atherly et al., 2006a; Nayar et al., 2014).

While the OT-I TCR transgenic mouse has been used to study CD8^{+} T cell biology for quite some time, a watershed paper by Zehn et.al. pushed the model

much further and characterized the response of OT-I T cells to altered peptide ligands (APLs) in vivo (Zehn et al., 2009). This provided the foundation for our experiments studying graded TCR signaling. We wanted to take a reductionist approach where we could use a clonal population of $CD8^+$ T cells and look at their response to varying strengths of TCR stimulus by using the described APLs or blocking ITK activity using knockout mice or a small molecule inhibitor. This system also allowed us to compare the effect of antigen dose and affinity on different early response genes, teasing apart contributions from both inputs. This model system shares all the caveats of T cell stimulation in cell culture, however, in these experiments primary APCs are used rather than supraphysiological stimulation of the TCR by antibody crosslinking, a method that has been mostly used in signaling experiments because of the difficulty in looking at signaling dynamics using single-cell methods like flow cytometry. We wanted to use new technological methods that would allow single-cell detection while also keeping intact the APC:T cell relationship. Using this model system to carefully test TCR signal transduction, we set out with one main objective: **Determine the mechanism by which TCR signaling drives graded IRF4 expression in $CD8^+$ T cells**.

It was known that *Itk*$^{-/-}$ T cells had a defect in calcium signaling. T cells stimulated with CD3 antibody were inhibited in their ability to flux calcium (Miller et al., 2004) and elegant experiments using T cells expressing a kinase dead mutant of ITK also revealed a calcium flux defect, specifically in the amplitude of signal observed with the indicator dye (Donnadieu et al., 2001). We reasoned

that this calcium signaling deficiency could be contributing to the lower IRF4 levels in ITK inhibited T cells. (Figure 1.2)

Itk$^{-/-}$ T cells are able to turn on p-Erk in response to TCR activation, suggesting that the MAPK pathway is unlikely to play a role. Excellent work by Das et.al. described a digital signaling network controlling the MAPK pathway in T cells. Using modeling and experimental data, they showed that CD69 has a binary response to Ras signaling and also that the Ras pathway has a positive feed-forward loop leading to digital p-Erk activation (Das et al., 2009). We posited that CD69 would be regulated differently by TCR signaling, when compared to IRF4, and is likely not regulated by ITK activity in the same way. (Figure 1.2)

Figure 1.2: Our hypothesis that TCR signal strength and ITK activity control graded IRF4 expression through the calcium-sensitive transcription factor NFAT.

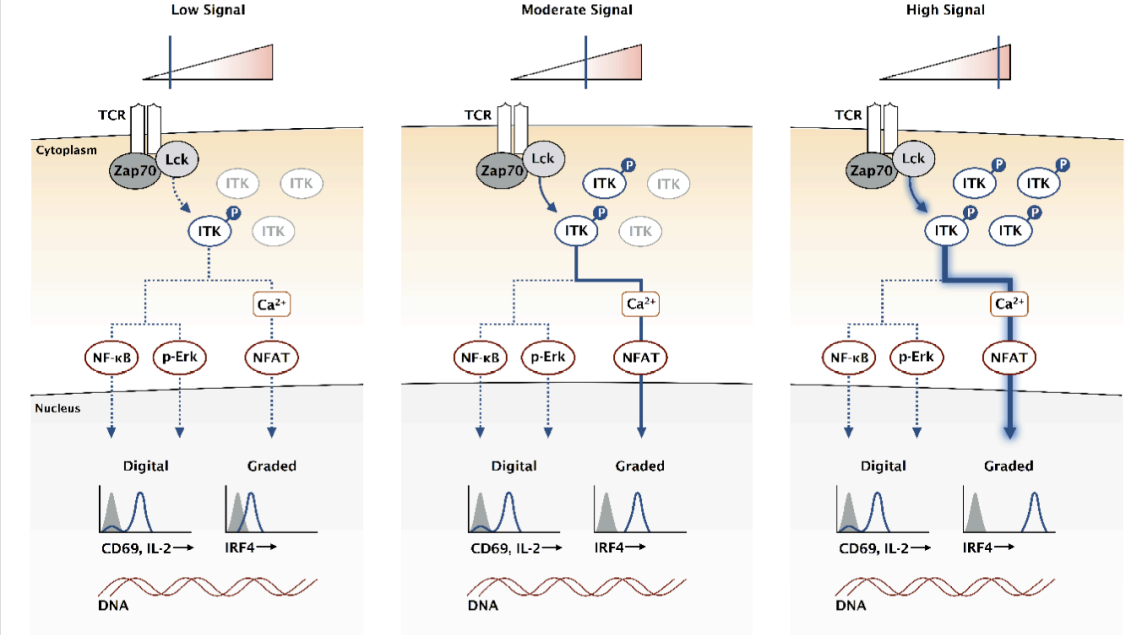


Figure 1.2: Our hypothesis that TCR signal strength and ITK activity control graded IRF4 expression through the calcium-sensitive transcription factor NFAT.

While the expression of genes like CD69 and IL-2 is not directly proportional to the signaling input of the TCR, other genes like IRF4 have a graded response where expression continues to increase with higher affinity TCR stimulation. High-affinity antigens increase the during of TCR binding, increasing the stability of Lck and ZAP70, which amplifies downstream signaling by creating a larger pool of activated LAT signaling complexes. This creates a larger pool of activated ITK, represented here for simplification without the other signaling components in the pathway. We propose that the calcium signaling pathway is uniquely sensitive to ITK and can provide a mechanism for graded IRF4 expression.

In another context of T cell function, Jain et. al. described a striking phenotype when *Ctla4*$^{-/-}$ mice were crossed to *Itk*$^{-/-}$. The disease pathology caused by infiltration of autoreactive T cells into multiple tissues, eventually leading to death, was completely prevented in the double knockout mice. The T cells retained their autoreactive proliferative capacity, however, now coupled with an inability to migrate out of the secondary lymphoid organs. Thus, disease prevention was due to the lack of pathological infiltration into lung, liver, and pancreatic tissue. Using 2-photon microscopy, labeled *Itk*$^{-/-}$ T cells were transferred into WT mice and then imaged in the lung. The cells lacking ITK were observed in the endothelium, rounded in shape and unable to crawl along the walls of the blood vessles, an early step in TEM (Jain et al., 2013). King et. al. connected TCR signal strength with autoreactive tissue pathology in the RIP-mOVA / OT-I diabetes (T1D) induction model, where Ova antigen is expressed in the β-islet cells of the pancreas. They described a sharp threshold in TCR affinity that lead to T1D induction with immunization of transferred OT-I T cells with high affinity antigen leading to pancreatic infiltration and low affinity antigen unable to cause disease (King et al., 2012). These experiments lead us to speculate that ITK-dependent regulation of IRF4 could be involved in the autoreactive T cell migratory defect.

Thesis Objectives

Based on these studies, **we hypothesized that changes in IRF4 expression, driven by the strength of TCR stimulus, are controlled by changes in the calcium signaling pathway. We also hypothesized that TCR and CD28 signaling synergized to control IRF4 expression in the context of autoreactive T cell migration.** These hypotheses are described in the following two chapters:

Chapter III: TCR signal strength controls dynamic NFAT activation threshold and graded IRF4 expression in $CD8^+$ T cells.

This chapter involves experiments testing our hypothesis that IRF4 regulation is mediated by calcium signaling in activated $CD8^+$ T cells. We use multiple single-cell techniques to look at changes in the calcium signaling pathway and its connection to transcription through NFAT activation. We then connect NFAT activity to IRF4 upregulation.

Chapter IV: TCR signal strength controls selectin binding and autoreactive $CD8^+$ T cell migration.

This chapter involves experiments testing our hypothesis that selectin binding, an early step in T cell transendothelial migration, could be sensitive to changes in TCR signal strength. We then test contributions from CD28 costimulatory receptor signaling followed by in vivo functionality in an adoptive transfer model of autoreactive T cell migration.

CHAPTER II: MATERIALS AND METHODS

Mice:

Mice were bred and housed in specific pathogen-free conditions at the University of Massachusetts Medical School (UMMS) in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Chapter III:

OTI *Rag*$^{-/-}$ (B6.129S7-Rag1^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1) were purchased from Taconic (Hudson, NY). CD45.1 (B6.SJL-Ptprc^{a} Pep3^{b}/BoyJ) were purchased from The Jackson Laboratory (Bar Harbor, ME). OTI *Rag*$^{-/-}$ *Itk*$^{-/-}$ CD45.1 mice were generated by crossing *Itk*$^{-/-}$, OTI *Rag*$^{-/-}$ and CD45.1 mice. *Itk*$^{-/-}$ mice have been described previously (Liu et al., 1998). Nur77-GFP reporter mice (a gift from Dr. S. Swain, UMMS) were crossed to OTI *Rag*$^{-/-}$ mice. Nur77-GFP reporter mice have been described previously (Moran et al., 2011). For experiments, mice at 8-12 weeks of age were used.

Chapter IV:

OTI *Rag*$^{-/-}$ (B6.129S7-Rag1^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1) were purchased from Taconic (Hudson, NY). CD45.1 (B6.SJL-Ptprc^{a} Pep3^{b}/BoyJ) and RIP-mOVA (Tg(Ins2-TFRC/OVA)296Wehi) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OTI *Rag*$^{-/-}$ *Itk*$^{-/-}$ CD45.1 mice were generated by crossing *Itk*$^{-/-}$, OTI *Rag*$^{-/-}$ and CD45.1 mice. *Itk*$^{-/-}$ mice have been described previously (Liu et al., 1998). B7$^{-/-}$ (*Cd80*$^{-/-}$ *Cd86*$^{-/-}$) mice were a gift from J. Kang and have been described previously (Borriello et al., 1997). For experiments, mice at 8-12 weeks of age were used.

Cell culture:

Chapter III:

Splenocytes from OT-I Rag$^{-/-}$ mice (and the genetic variants) were stimulated ex-vivo with recombinant peptide (N4, T4, or G4) at the indicated doses for 24h. Antigen-presenting-cells (APCs) in the bulk splenocyte population were sufficient for stimulation. (Figures 1-2) Recombinant peptides were purchased from 21st Century Biochemicals (Marborough, MA). PRN694 was generously provided by Principia Pharmaceuticals (Zhong et al., 2015). For stimulation readouts earlier than 24h, OT-I T cells were stimulated at a 5:1 ratio of APCs to T cells. The APCs used were bulk splenocytes isolated from WT C57BL/6 mice, then pulsed with peptide prior to the addition of T cells. (Fig. 4) APCs used in Fig. 5 were stimulated with LPS (1ug/mL) and pulsed with peptide for 1h prior to addition of T cells. LPS, FK506, PMA, and Ionomycin were purchased from Sigma-Aldrich (St. Louis, MO).

Chapter IV:

Splenocytes from OT-I Rag$^{-/-}$ mice (and the genetic variants) were stimulated ex-vivo with APCs pulsed with recombinant peptide (N4, T4, or G4) at the indicated doses for 24-48h. APCs were bulk splenocytes from WT (C57BL/6) or B7$^{-/-}$ mice stimulated with LPS (1ug/mL) and pulsed with peptide for 1h prior to addition of T cells. Recombinant peptides were purchased from 21st Century Biochemicals (Marborough, MA). LPS was purchased from Sigma-Aldrich (St. Louis, MO).

PRN694 was generously provided by Principia Biopharma Inc (Zhong et al., 2015).

Antibodies and flow cytometry:

Chapter III:

IRF4 (eF660 and PE), CD8a (PE-eF610), Va2 (APC and APC-eF780), TCRb (APC-eF780), Eomes (PE and PE-Cy7), T-bet (PerCP-Cy5.5), CD45.1 (eF450 and APC-eF780), CD69 (FITC and PE-Cy7) were purchased from eBioscience / Thermo Fisher (San Diego, CA). CD44 (v500) and CD45.1 (BV510) were purchased from BD Biosciences (Billerica, MA). LIVE/DEAD Violet, LIVE/DEAD Aqua, goat anti-rabbit (PE) were purchased from Life Technologies (Grand Island, NY). Single cell suspensions were prepared from isolated spleens; RBCs were lysed; Fc receptors were blocked using supernatant from 2.4G2 hybridomas. Intracellular transcription factor staining was performed using eBioscience FoxP3 Transcription Factor Staining Buffer Set. Samples were analyzed on the LSR II flow cytometer (BD Bioscience) and data was analyzed on Flow Jo (Tree Star).

Chapter IV:

IRF4 (eF660 and PE), CD8a (PE-eF610), Va2 (APC and APC-eF780), TCRb (APC-eF780), CD226 (APC), CD25 (PE-Cy7), CD45.1 (eF450 and APC-eF780), CD69 (FITC and PE-Cy7) were purchased from eBioscience / Thermo Fisher (San Diego, CA). CD44 (v500), CD45.1 (BV510), and purified recombinant

mouse P-selectin – human IgG fusion protein were purchased from BD Biosciences (Billerica, MA). LIVE/DEAD Violet and goat anti-human IgG1 Fc (APC) were purchased from Life Technologies (Grand Island, NY). Single cell suspensions were prepared from isolated spleens; RBCs were lysed; Fc receptors were blocked using supernatant from 2.4G2 hybridomas. Intracellular transcription factor staining was performed using eBioscience FoxP3 Transcription Factor Staining Buffer Set. Samples were analyzed on the LSR II flow cytometer (BD Bioscience) and data was analyzed on Flow Jo (Tree Star).

Nuclear flow and RNA flow:

Chapter III:

Nuclei isolation and flow cytometry staining of OT-I cells stimulated in co-cultures were performed as previously described (Gallagher et al., 2018). Briefly, $CD8^+$ T cells were negatively enriched from OT-I spleens (Stemcell Technologies) and labeled with CellTrace Violet cell tracking and proliferation dye (Thermo Fisher) for 20m prior to co-culture with peptide-pulsed B6 splenocytes. For nuclei isolation, cells were treated and washed with sucrose and detergent buffers. The nuclei were fixed in 4% paraformaldehyde and then intranuclear staining was performed with a 0.3% Triton-X 100 detergent PBS buffer. NFAT1-AF488 antibody (clone D43B1) was purchased from Cell Signaling Technology (Danvers, MA). The PrimeFlow assay kit, from Thermo Fisher, was used for RNA flow. *Cd69* and *Irf4* probe sets were ordered for AF647.

ChIP analysis:

Chapter III:

NFAT1 ChIP-Seq data (GSE64409) from Martinez *et. al*. (Martinez et al., 2015a) was analyzed using Integrative Genomics Viewer (Broad Institute, Cambridge, MA) (Robinson et al., 2011). A snapshot was taken of the *Irf4* gene locus.

RIP-mOVA Adoptive Transfer Experiments:

Chapter IV:

For diabetes induction, 5 x 10^6 OT-I (*Itk*$^{+/+}$ or *Itk*$^{-/-}$) CD8 T cells were adoptively transferred intravenously into RIP-mOVA mice 1 day prior to immunization with a PBS solution containing 50µg of T4 peptide and 25µg of LPS by intraperitoneal injection, described previously (King et al., 2012). Urine glucose was monitored daily with test strips purchased from Roche Diagnostics. (Basel, Switzerland) Mice were sacrificed when urine glucose levels exceded 1000 mg/dL. For the 3-day proliferation experiment, the OT-I T cells were labeled with CFSE (Life Technologies) and transferred and immunized in the same way. After 3d, the spleens were harvested for flow cytometry.

Statistical analysis:

Graphs represent mean ± SEM. Statistical significance indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (NS, $p > 0.05$) based on one-way ANOVA followed by Dunnett's test or unpaired Student *t* test. Data analysis performed using GraphPad Prism 7.0 (GraphPad, San Diego, CA).

CHAPTER III: TCR SIGNAL STRENGTH CONTROLS DYNAMIC NFAT ACTIVATION THRESHOLD AND GRADED IRF4 EXPRESSION IN $CD8^+$ T CELLS

Attributions and Copyright Information

The data described in this chapter was submitted for publication in *Science Signaling* in March of 2019.

TCR signal strength controls dynamic NFAT activation threshold and graded IRF4 expression in $CD8^+$ T cells

James M. Conley, Michael P. Gallagher, and Leslie J. Berg

Specific Contribution to Figures: James Conley performed, designed, and analyzed all experiments described in figure 3.1 through 3.7, with the exception of those described in Figure 3.6A-C which were done by Michael Gallagher.

Introduction

A central process of the adaptive immune response to infection is the activation and expansion of cytotoxic T cells. Characterized by their expression of the co-receptor CD8, these lymphocytes are critical for identification and removal of intracellular pathogens. Activation of naïve $CD8^+$ T cells is primarily driven by binding of MHC:peptide antigen to their T cell receptor (TCR). Upon receptor binding, a signaling cascade is triggered which directs the cell to respond by reorganizing its cytoskeleton, upregulating glycolytic pathways, and preparing for cell division and migration. The cell rapidly changes its transcriptional program and thousands of new genes are expressed to meet this new functional demand (N. Zhang and Bevan, 2011). While numerous studies have characterized the activated T cell transcriptome, detailed insight into pathways that link proximal TCR signaling dynamics to graded gene expression has remained elusive.

TCRs can bind to MHC:peptide complexes with wide variations of ligand binding kinetics. Antigen abundance can also vary greatly, as a result of differences in pathogen burden and differing efficiencies of antigen presentation. Graded or variable responses to changes in TCR signal strength are known to generate diverse functional outcomes in responding T cells, a phenomenon best illustrated by the selection processes that shape the repertoire of developing T cells in the thymus (Klein et al., 2014). The responses of peripheral T cells to infection or immunization are also modulated by variations in TCR signal strength. When antigen doses are held constant, CD8 T cells with higher affinity

TCRs for MHC:peptide show a greater magnitude of expansion, and exhibit enhanced production of IFNγ relative to low affinity clones (Zehn et al., 2009). However, it remains unclear whether higher antigen doses are able to compensate for lower TCR affinity during peripheral T cell activation, or whether intrinsic differences in TCR interactions with MHC:peptide establish limits on the downstream outcomes of T cell activation.

The transcription factor Interferon Regulatory Factor 4 (IRF4) has been extensively characterized in lymphocytes(Huber and Lohoff, 2014). For $CD8^+$ T cells, the levels of IRF4 expression following T cell activation determine the magnitude of terminal effector cell (TEC) expansion in vivo (Man et al., 2013; Nayar et al., 2014; Yao et al., 2013). IRF4 expression is nearly undetectable in naïve and memory T cells, but is rapidly induced following TCR stimulation. Along with its frequent binding partner BATF, IRF4 upregulates a host of genes in newly-activated $CD8^+$ T cells, specifically genes critical for the metabolic switch to glycolysis (Kurachi et al., 2014; Man et al., 2013). In T_H2 cells, strength of TCR stimulation correlated with BATF-IRF4 binding to different enhancer regions of the genome, leading to variations in gene expression patterns (Iwata et al., 2017). While graded expression of IRF4, which changes the magnitude of the effector response, is dependent on TCR signal strength, the signaling mechanism regulating the control of IRF4 expression levels in $CD8^+$ T cells is not completely understood.

Our interest in the signaling pathway regulating graded IRF4 expression also derived from our previous findings that this process is controlled by the relative activity of the Tec kinase ITK (Nayar et al., 2012). A key molecule in TCR signal transduction, ITK is activated by the Src kinase Lck and recruited to the LAT signaling complex along with its substrate, phospholipase C (PLCγ). Once activated, ITK phosphorylates and activates PLCγ. Activated PLCγ cleaves the membrane phospholipid PIP_2 into the two secondary messengers diacylglycerol (DAG) and inositol triphosphate (IP3), which both help propagate downstream signaling pathways to regulate gene expression (Andreotti et al., 2018). To understand the signaling mechanisms that contribute to the graded levels of IRF4, we used the OT-I TCR transgenic mouse line along with altered peptide ligands (APLs) that change the signaling activity of the TCR without compromising MHC:peptide binding. In addition to using APLs, we used multiple methods to inhibit or inactivate the signaling molecule ITK.

Using this reductionist approach, we varied both the MHC:peptide dose and the affinity of the TCR interaction with individual MHC:peptide complexes. Using this system to fine tune TCR signaling, we then employed several single-cell assays to measure the consequences of varying TCR signal activity, thereby providing insight into the pathways contributing to graded expression of IRF4. We found that $CD8^+$ T cells are able to integrate the digital activation of NFAT into graded IRF4 protein expression by varying the kinetics of activation. Furthermore, we showed that ITK activity contributes to TCR signaling by accelerating the rate of this response. Thus, under conditions of weak TCR

signaling and/or in the absence of ITK activation, delayed kinetics of NFAT activation and *Irf4* mRNA expression create a population of $CD8^+$ T cells that, as shown previously (Atherly et al., 2006a; Nayar et al., 2014; 2015), has reduced proliferative capacity.

Results

Weaker TCR signaling reduces maximum effector-associated gene expression in CD8+ T cells

In the first 24h after TCR stimulation, $CD8^+$ T cells upregulate a variety of proteins that play key roles in T cell proliferation and cell trafficking. Two factors, CD69 and IRF4, are rapidly increased. Here, we used the TCR transgenic model OT-I to study $CD8^+$ T cell activation in response to different peptide ligands that stimulate this TCR with differing potencies. The cognate peptide for the OT-I TCR is derived from chicken Ovalbumin, with the amino acid sequence of SIINFEKL (N4) (Hogquist et al., 1994). By substituting amino acids at the fourth position, TCR affinity for the MHC:peptide complex can be decreased without affecting peptide binding to MHC Class I (Zehn et al., 2009). For our studies, we used the SIITFEKL peptide (T4), which is approximately 100-fold less potent, and the SIIGFEKL peptide (G4), which is approximately 1000-fold less potent, than N4 (Rosette et al., 2001; Zehn et al., 2009).

Initially, we performed dose response experiments in which each of the three peptides was added to unfractionated splenocytes from OT-I TCR transgenic x *Rag2*$^{-/-}$ (hereafter referred to as OT-I) mice, and cells were assessed 24h later by flow cytometry. For CD69 expression, as expected, a bimodal response was observed. At low peptide doses of T4 peptide, for instance, no CD69 upregulation was observed. As the peptide dose increased, a threshold was reached where a subset of cells transitioned from low CD69 expression to high expression (Figure 3.1A, left panel). At these intermediate doses, a mixture of $CD69^+$ and $CD69^-$ cells was observed. Plotting the percent CD69 positive

fraction at each peptide dose generated a steep dose response curve, indicative of an all-or-nothing binary response (Figure 3.1A, right panel).

In contrast, simultaneous examination of IRF4 expression in the same cell populations showed a markedly distinct pattern of expression. Like CD69, low peptide doses do not turn on IRF4. However, at higher peptide doses, IRF4 levels continue to increase as peptide dose increases (Figure 3.1B). If CD69 expression is used as a minimal marker for activation, IRF4 levels continue to rise with higher peptide doses, even after the peak magnitude of CD69 is achieved. Simultaneously overlaying the %CD69+ and IRF4 MFI line plots on the same graph illustrates this comparison, as highlighted by the area shaded (Figure 3.1C). The shallow slopes for the dose response curves for IRF4 expression, as well as the variable slopes of these curves for peptides of different potencies, indicate that IRF4 regulation is not binary in nature; rather, IRF4 expression levels are tunable by TCR signal input. Correspondingly, a significant difference in maximum IRF4 expression levels are seen when comparing the responses to the three different peptides, with maximal expression proportional to TCR affinity (Figure 3.1D).

To determine whether differences in peak expression levels of IRF4 in response to stimulation with different potency peptides might result from altered kinetics of IRF4 gene expression, we examined CD69 and IRF4 levels on

Figure 3.1. CD69 and IRF4 respond differently to graded TCR signaling in activated CD8$^+$ T cells.

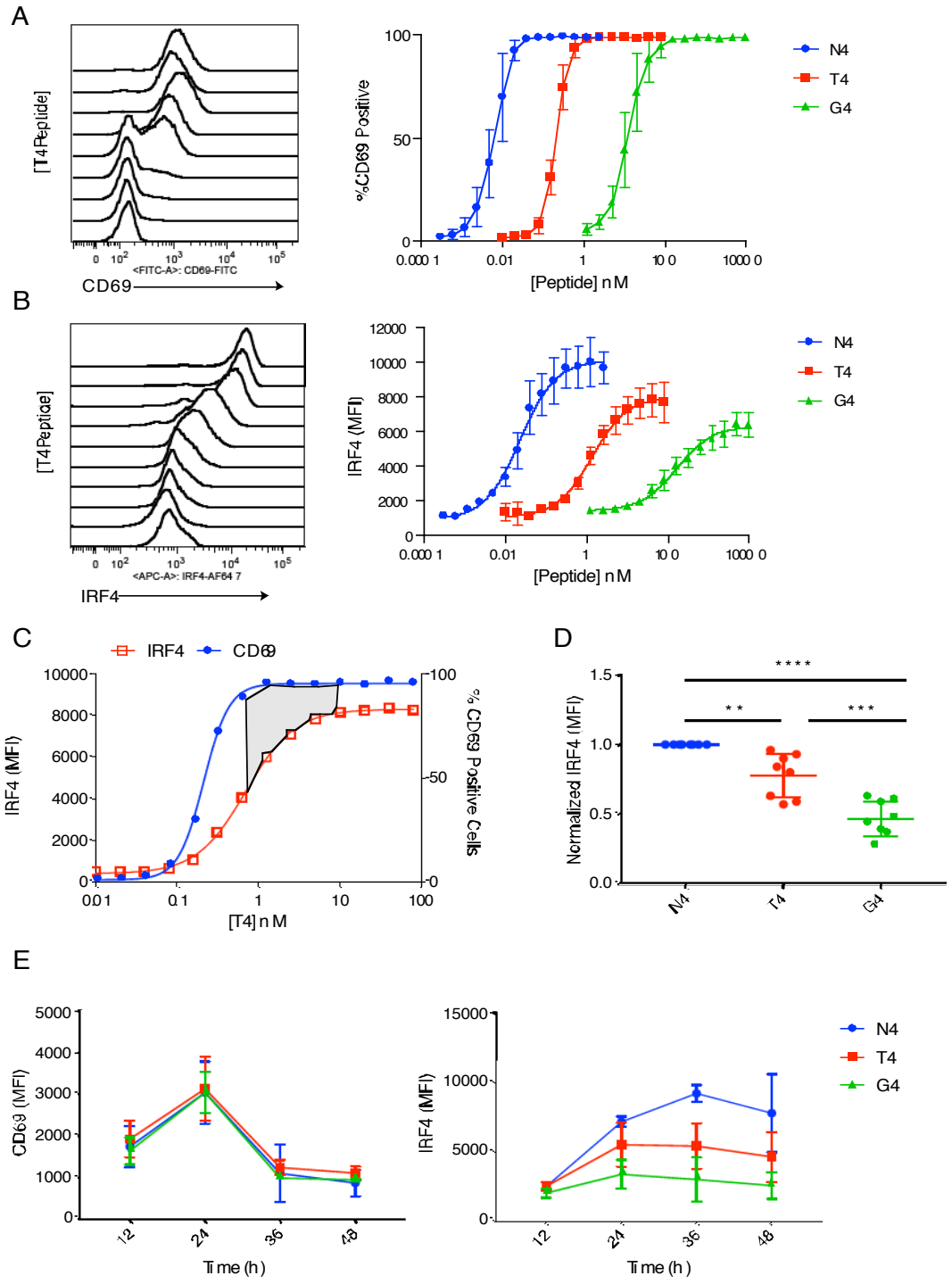


Figure 3.1. CD69 and IRF4 respond differently to graded TCR signaling in activated CD8$^+$ T cells.

(**A**) Representative histogram plots of CD69 staining on OT-I cells stimulated with different doses of T4 peptide for 24h. Cells were gated on live CD8$^+$ TCRβ$^+$. Plots of %CD69+ values are shown for all three peptides at right.

(**B**) Representative histogram plots of IRF4 intracellular staining in OT-I cells stimulated with different doses of T4 peptide for 24h. Cells were gated on live CD8$^+$ TCRβ$^+$. Plots of IRF4 MFI values are shown for all three peptides at right.

(**C**) IRF4 MFI and %CD69+ values for the T4 peptide dose response were plotted on the same graph. The area shaded in gray emphasizes the concentration of antigen that yields maximum CD69 expression but is still on the upslope for IRF4 expression.

(**D**) IRF4 MFI values were normalized to N4 stimulation over multiple experiments. These data show results of stimulations with 1nM N4, 100nM T4, and 1μM G4, which are the relative concentrations eliciting maximum IRF4 expression for each peptide. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (one-way ANOVA followed by Dunnett's test for N4 comparisons. Unpaired student *t* test for T4 and G4 comparison)

(**E**) OT-I T cells were stimulated for 12-48h with 1nM N4, 100nM T4, or 1μM G4. MFI values for CD69 (left) and IRF4 (right) are plotted over time.

Data are representative of three to five experiments.

stimulated OT-I cells at 12, 24, 36, and 48h post-stimulation. These data clearly indicate that the affinity-based maximum expression of IRF4 was not due to a delay in the timepoint at which maximal IRF4 expression was achieved. Instead, we observed maximum levels of IRF4 at 24-36h post-stimulation, regardless of the peptide used to stimulate the T cells. Similar kinetics of CD69 induction were also observed with all 3 peptides (Figure 3.1E). Overall these results show that IRF4 expression levels are a sensitive measure of TCR signal strength, and are sensitive to both the dose of antigen as well as the strength of binding of each TCR to peptide-MHC.

We next examined other genes known to be regulated by TCR signaling. For these studies, we first chose Nur77 (*Nr4a1*) induction, a response commonly used to monitor TCR signal strength both in vitro and in vivo. (Baldwin and Hogquist, 2007; Moran et al., 2011; Woronicz et al., 1994) OT-I mice were crossed to the Nur77-GFP reporter line (Moran et al., 2011), and OT-I $CD8^+$ T cells were stimulated in bulk splenocyte cultures with varying doses of each of the three Ova peptides (N4, T4, and G4). Unlike the pattern of expression observed for IRF4, Nur77-GFP peak expression levels were not dependent on the peptide potency (Fig. 3.2A, left and middle panels). In each case, higher doses of lower potency peptides were able to achieve the same levels of GFP expression as the strongest N4 peptide. This pattern of expression was independent of the timepoint examined, as timecourse studies showed overlapping kinetics of Nur77-GFP expression regardless of the peptide used to stimulate the T cells (Figure 3.2A, right panel). Thus, while Nur77 reporter

expression is closely linked to TCR activation in vitro and in vivo, we did not observe the same affinity based maximum expression relationship that was observed with IRF4 (Figure 3.2A).

In contrast to Nur77-GFP, examination of CD25, the IL-2 receptor alpha chain, showed a pattern resembling IRF4, in that maximum expression levels of CD25 were proportional to peptide potency (Figure 3.2B). The graded expression of CD25 was even more pronounced over time with large differences observed at 48h post-stimulation. Eomesodermin (Eomes), a transcription factor associated with memory CD8 T cells, has been shown to be downregulated by IRF4 (Nayar et al., 2012). Therefore, we predicted an inverse relationship between Eomes and IRF4 expression in stimulated OT-I T cells. Consistent with this, we observed Eomes expression increasing at the low peptide doses just above the threshold needed to induce CD69 upregulation; as peptide doses increased, leading to increasing levels of IRF4, Eomes expression was reduced in a dose-dependent manner. Additionally, peak expression levels of Eomes were inversely related to peptide potency, as the highest levels of Eomes were observed after stimulating OT-I cells with the weakest potency G4 peptide (Figure 3.2C). These data confirm our findings that some gene expression responses are highly tunable by TCR signal strength, and depend on the binding interactions of individual MHC:peptide complexes with the TCR, and not just on the relative dose of antigenic peptide present on antigen-presenting-cells.

Figure 3.2. Graded TCR signaling induces distinct patterns of Nur77, CD25, and Eomes expression.

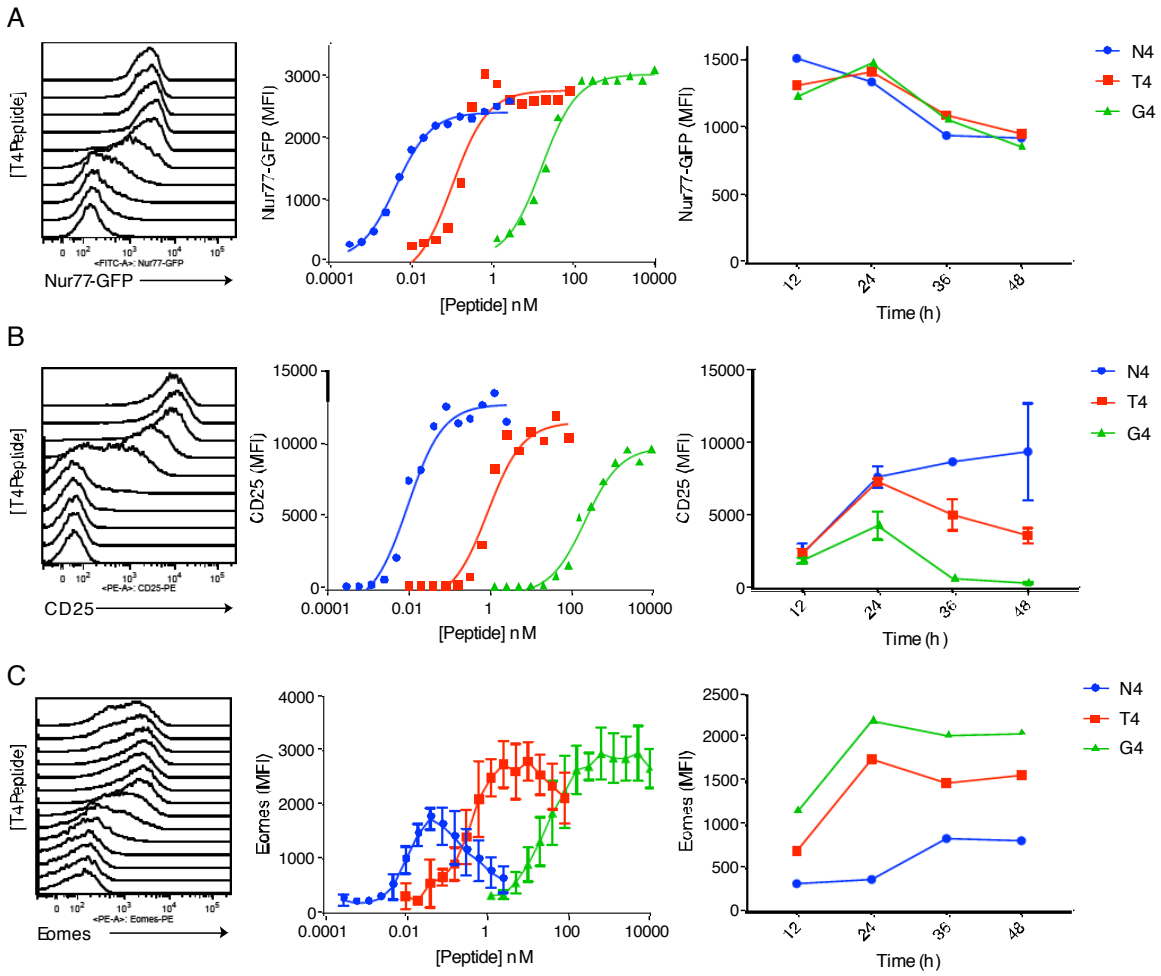


Figure 3.2. Graded TCR signaling induces distinct patterns of Nur77, CD25, and Eomes expression.

(**A**) OT-I Nur77-GFP cells were treated with varying doses of N4, T4, or G4 peptides for 24h. Representative histograms for Nur77-GFP are shown for the T4 peptide (left). GFP MFI is plotted for each peptide dose (middle). Cells were treated with 1nM N4, 100nM T4, or 1μM G4 from 12-48h and MFI for each peptide is plotted over time.

(**B**) OT-I cells were treated with varying doses of N4, T4, or G4 peptides for 24h. Representative histograms of CD25 staining are shown for the T4 peptide (left). CD25 MFI is plotted for each peptide dose (middle). Cells were treated with 1nM N4, 100nM T4, or 1μM G4 from 12-48h and MFI for each peptide is plotted over time.

(**C**) OT-I cells were treated with varying doses of N4, T4, or G4 peptides for 24h. Representative histograms of Eomes intracellular staining are shown for the T4 peptide (left). Eomes MFI is plotted for each peptide dose (middle). Cells were treated with 1nM N4, 100nM T4, or 1μM G4 from 12-48h and MFI for each peptide is plotted over time.

Data are representative of two to three experiments.

ITK activity drives graded IRF4 expression in CD8+ T cells

To address in greater detail the TCR signals responsible for graded expression of genes based on TCR signal strength, we chose to focus on the regulation of IRF4. We reasoned that binary modes of expression, such as that seen for CD69, had previously been characterized. Specifically, Das et. al. demonstrated a positive feed-forward pathway accounting for the all-or-nothing response of *Cd69* to TCR stimulation. In this study, the authors showed that following TCR stimulation, the initial production of the second messenger DAG activated the Ras GTP-exchange factor Ras-GRP, leading to the generation of activated Ras molecules. These Ras-GTP molecules bound to a second Ras GTP-exchange factor SOS, acting as an allosteric modulator of SOS activity and promoting a massive enhancement in the production of activated Ras. A consequence of this feed-forward loop was that low signaling thresholds could rapidly produce maximal activation of the Ras-MAPK pathway, a key inducer of CD69 expression. The authors then linked this pathway to the bimodal expression of CD69 (Das et al., 2009). In contrast to this well-characterized pathway producing bimodal expression of CD69, the signals accounting for graded expression of IRF4 in response to variations in TCR signal strength have not been described.

While changes in IRF4 expression were known to be dependent on ITK activity in the context of low dose αCD3 stimulation (Nayar et al., 2012), to pursue this connection further, we generated *Itk*$^{-/-}$ OT-I TCR transgenic mice. Stimulation of splenocytes from these mice with the three Ova peptide variants showed that CD69 upregulation was nearly unaffected by the absence of ITK

when T cells were stimulated with varying doses of N4 or T4 peptide. Stimulation with the weakest potency peptide G4 showed a modest impairment of CD69 upregulation, but the maximal response of CD69 expression could be restored by modest increases in peptide dose (Figure 3.3A).

In contrast to CD69, IRF4 upregulation was markedly reduced in stimulated *Itk*$^{-/-}$ OT-I T cells, particularly in response to T4 and G4 peptides (Figure 3.3B-C). Interestingly, the overall peptide concentration-dependence of IRF4 expression was not changed; for example, for T4 peptide, the EC_{50} for WT OT-I cells was 1.3nM and was 1.0nM for *Itk*$^{-/-}$ cells. However, the maximum level of IRF4 expression was significantly decreased, in addition to being substantially delayed (Figure 3.3D-E). To further explore the relationship between ITK and maximum IRF4 expression, we treated OT-I cells with a small molecule ITK inhibitor PRN-694 (Zhong et al., 2015). Inhibition of ITK significantly reduced IRF4 expression in WT OT-I cells stimulated with either N4 or T4 stimulated peptide, with similar IC_{50}s (20-40nM) (Figure 3.3F). Further evidence that ITK activity could fine tune IRF4 expression was generated in an additional experiment, where cells were treated with multiple doses of T4 peptide, and for each peptide dose, cells were treated with a series of doses of PRN-694 in an 8x8 matrix format (Figure 3.3G). Quantification of these dose-response experiments demonstrates a clear relationship between ITK activity and maximum IRF4 expression, as is evident by the stratified plateaus of the dose response curves. PRN694 also inhibited CD25 expression at a similar potency as for inhibition of IRF4 upregulation, but did not inhibit CD69 or Nur77-GFP

expression (Figure 3.3H). Overall, these studies demonstrated that modulation of TCR signal strength by reducing TCR affinity for MHC:peptide or by inhibiting ITK activity led to reduced IRF4 expression. Furthermore, these data also showed that low affinity peptides were unable to induce the same levels of IRF4 expression as higher affinity peptides, regardless of the dose of peptide provided.

Figure 3.3. ITK inhibition reduces maximum IRF4 expression in CD8$^+$ T cells in a graded manner.

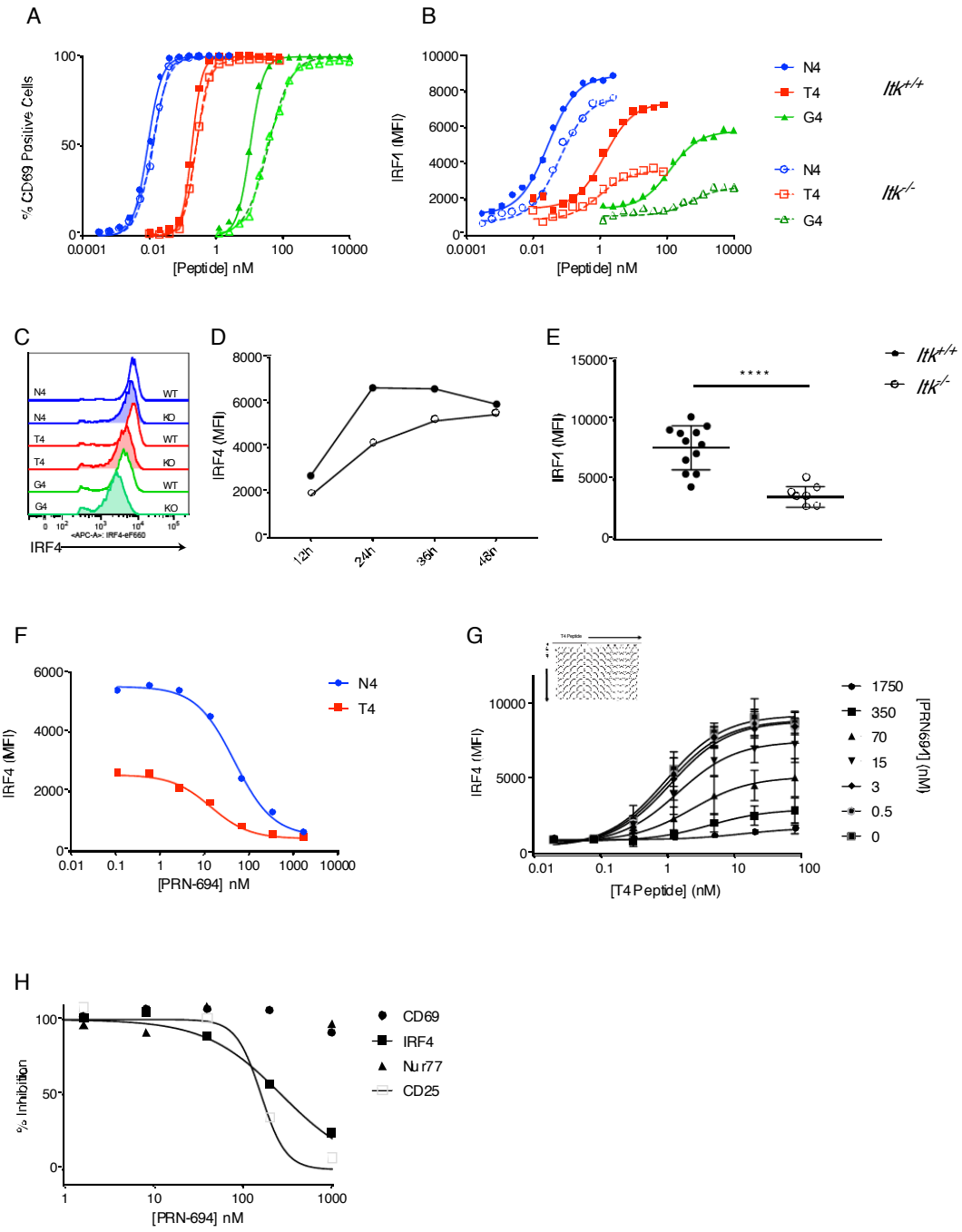


Figure 3.3. ITK inhibition reduces maximum IRF4 expression in $CD8^+$ T cells in a graded manner.

(**A-B**) OT-I WT ($Itk^{+/+}$) or OT-I $Itk^{-/-}$ ($Itk^{-/-}$) T cells were stimulated each peptide for 24h at multiple doses. %CD69+ (A) and IRF4 MFI (B) are plotted for each peptide concentration.

(**C**) Representative histograms of IRF4 intracellular staining for 1nM N4, 100nM T4, and 1μM G4 peptide stimulations of $Itk^{+/+}$ or $Itk^{-/-}$ cells.

(**D**) $Itk^{+/+}$ or $Itk^{-/-}$ OT-I cells were stimulated with 100nM T4 peptide at timepoints from 12-48h, and IRF4 MFI is plotted.

(**E**) IRF4 MFI values of $Itk^{+/+}$ or $Itk^{-/-}$ cells stimulated with 100nM T4 for 24h. **** $p \leq 0.0001$ (unpaired student *t* test)

(**F**) OT-I T cells were stimulated with 1nM N4 and 100nM T4 peptide while simultaneously treated with varying doses of the ITK inhibitor PRN694 for 24h. Cells were stained for intracellular IRF4 and MFI of IRF4 staining under each condition is displayed.

(**G**) OT-I T cells were stimulated with a range of doses of T4 peptide in a matrix using varying doses of PRN694. After 24h the cells were stained for IRF4, and the IRF4 MFI for each condition is displayed. Each curve represents a dose response of T4 peptide at a single concentration of PRN694.

(**H**) OT-I T cells were stimulated with 100nM T4 peptide with varying doses of PRN694 for 24h. The cells were stained for IRF4, CD69, and CD25. For Nur77,

the Nur77-GFP reporter was used. The MFI values were normalized to a positive and negative control to obtain % Inhibition.

Data are representative of three to five experiments.

Calcium signaling drives graded IRF4 expression in CD8+ T cells

To determine the component(s) of TCR signaling that could account for the graded expression of IRF4 in response to variable TCR signaling, as well as the importance of ITK signaling in this response, we considered the major transcription factors activated by the TCR. Due to the demonstrated role of ITK in this phenomenon, we focused on pathways downstream of PLC-γ1, the MAPK, NF-κB, and calcium/NFAT signaling pathways (Brownlie and Zamoyska, 2013) (Macian, 2005; Paul and Schaefer, 2013). As previous studies have demonstrated that *Itk*$^{-/-}$ T cells have a substantial defect in calcium signaling amplitude (Miller et al., 2004) (Donnadieu et al., 2001), we initially hypothesized that calcium could directly contribute to graded IRF4 expression in T cells.

To address the signaling pathways that could be contributing to increased IRF4 expression, we utilized two common pharmacological agents commonly used to stimulate T cells in vitro, phorbol 12-myristate 13-acetate (PMA) and Ionomycin. PMA is a small molecule mimetic of DAG that directly activates protein kinase C and Ras-MAPK signaling, whereas the calcium ionophore, Ionomycin, induces calcium signaling in T cells. Unlike the usual strategy of combining these two agents together to stimulate T cells, we separately treated WT OT-I cells with varying doses of PMA or Ionomycin alone. After 24h, IRF4 expression was assessed by flow cytometry (Figure 3.4A). After treatment with PMA, we observed a binary response of IRF4 expression, with a modest induction of IRF4 that achieved a stable plateau. In contrast, treatment with Ionomycin recapitulated the graded induction of IRF4 expression. Only in

combination do the two agents yield comparable IRF4 expression to that observed with strong peptide antigen-dependent stimulation (Figure 3.4B). Furthermore, the ability of Ionomycin to promote a graded induction of gene expression was unique to IRF4, as neither CD69, Nur77-GFP, nor CD25 showed a comparable upregulation induced by calcium signaling alone (Figure 3.5A-C). The calcineurin inhibitor FK-506 was also able to block IRF4 upregulation, emphasizing the dependence of this response on calcium signaling (Figure 3.4C). These data indicated that IRF4 is regulated by multiple signaling pathways, but that the calcium pathway is responsible for the graded expression of IRF4 in response to variations in TCR signal strength.

Figure 3.4. Multiple signaling inputs drive IRF4 expression with calcium signaling controlling graded expression.

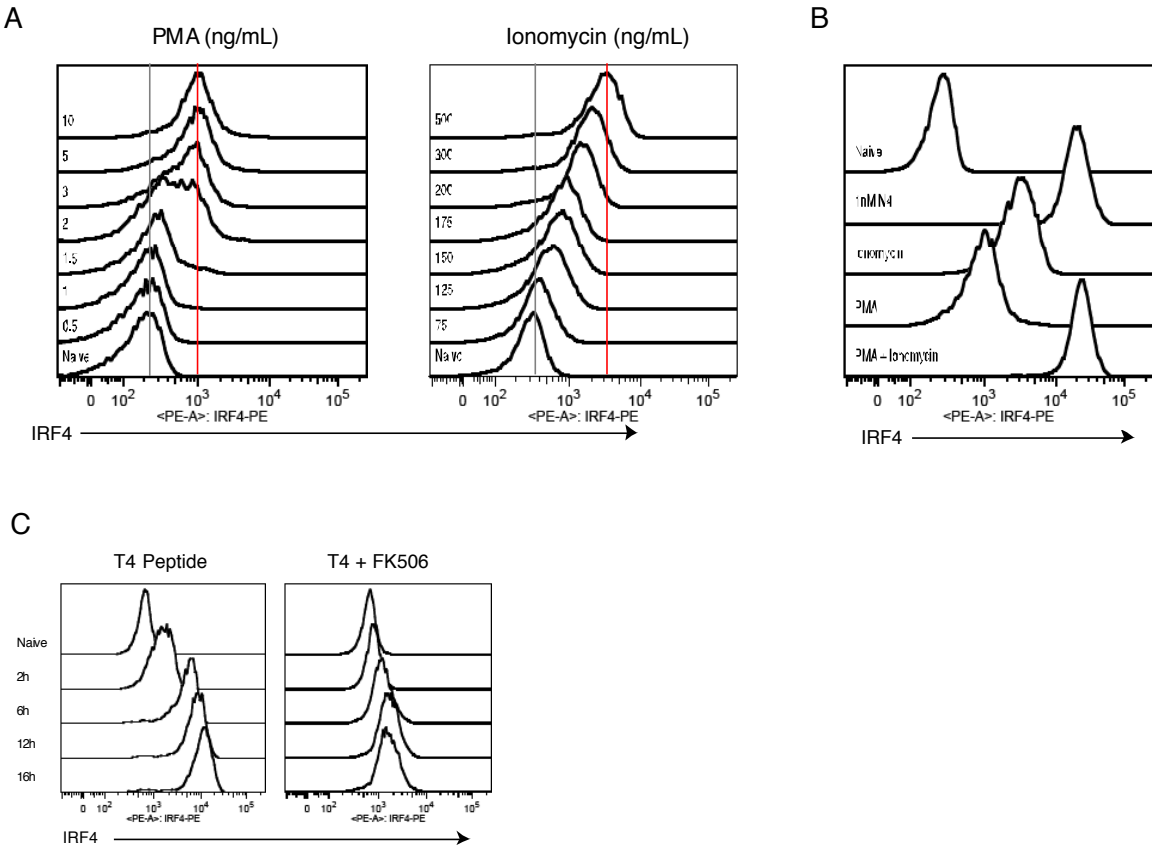


Figure 3.4. Multiple signaling inputs drive IRF4 expression with calcium signaling controlling graded expression.

(**A**) OT-I T cells were treated with varying doses of PMA or Ionomycin for 24h, and cells were stained for intracellular IRF4. Representative histograms of IRF4 staining are shown.

(**B**) Representative histograms of IRF4 staining for OT-I cells left unstimulated (Naïve), or stimulated with 500ng/mL Ionomycin, 10ng/mL PMA, a combination of both, or 1nM N4 for 24h.

(**C**) Representative histograms of IRF4 staining for OT-I cells treated with 100nM T4 for timepoints from 2-16h in the absence (left) or presence of 100nM FK506.

Data are representative of three to five experiments.

Figure 3.5. PMA and Ionomycin together drive graded IRF4 expression while Nur77 and CD69 show modest expression with PMA alone.

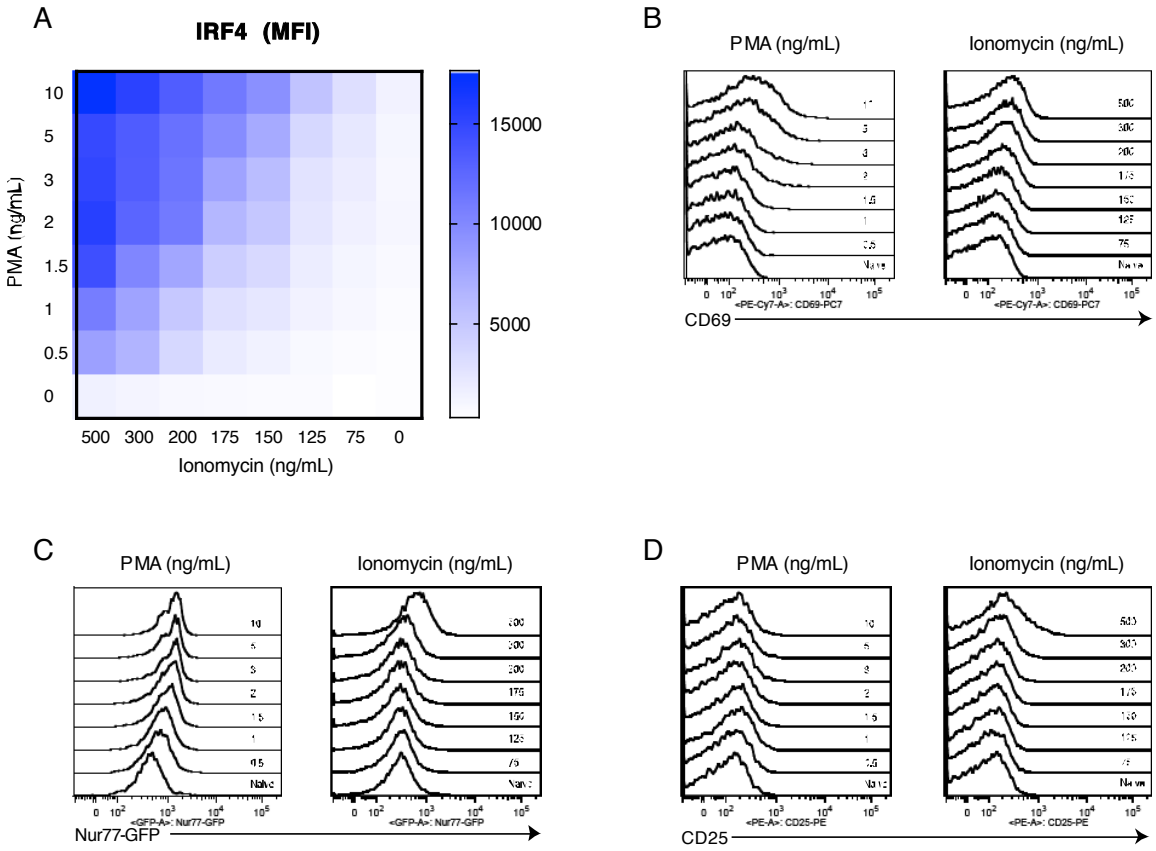


Figure 3.5. PMA and Ionomycin together drive graded IRF4 expression while Nur77 and CD69 show modest expression with PMA alone.

(**A**) Representative heat map of IRF4 MFI values for OT-I T cells treated with various doses of PMA and Ionomycin in combination for 24h.

(**B-D**) Representative histograms for CD69 (B), Nur77-GFP (C), and CD25 (D) after treatment with PMA or Ionomycin for 24h.

Data are representative of two to three experiments.

Reducing TCR signal strength digitally regulates NFAT activation, a transcription factor that binds to the Irf4 promoter

We hypothesized that the calcium-mediated regulation of IRF4 expression worked through NFAT activation. NFAT is a family of transcription factors that are regulated indirectly by calcium flux through the removal of inhibitory phosphorylation sites by the calcium-activated phosphatase calcineurin (Dolmetsch et al., 1998; Marangoni et al., 2013). Normally, NFAT is highly phosphorylated and sequestered in the cytoplasm. When calcineurin is active, these sites are removed, unmasking the nuclear localization domain and allowing nuclear transport where it can then bind to DNA along with other transcription factor binding partners, often AP-1 (Hogan et al., 2003; Müller and Rao, 2010). To test the effects of graded TCR signaling on NFAT activation in CD8 T cells, we utilized a nuclear flow cytometry assay to assess nuclear NFAT levels in activated OT-I T cells (Gallagher et al., 2018). For these experiments, OT-I $CD8^+$ T cells were isolated, labeled with fluorescent cell tracking dye, and then mixed with unlabeled splenocytes plus varying doses of the three peptide antigens. After 30 minutes, nuclei were prepared and stained with an antibody to NFAT1. Histograms of NFAT1 staining in nuclei of OT-I cells stimulated with varying concentrations of the T4 peptide variant showed that NFAT1 activation was not graded in nature, but rather, exhibited a binary response (Figure 3.6A, left panel). As the dose of peptide increased, the proportion of OT-I cells with nuclear NFAT1 increased in a dose-dependent manner (Figure 3.6A, right panel). While NFAT activation was binary at the single-cell level, the overall pattern of NFAT1 activation seen in the population of OT-I cells as a whole reflected both the dose

of peptide as well as the binding strength of each peptide variant. When plotted as a percentage of NFAT1+ nuclei, the data for NFAT1 activation show a striking similarity to the data examining IRF4 protein expression under these same conditions. Thus, at this early 30 minute timepoint, the OT-I population shows a maximum level of NFAT1-positive nuclei that is proportional to peptide affinity with N4>T4>G4.

We then examined the effect of ITK activity on NFAT activation. As shown in Figure 3.6B (left panel), ITK inhibition by PRN694 retained a bimodal response of NFAT1 activation following stimulation of OT-I cells with antigen-presenting-cells and peptide. Instead, inhibition of ITK reduced the percentage of NFAT1+ nuclei at each peptide dose tested (Figure 3.6B). These results also mirror the effects of ITK deficiency on the expression of IRF4 protein in response to varying doses of T4 peptide. To generate a more detailed view of the NFAT1 activation response, we performed a timecourse experiment, including timepoints ranging from 5 minutes to 60 minutes post-stimulation. These data confirmed our initial observations that varying TCR signal strength had a dramatic effect on the kinetics of NFAT1 nuclear localization (Figure 3.6C). Whereas higher doses of N4 peptide elicited a more rapid rise in the percentage of NFAT1+ nuclei, stimulation with lower doses of this peptide delayed the accumulation of NFAT1+ nuclei. Strikingly, the weaker peptide variant T4 was unable to induce NFAT1 nuclear localization in any cells, regardless of the peptide dose, within the first 15 minutes of stimulation (Figure 3.6C, right panel). This lag in NFAT nuclear localization is consistent with the delay in calcium flux onset observed in single

OT-I cells stimulated with lower doses of the N4 peptide antigen (Dura et al., 1AD). We reasoned that this delay in NFAT activation could dictate the graded levels of IRF4 protein seen at later timepoints. Extensive characterization of NFAT DNA binding sites in activated $CD8^+$ T cells has been reported by A. Rao and colleagues (Martinez et al., 2015a). Analysis of these published data for NFAT binding sites at the *Irf4* promoter region revealed a robust peak 1.2kB upstream of exon 1 (Figure 3.6D). Interestingly, this NFAT binding peak is present in cells that expressed an NFAT protein carrying amino acid substitutions that interfere with its binding to AP-1. These data confirm that NFAT is able to bind to the *Irf4* regulatory region as a homodimer in the absence of its usual binding partner, consistent with its robust control through calcium signaling alone.

Figure 3.6. Reducing TCR signal strength digitally regulates activation of NFAT, a transcription factor that binds to the *Irf4* promoter.

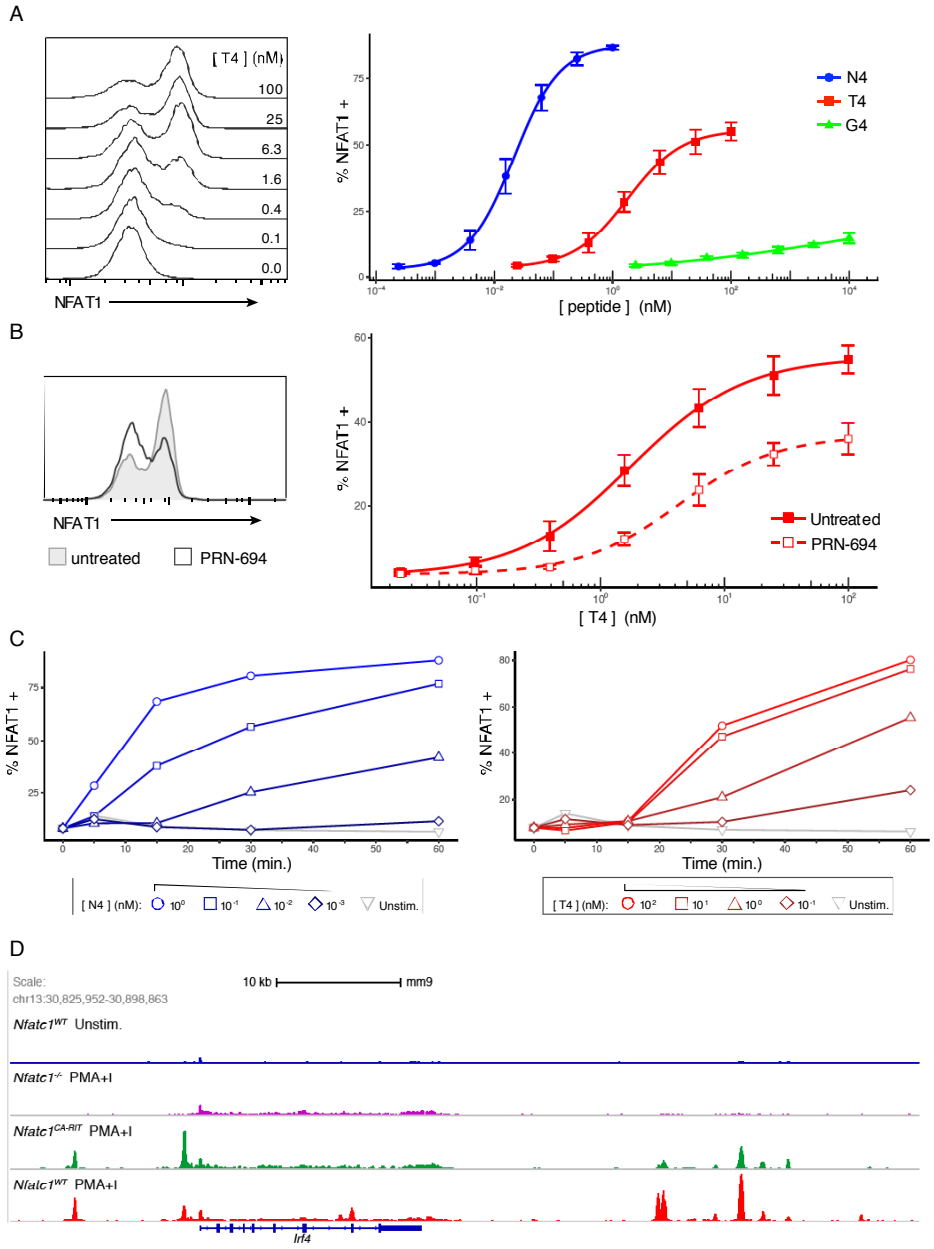


Figure 3.6. Reducing TCR signal strength digitally regulates activation of NFAT, a transcription factor that binds to the *Irf4* promoter.

(**A**) Representative histograms of NFAT1 fluorescence in OT-I nuclei isolated after T cells were stimulated with B6 splenocytes pulsed with indicated doses of T4 peptide for 30m (left). Line plots of %NFAT^{+} nuclei after 30m of stimulation with B6 splenocytes pulsed with indicated doses of either N4, T4 and G4 peptides (right). OT-I nuclei were identified as CellTrace Violet^{+} events.

(**B**) Representative histograms of NFAT1 fluorescence in OT-I nuclei after cells were stimulated for 30m with B6 splenocytes pulsed with 25 nM T4 peptide with or without 50nM PRN694 treatment (left). Line plots of %NFAT+ (nuclear NFAT) values are shown for the T4 peptide dose response with and without 50nM PRN694 treatment (right). Nuclei were gated on CellTrace Violet^{+} events.

(**C**) Line plots of %NFAT+ OT-I nuclei over a 60m timecourse after cells were stimulated B6 splenocytes pulsed with varying doses of either N4 (left) or T4 (right) peptides as indicated.

(**D**) NFAT1 ChIP-Seq data (GSE64409) on activated CD8 T cells from Martinez *et al* (Martinez et al., 2015b) were visualized using IGV software and a snapshot was taken of the *Irf4* locus. The data represents 4 samples: (1) WT T cells, transduced with Mock construct, unstimulated (2) *Nfatc1*$^{-/-}$ T cells, transduced with Mock construct, stimulated with PMA/Ionomycin for 1h (3) *Nfatc1*$^{-/-}$ T cells, transduced with CA-RIT-NFAT (constitutively active NFAT unable to bind AP-1), stimulated with PMA/Ionomycin for 1h (4) WT T cells, transduced with Mock construct, stimulated with PMA/Ionomycin for 1h.

Data are representative of two or three experiments (A-C).

Graded TCR signaling alters the kinetics of Irf4 mRNA upregulation

IRF4 upregulation is detectable by 6h post-stimulation and generally peaks at 24h, with maximum levels of expression differing between T cells stimulated by ligands of different potencies. These data suggested that evaluating the kinetics of early *Irf4* mRNA upregulation could be informative in understanding how cells stimulated with distinct TCR signal strengths ultimately accumulate different amounts of IRF4 protein. Using RNA flow cytometry, we measured *Irf4* mRNA in activated OT-I T cells over the first 16h post-stimulation. Cells stimulated with 100nM of T4 peptide rapidly upregulated *Irf4* mRNA by 2h, and levels of this mRNA were relatively constant for 16h post-stimulation (Figure 3.7A-B). We also observed a sharp transition of cells going from *Irf4* mRNA negative to positive in the time from 0-2h post-stimulation. Consistent with the IRF4 protein data, FK506 completely blocks upregulation of *Irf4* mRNA. These findings emphasize the important role of calcium signaling and NFAT activation in *Irf4* mRNA transcription, a result consistent with previous data demonstrating NFAT binding to the *Irf4* gene locus in activated T cells (Man et al., 2017).

In contrast, inhibition of ITK signaling by treatment of cells with PRN694 delayed, but did not abolish, the upregulation of *Irf4* mRNA. Similar to our results examining NFAT activation, we observed two distinct populations of OT-I cells at 2h post-stimulation in the presence of PRN694, one population positive for the *Irf4* mRNA and one population remaining negative. Compared to the cells stimulated in the absence of the ITK inhibitor, those treated with PRN694 showed a slower transition to having *Irf4* mRNA, along with a broader histogram peak, indicative of greater variability in expression within the population. The role for

ITK in accelerating the rate of *Irf4* mRNA upregulation was also confirmed by examination of stimulated WT versus *Itk*$^{-/-}$ OT-I T cells. Again, along with delayed kinetics of *Irf4* mRNA expression in the absence of ITK, we observed a bimodal pattern of *Irf4* mRNA staining (Figure 3.7C). Finally, as expected based on the protein expression data, we found no major effect on *Cd69* mRNA expression in the absence of ITK (Figure 3.7C).

Reduced TCR signaling consistently yielded a reduction in peak expression of IRF4 protein, based on the IRF4 histogram MFI, while also showing a broader distribution within the population, with a broader histogram peak observed in ITK-inhibited T cells. We wanted to measure this increase in histogram width. To quantify this variability in expression we plotted the coefficient of variance (CV) for IRF4 MFI of OT-I cells stimulated with 100nM T4 peptide, in the absence or the presence of varying different doses of PRN694 (Figure 3.7D). ITK inhibition significantly increased the CV of both IRF4 protein and *Irf4* mRNA, suggesting increased heterogeneity of expression across the clonal population. This was not a consequence of failing to activate a subset of cells, as >95% of cells were CD69+ under each of these conditions.

Figure 3.7. Reducing TCR strength, through ITK inhibition, delays *Irf4* mRNA upregulation and increases variability across the population.

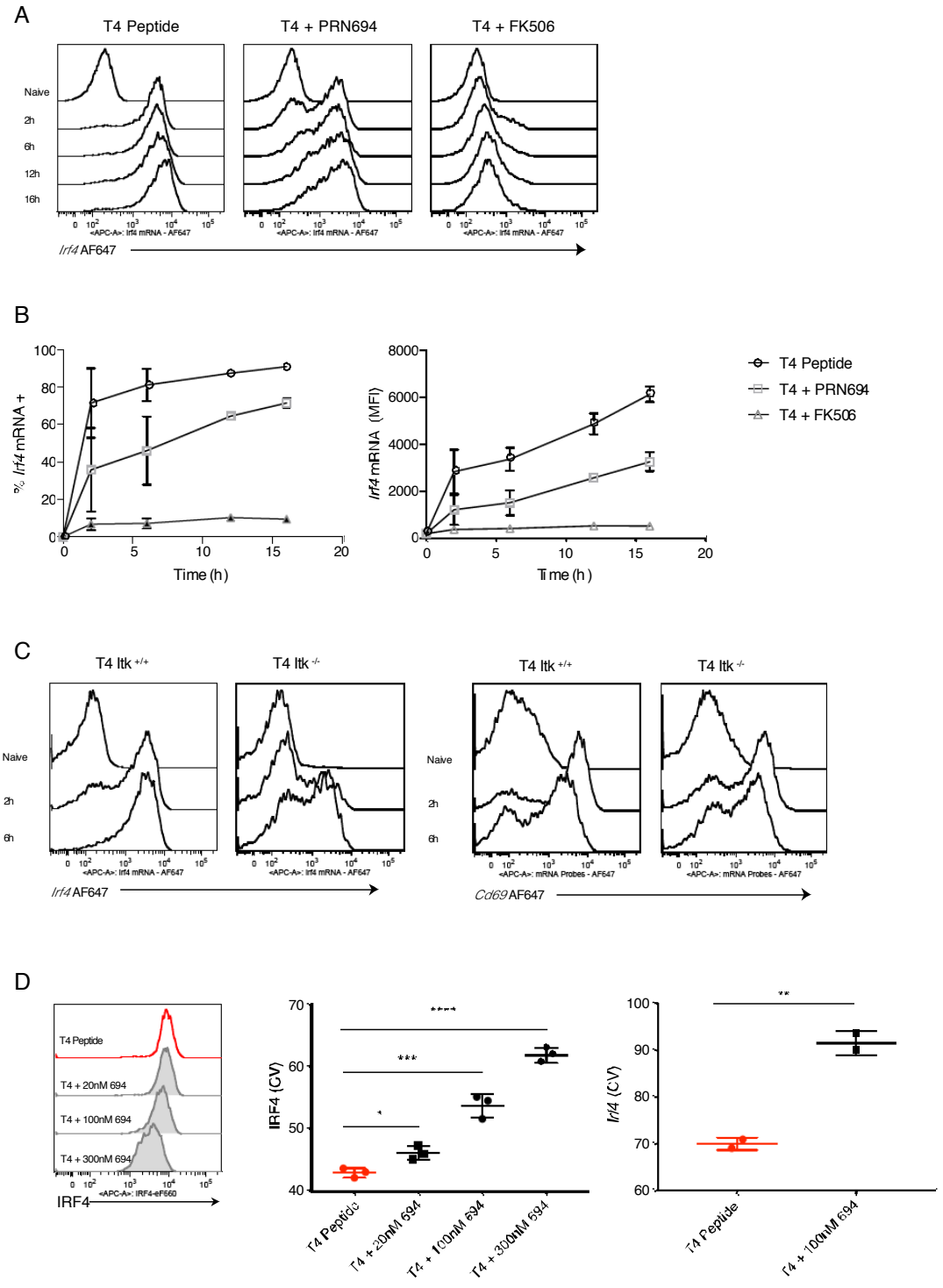


Figure 3.7. Reducing TCR strength, through ITK inhibition, delays *Irf4* mRNA upregulation and increases variability across the population.

(**A-B**) OT-I T cells were treated with 100nM T4 peptide for timepoints from 2-16h either alone or in the presence of 100nM PRN694 or 100nM FK506. (A) Representative histogram plots of *Irf4* mRNA expression. (B) Data are plotted as %*Irf4* mRNA positive (left) or as *Irf4* mRNA MFI (right) over time.

(**C**) $Itk^{+/+}$ or $Itk^{-/-}$ cells OT-I T cells were treated with 100nM T4 peptide for 2 or 6h. Representative histograms for *Irf4* and *Cd69* mRNA expression ar shown.

(**D**) Representative histograms for OT-I T cells stimulated with 100nM T4 peptide in the absence or presence of various doses of PRN694 treatment for 24h, and cells were stained for intracellular IRF4. The coefficient of variance (CV) of IRF4 staining was plotted for each treatment group. * $p \leq 0.1$ ** $p \leq 0.01$ *** $p \leq 0.001$ **** $p \leq 0.0001$ (one-way ANOVA followed by Dunnett's test for the left panel and unpaired student *t* test for the right panel)

Data are representative of two to three experiments.

Discussion

Understanding the molecular mechanisms that control graded signaling through the TCR has been a central effort in T cell biology. A component of this effort has been studies directed at elucidating the contribution of the Tec kinase ITK to this process. Here, we utilized a combination of assays providing single cell data to dissect the signaling inputs that control the graded expression of the transcription factor IRF4, a key factor regulating the magnitude of the $CD8^+$ effector T cell response to infection. Our studies revealed a process in which a clonal population of naïve CD8 T cells integrated different strengths of TCR stimulation into binary NFAT1 activation responses. Weaker TCR stimulation led to a delay in the kinetics of NFAT nuclear localization that correlated with slower upregulation of *Irf4* mRNA, ultimately resulting in reduced expression of the IRF4 protein. As a consequence of this mechanism, a population of $CD8^+$ T cells that receives weak TCR signaling is destined to undergo limited clonal expansion (Man et al., 2013; Nayar et al., 2015; 2014; Yao et al., 2013).

Our results are in agreement with the recent studies of Richard *et. al*. Using state-of-the-art single cell RNA sequencing and proteomic techniques, the authors showed that the strength of TCR signaling controlled the rate at which individual cells were activated within a clonal population (Richard et al., 2018), and was responsible for greater variability in gene expression between individual cells in low affinity clonal populations. However, in analyses of cytolytic effector functions several days post-stimulation, this delay in kinetics among weakly stimulated naïve $CD8^+$ T cells did not ultimately impair their cytolytic capacity,

results that are in line with our observations that only some genes (e.g., *Irf4*, *Il2ra*) are sensitive to this delay in activation and are likely driving the magnitude of clonal expansion, while others (e.g., *Cd69*) are not.

Our results also expand our knowledge of how the Tec kinase ITK regulates the strength of TCR signaling. Many studies have documented that ITK is not required for T cell activation per se. Instead, T cells lacking ITK have selective defects in some T cell responses, while other responses appear unaffected by the absence of ITK. For instance, naïve *Itk*$^{-/-}$ CD4 T cells are defective in producing IL-17A or IL-9, when stimulated under T_H17 or T_H9 polarizing conditions, respectively (Gomez-Rodriguez et al., 2016; 2014). Yet, *Itk*$^{-/-}$ mice are proficient at clearing infections of *Leishmania major*, and viruses such as LCMV, Influenza A, and Vaccinia, responses that require effective T_H1 and cytotoxic CD8+ T cell responses, respectively (Atherly et al., 2006a; 2006b; Bachmann et al., 1997; Schaeffer et al., 2001). Interestingly, these data also correspond nicely to the recent findings of Richard, et al, which found that expression of genes required for cytotoxic T cell function were independent of the strength of the initial T cell stimulation.

Our studies indicate that ITK functions as an accelerator in the TCR signaling pathway. In this regard, ITK is not required for TCR signaling, but acts to accelerate the process by which TCR stimulation produces a calcium response capable of inducing NFAT nuclear translocation. We speculate that ITK functions to increase the number of activated PLC-γ1 molecules produced by each stimulated TCR. As our data also show that NFAT nuclear translocation is

an all-or-nothing response to TCR stimulation, we envision a sharp threshold of IP_3 production by activated PLC-γ1 that is required to induce NFAT activation. When ITK signaling is engaged, the rate at which this threshold is achieved can be dramatically increased. It is likely that other key transcription factors activated by TCR stimulation have alternative modes of response to variations in TCR signal strength, possibly explaining why some genes expression responses, but not all, are sensitive to ITK signaling.

Our data indicate that NFAT is not the sole transcription factor contributing to IRF4 expression after TCR stimulation. As shown by the modest upregulation of IRF4 in response to PMA alone, as well as the combinatorial effect of PMA and Ionomycin added together, we conclude that additional DAG-dependent factors are required for optimal IRF4 upregulation in response to TCR signaling. One likely pathway contributing to the NFAT-independent component of IRF4 expression is NF-κB, as previous studies have suggested a role for both canonical NF-κB p65, as well as c-Rel in *Irf4* expression (Grumont and Gerondakis, 2000). While these findings reveal the complex and multifactorial regulation of genes induced by TCR stimulation, they also highlight the utility of reductionist experiments, such as those described here, in identifying key inputs responsible for differing responses to variations in TCR signal strength. Our studies also demonstrate that increasing ligand dose or density on antigen-presenting-cells cannot necessarily overcome signaling deficits due to weakly stimulatory ligands. Thus, increasing the concentration of weakly potent peptide antigens is unable to generate the levels of IRF4 in naïve $CD8^+$ T cells

that can be achieved by more potent ligands. Understanding the complete program of genes that share this characteristic of *Irf4* will provide critical information in the development of improved vaccines and T cells used for adoptive immunotherapy, to maximize desired effector and/or memory T cell responses.

CHAPTER IV: TCR SIGNAL STRENGTH DRIVES SELECTIN BINDING AND AUTOREACTIVE CD8$^+$ T CELL MIGRATION

Introduction

During the adaptive immune response, activated lymphocytes migrate from the secondary lymphoid organs to the site of infection to prevent further expansion of the invading pathogen. A complex network of cytokine and chemokine signaling gradients guide these cells to the damaged area, aided by the innate immune response, which uses pathogen-associated molecular patterns (PAMPs) to activate resident myeloid cells to secrete these factors. These soluble proteins act on the resident endothelial cells causing them to produce a variety of adhesion molecules, facilitating recruitment of the circulating lymphocytes. The multi-step process where lymphocytes are recruited from the vasculature to the inflamed tissue, transendothelial migration (TEM), is a critical step in the immune response. First, the lymphocytes are captured and bound to the apical endothelial surface, controlled by selectin interactions, allowing the cell to "roll" along the membrane while resisting the shearing force of the blood. Tighter integrin-based interactions follow, causing the cell to arrest and "crawl" along the endothelial cell searching for junctions between cells. Then through diapedesis, the lymphocyte migrates from the blood vessel lumen to the connective tissue (Vestweber, 2015).

In the context of autoimmune disease, autoreactive T cells that are activated and expanded in the secondary lymphoid organs, require TEM to enter target organ tissue in the absence of pathogen-associated inflammatory cues that normally facilitate extravasation. While the signaling requirements for autoreactive T cell migration are not completely understood, there is evidence

that signaling through both the T cell receptor (TCR) and costimulatory molecules (i.e. CD28) are playing a role. In mice lacking the tolerance ligand cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a robust expansion of autoreactive T cells is observed, leading to multiple organ damage and death of the mice within 4 weeks (Chambers et al., 1997; Ise et al., 2009). When the *Ctla4*$^{-/-}$ mice were crossed to a knockout of Inducible T cell Kinase (ITK) (*Itk*$^{-/-}$), a tyrosine kinase important in TCR signal transduction (Andreotti et al., 2018; Berg et al., 2005; Conley et al., 2016), the disease pathology was completely prevented (Jain et al., 2013). However, the T cells in the double knockout mice remained autoreactive, had a highly activated phenotype and expanded to large numbers in secondary lymphoid organs and blood. Two-photon imaging showed impaired interactions of *Ctla4*$^{-/-}$*Itk*$^{-/-}$ T cells with lung endothelial cells after adoptive transfer into naïve wild-type recipients. These data suggested a requirement for robust TCR signaling to prime autoreactive T cells for effective TEM. This requirement for ITK is not observed in the context of infection, as *Itk*-deficient T cells are able to clear infections in tissue restricted models like Influenza A (Fan et al., 2012). Furthermore, the importance of strong TCR signaling for the development of pathogenic autoreactive tissue-infiltrating T cells was also observed in a study using the RIP-mOVA mouse, where ovalbumin antigen is expressed in the pancreas. In this study, CD8 T cell-mediated induction of diabetes was only seen in mice that were adoptively transferred with ovalbumin-specific (OT-I TCR transgenic) T cells stimulated with high-affinity peptide antigen, a process that was dependent on the integrin VLA-4 (King et al., 2012).

The process of TEM requires a number of cell surface receptors that facilitate intercellular contact as well as intracellular signal transduction. In T cell TEM, P-selectin (and E-selectin) from the endothelium binds P-selectin glycoprotein ligand-1 (PSGL1) and CD44 on the activated lymphocyte promoting the “rolling” process. Then, integrins such as intercellular adhesion molecule 1 (ICAM1 and VCAM1) bind to ligands, lymphocyte function-associated antigen 1 (LFA1 and VLA-4), on the T cell. Integrin binding is a stronger interaction compared to that of the selectins for their ligands, leading to T cell “crawling” on the endothelium and later diapedesis (Hogg et al., 2011; McEver and Zhu, 2010; Zarbock et al., 2011). In the lymph node, T cells upregulate CD44 expression after antigen stimulation. Naïve T cells constitutively express PSGL1, however, after stimulation, they modify the receptor by adding carbohydrate molecules to the extracellular portion of the receptor. This post-translational modification increases the binding of PSGL1 for its cognate ligand P-selectin (McEver and Cummings, 1997; Norman et al., 1995) Both PSGL1 and CD44 function in the rolling stage of TEM.

The glycoprotein, T cell immunoglobulin and mucin domain 1 (TIM-1), is another P-selectin ligand. Largely expressed in activated T_H1 and T_H17 cells, the receptor mediates trafficking in multiple infection and autoimmune models (Angiari et al., 2014). Activated T cells also express the integrin LFA1, which is critical for the crawling process (Hogg et al., 2011; Walling and Kim, 2018). The costimulatory ligand DNAX accessory molecule 1 (DNAM-1, CD226), while normally associated with T and NK cell cytotoxicity (Gilfillan et al., 2008; Welch et

al., 2012), has also been linked to autoreactive T cell function (Ayano et al., 2015). Blocking of CD226 signaling with an antibody inhibited T_H1 expansion and tissue pathology in a mouse model of autoimmunity, experimental autoimmune encephalomyelitis (EAE) (Dardalhon et al., 2005).

The upregulation of these surface receptors on T cells is predominantly mediated by TCR signaling following antigen encounter. TCR signaling also modulates LFA1 function at multiple levels (Beinke et al., 2010). For example, one of the critical steps for strong binding of LFA1 to its ligand ICAM-1 is the TCR-mediated signal that promotes a conformational change in LFA1, converting its extracellular domain from the bent to the extended conformation. In addition to TCR signaling, this change can also be triggered by signaling through some chemokine receptors (GPCRs) or by P-selectin binding. However, TCR signaling likely also contributes to LFA1 function by mechanisms other than the signal-induced conformational change in the LFA1 extracellular domain. Vesicular trafficking of LFA1 to the plasma membrane is dependent on the small GTPase RAP1. RAP1 is activated by a family of GEFs, one of which, CALDAG-GEF1, is regulated by phospholipase C (PLC), a lipase central in TCR signaling that cleaves plasma membrane-associated phospholipid PIP_2 into diacylglycerol (DAG) and inositol triphosphate (IP3) (Ghandour et al., 2007; Katagiri et al., 2004). PLCγ is the target of ITK, suggesting that ITK could play a role in this process. Indeed, *Itk*$^{-/-}$ T cells are defective in LFA1-mediated adhesion and T_H1 and T_H17 cells treated with a small molecule ITK inhibitor (PRN694) have impaired P-selectin binding compared to control T cells. (Cho et al., 2015;

Finkelstein et al., 2005; Zhong et al., 2015). Upstream TCR signaling molecules Lck, ZAP70, and SLP-76 are also associated with integrin signaling (Baker et al., 2009; Nika et al., 2010).

Using the OT-I TCR transgenic mouse line, in which $CD8^+$ T cells express a TCR specific for the chicken ovalbumin peptide SIINFEKL, we investigated the role of TCR signal strength on adhesion and migration molecule expression, and on autoreactive T cell tissue infiltration. To vary TCR signal strength, we took advantage of the altered peptide ligands (APLs) identified for the OT-I TCR (Zehn et al., 2009). Additionally, we addressed the contribution of ITK signaling to the optimal expression of adhesion molecules and T cell migration *in vivo*. We found that, similar to our previous observations with $CD4^+$ T cells, $CD8^+$ T cells depend on ITK activity for robust selectin binding and autoreactive T cell migration. We also found that high expression of multiple receptors contributing to TEM, including modified PSGL1, CD44, and CD226 require CD28 costimulation along with strong TCR signaling, indicating that TCR and CD28 signaling pathways cooperate to induce maximal expression of these genes.

Results

Reduced TCR signaling inhibits selectin ligand expression and function in CD8$^+$ T cells

In the first 24-48h after TCR stimulation, CD8$^+$ T cells upregulate a variety of proteins that play key roles in T cell trafficking. One factor, CD44, is increased and remains stable. Another factor, PSGL1, is post-translationally modified with carbohydrate molecules to increase its ability to bind P-selectin. Here, we used the TCR transgenic model OT-I to study CD8$^+$ T cell activation in response to different peptide ligands that stimulate this TCR with differing potencies. The cognate peptide for the OT-I TCR is derived from chicken Ovalbumin, with the amino acid sequence of SIINFEKL (N4) (Hogquist et al., 1994). By substituting amino acids at the fourth position, TCR affinity can be decreased without affecting MHC Class I binding (Zehn et al., 2009). The SIITFEKL (T4) peptide is approximately 100-fold less potent, and SIIGFEKL (G4) is approximately 1000-fold less potent, than N4 (Rosette et al., 2001; Zehn et al., 2009)

Initially, we performed a simple experiment in which each of the three peptides were added to bulk splenocytes, acting as antigen presenting cells (APCs) then combined with CD8$^+$ T cells purified from the spleens of OT-I TCR transgenic x *Rag2*$^{-/-}$ (hereafter referred to as OT-I) mice. The T cells were stimulated for 48h and analyzed for P-selectin binding. Because PSGL1 modification, not expression, is central to its function, we used a purified form of P-selectin, which is fused to the Fc region of human IgG1, allowing for detection with a fluorescently conjugated secondary antibody. After 48h, the cells were stained with the recombinant protein and assessed by flow cytometry as an assay for PSGL1 binding. At doses of peptide capable of inducing maximum

CD69 expression (Figure 4.1A, left panel), all three peptides bound P-selectin, with their histograms greater than observed in naïve cells. However, the T4 stimulated cells had a large subpopulation, which bound more P-selectin (Figure 4.1A, right panel). This subpopulation likely expresses the glycosylated form of PSGL1 and was present to a lesser extent in G4 and N4 stimulated cells. It is unclear how kinetically stable this population is and if the high-affinity N4 stimulation condition yields the same population earlier in the response that degrades over time.

We next examined if this high affinity P-selectin population was sensitive to ITK inhibition by treating the T4 stimulated cells simultaneously with the small molecule inhibitor of ITK, PRN694 (Zhong et al., 2015). Reducing TCR signal strength with ITK inhibition blocked the generation of the high P-selectin binding T cells. (Figure 4.1B) This suggested that the modified PSGL1 ligand was dependent on the strength of TCR signaling.

To determine if the alternate ligand, CD44, for P-selectin was also sensitive to changes in TCR signal strength, we stimulated OT-I T cells with

Figure 4.1. Medium affinity TCR stimulation generates high P-selectin binding and CD44^{hi} T cells, a subpopulation dependent on ITK activity.

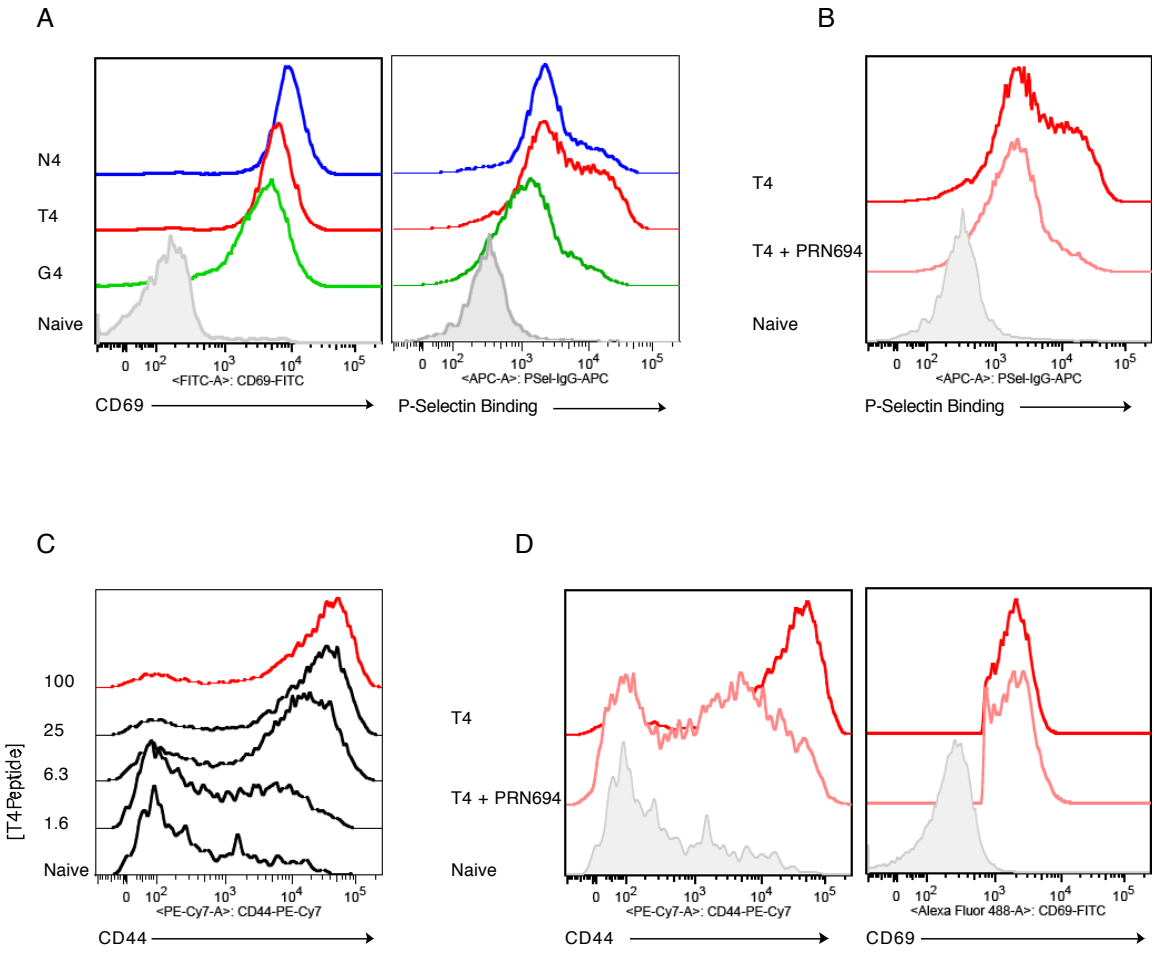


Figure 4.1. Medium affinity TCR stimulation generates high P-selectin binding and CD44^{hi} T cells, a subpopulation dependent on ITK activity.

(**A**) Representative histogram plots of CD69 staining (left) and P-selectin binding (right) on OT-I cells stimulated with 1nM N4, 100nM T4, and 1μM G4 peptide for 48h. Cells were stained with recombinant P-selectin-human IgG fusion protein as an assay for PSGL1 binding.

(**B**) Representative histogram plots of P-selectin binding in OT-I cells stimulated with T4 peptide with and without 100nM PRN694 for 48h.

(**C**) Representative histogram plots of CD44 staining in OT-I cells stimulated with different doses of T4 peptide for 48h.

(**D**) Representative histogram plots of CD44 (left) and CD69 (right) staining in OT-I cells stimulated with T4 peptide with and without 100nM PRN694 for 48h. Cells were gated on live CD8$^+$ TCRβ$^+$. Data are representative of two to three experiments.

multiple concentrations of the T4 peptide. Consistent with CD69 upregulation, CD44 was also increased in the T cells after 48h. (Figure 4.1C) CD44 expression was also sensitive to ITK inhibition, with a decrease in MFI observed in the PRN694 treated cells. (Figure 4.1D, left panel) While there seems to be a bimodal response with $CD44^-$ cells transitioning to $CD44^+$ cells with increasing antigen dose, the ITK inhibited cells show a unique effect where a portion of the population does not turn on CD44 while the rest expresses lower amounts with higher variability among the $CD44^+$ population. However, these cells were all able to make CD69. (Figure 4.1D, right panel) This is consistent with the variability we observed in IRF4 expression in T cells treated with PRN694. (Chapter 3) We also tested the ITK-dependence on TIM-1 activation, another ligand for P-selectin expressed on T cells (Angiari et al., 2014). In $CD4^+$ T cells stimulated with αCD3/CD28 for 72h, PRN694 inhibited TIM-1 surface expression. Interestingly, TIM-1 surface expression was sensitive to calcium signaling, with a large increase in surface expression observed after Ionomycin treatment. (Hyoung-Soo Cho, unpublished) These data confirm our hypothesis that reduced TCR signaling, whether through lower affinity antigen or ITK inhibition, inhibits the T cells ability to adhere to the endothelium through P-selectin binding, the first critical step in TEM.

TCR and CD28 costimulation additively drive selectin binding and effector function in $CD8^+$ T cells

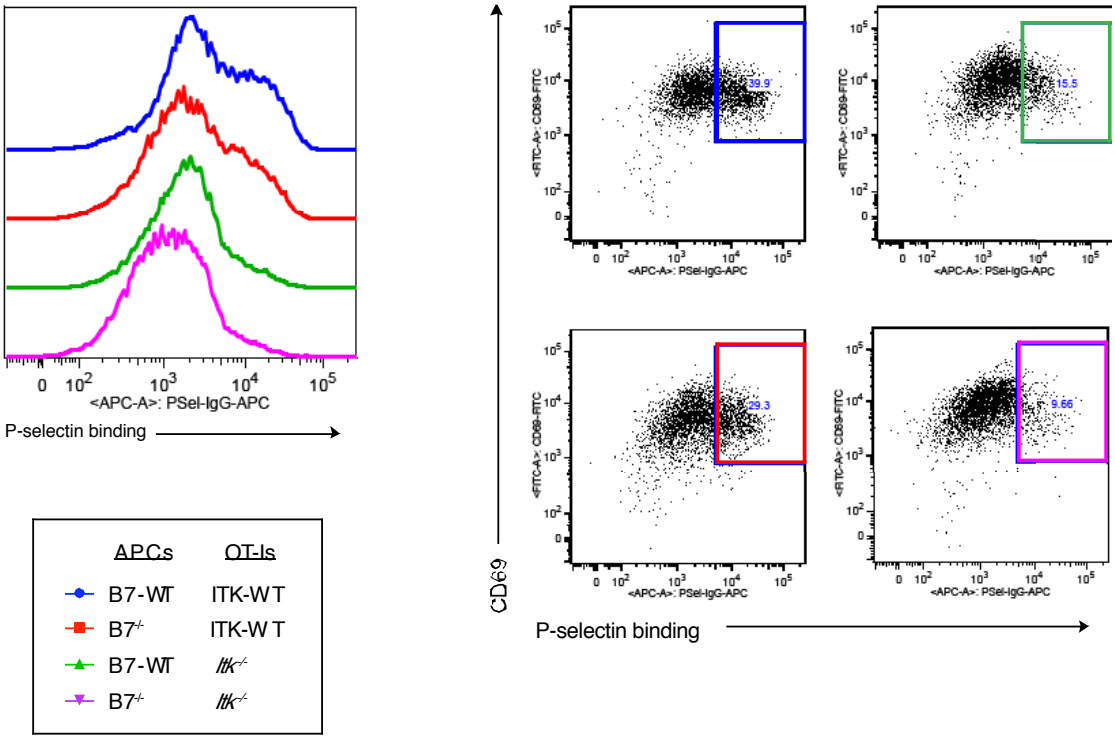
We next wanted to determine the contribution of CD28 costimulation on the TCR-mediated selectin binding regulation. To test this we used splenocytes isolated from *Cd80*$^{-/-}$*Cd86*$^{-/-}$ mice (hereafter referred to as $B7^{-/-}$) as APCs. We

were able to directly compare T cells with and without *Itk* stimulated with APCs with and without B7, the ligand for CD28. While minimal inhibition of the highly bound P-selectin T cells was observed with CD28 inhibition alone, a large defect in the *Itk*$^{-/-}$ T cells is seen, consistent with the PRN694 data. Reduction of both TCR and CD28 signaling further blocked this population, suggesting an additive effect of the signaling pathways on P-selectin binding. (Figure 4.2A, left panel) Similar to IRF4, this effect of P-selectin binding inhibition is independent of the cells ability to upregulate CD69. (Figure 4.2A, right panel) The additive effect was even more pronounced with CD44 expression. (Figure 4.2B) Consistent with PRN694 data, both a reduction in the cells ability to produce CD44 and the amount of protein made across the population is observed. This effect is even greater with both TCR and CD28 signaling reduced.

While IRF4 has been well characterized as a TCR-dependent mediator of CD8^{+} T cell effector function (Man et al., 2013; Nayar et al., 2015; 2014; Yao et al., 2013), it is unclear if CD28 costimulation plays a role in its rapid upregulation in stimulated CD8^{+} T cells. Using the B7$^{-/-}$ APCs, we were able to look at IRF4 expression as well as CD25, the high affinity Il-2 receptor, another factor sensitive to graded TCR signaling. (Figure 4.3) Here the OT-I T cells are stimulated with multiple doses of the three peptide antigens N4 (Figure 4.3A-B),

Figure 4.2. Maximum P-selectin binding and CD44^{hi} cells require both high TCR and CD28 signaling in activated CD8^{+} T cells.

A



B

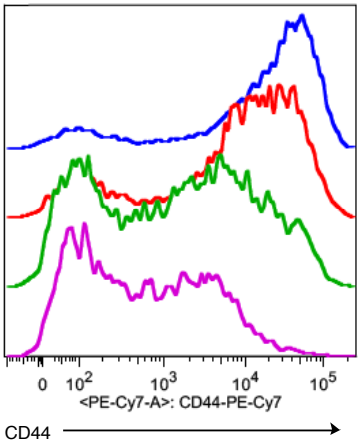


Figure 4.2. Maximum P-selectin binding and CD44^{hi} cells require both high TCR and CD28 signaling in activated CD8^{+} T cells.

(**A**) Representative histogram plots of P-selectin binding (left) on OT-I (WT and *Itk*$^{-/-}$) cells stimulated with 100nM T4 peptide-pulsed APCs (WT and B7$^{-/-}$) for 48h. Cells were stained with recombinant P-selectin-human IgG fusion protein as an assay for PSGL1 binding. Plots of CD69 versus P-selectin binding (right).
(**B**) Representative histogram plots of CD44 staining on OT-I (WT and *Itk*$^{-/-}$) cells stimulated with 100nM T4 peptide-pulsed APCs (WT and B7$^{-/-}$) for 48h.
Cells were gated on live CD8^{+} TCRβ^{+}. Data are representative of two to three experiments.

Figure 4.3. Maximum IRF4 and CD25 expression requires both high TCR and CD28 signaling in activated CD8$^+$ T cells.

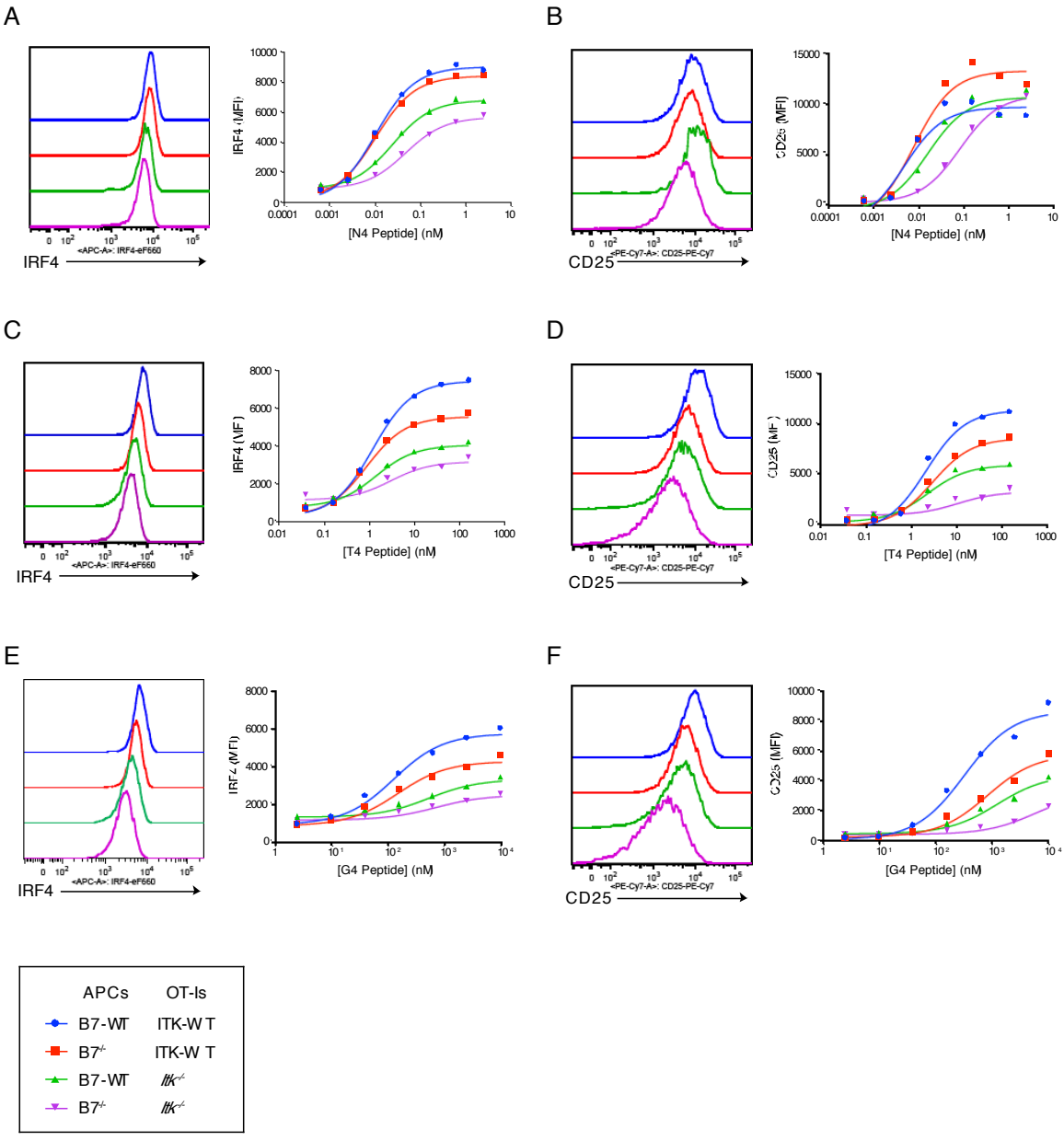


Figure 4.3. Maximum IRF4 and CD25 expression requires both high TCR and CD28 signaling in activated CD8$^+$ T cells.

(**A-F**) Representative histogram plots of IRF4 (left) and CD25 (right) on OT-I (WT and *Itk*$^{-/-}$) cells stimulated with peptide-pulsed APCs (WT and B7$^{-/-}$) for 24h. Cells were gated on live CD8$^+$ TCRβ$^+$. Plots of MFI values for all 4 conditions are shown for a dose response of each peptide. (**A, B**) N4 (**C, D**) T4 (**E, F**) G4.

Data are representative of two to three experiments.

T4 (Figure 4.3C-D), and G4 (Figure 4.3E-F). Consistent with the selectin binding data, we observed an additive effect with dual inhibition of ITK and CD28. With high affinity N4 stimulation, CD28 signaling was not required for maximum IRF4 expression. (Figure 4.3A, left panels) Consistent with our previous observations, it is lower affinity antigen that requires robust ITK activity. Here we also observed that this is true for CD28 signaling, with very little IRF4 and CD25 upregulation observed in the absence of ITK and CD28 function. (Figure 4.3C-F)

TCR and CD28 signaling drive expression of CD226 in activated CD8$^+$ T cells

We next examined if the costimulatory factor CD226 was also driven by the strength of TCR stimulation in a similar manner as CD44. The transmembrane glycoprotein is associated with T and NK cell cytotoxicity, however, the cognate ligands for CD226, poliovirus receptor (PVR, CD155) and Nectin-2 (CD112) are also expressed on the endothelium. The receptors are known to regulate monocyte extravasation (Reymond et al., 2004). CD226 is also known to associate with the integrin LFA1 and the tyrosine kinase Fyn in T cells suggesting a signaling role in TEM (Shibuya et al., 2003). We tested CD226 expression in OT-I T cells stimulated with multiple doses of the T4 peptide. (Figure 4.4A) Similar to CD69, a bimodal response was observed with cells transitioning from CD226$^-$ to CD226$^+$ at the concentration of antigen sufficient to activate the T cells. However, unlike CD69, CD226 is completely blocked by ITK inhibition, here with treatment of PRN694. (Figure 4.4B) CD226 upregulation is also dependent on CD28 costimulation with an additive inhibition observed with

Figure 4.4. Maximum CD226 expression requires both high TCR and CD28 signaling in activated CD8$^+$ T cells.

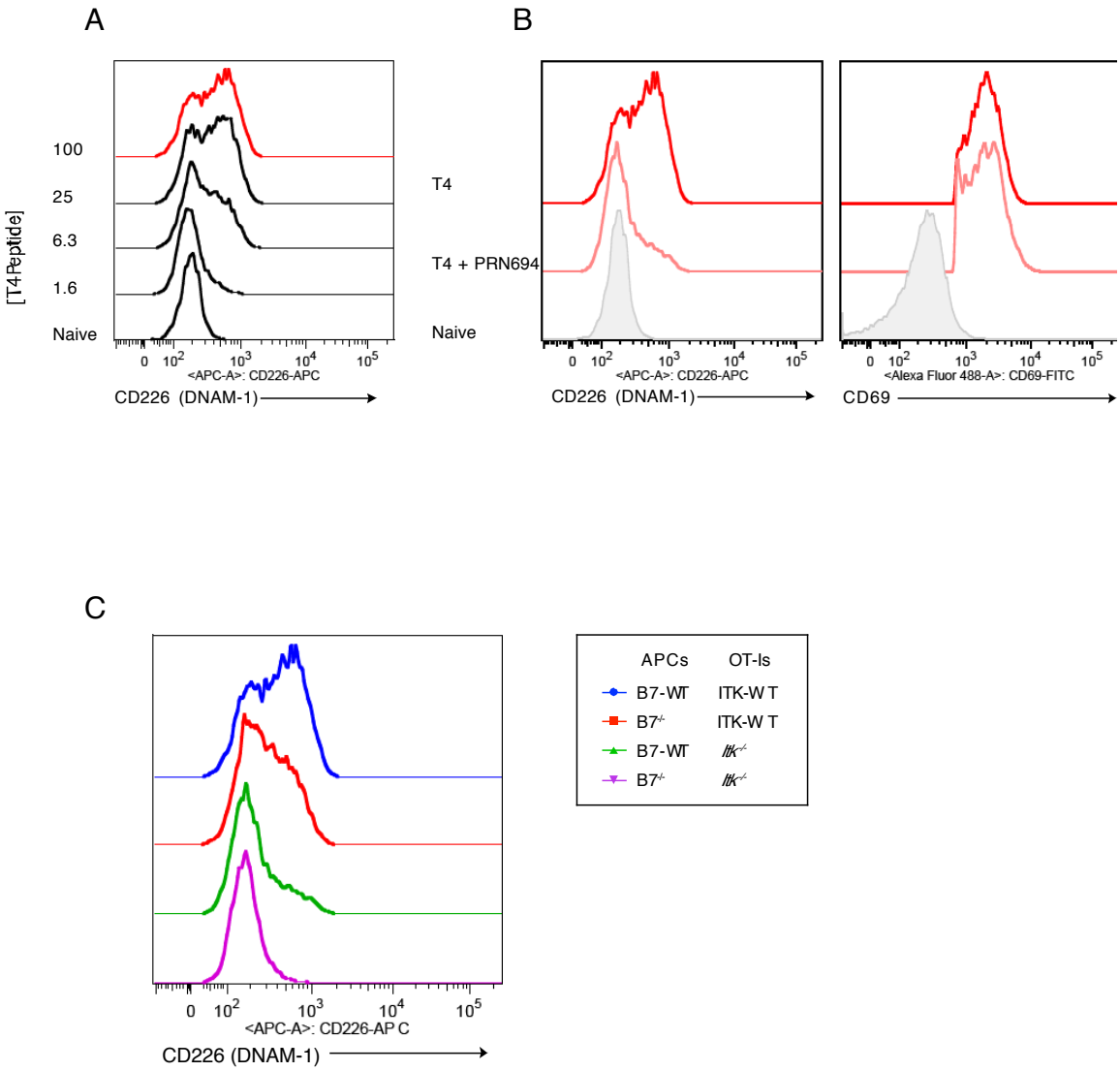


Figure 4.4. Maximum CD226 expression requires both high TCR and CD28 signaling in activated CD8$^+$ T cells.

(**A**) Representative histogram plots of CD226 staining in OT-I cells stimulated with different doses of T4 peptide for 48h.

(**B**) Representative histogram plots of CD226 (left) and CD69 (right) staining in OT-I cells stimulated with T4 peptide with and without 100nM PRN694 for 48h.

(**C**) Representative histogram plots of CD226 staining on OT-I (WT and *Itk*$^{-/-}$) cells stimulated with 100nM T4 peptide-pulsed APCs (WT and B7$^{-/-}$) for 48h.

Cells were gated on live CD8$^+$ TCRβ$^+$. Data are representative of two to three experiments.

stimulation of *Itk*-deficient OT-Is with APCs lacking B7. (Figure 4.4C) This data suggests that multiple inputs from the TCR and CD28 signaling pathways are likely contributing to the transcriptional regulation of pro-migratory molecules that are required for TEM: PSGL1, CD44, TIM-1, and CD226.

ITK inhibition partially blocks autoreactive CD8$^+$ T cell function in adoptive transfer model

We next wanted to test the ability of these CD8$^+$ T cells to migrate in vivo using a model system that required T cell extravasation to a site of antigen in the absence of an inflammatory environment. In the RIP-mOVA mouse, the rat insulin promoter (RIP) drives a transgene encoding membrane bound ovalbumin antigen (mOVA), restricting expression mainly to the β-islet cells of the pancreas (Blanas et al., 1996). Through adoptive transfer, OT-I T cells can recognize their cognate antigen in these islet cells and target them for CD8$^+$ T cell mediated cell death. The OT-I T cells can spontaneously lead to pathology, however, introduction of antigen in the lymphoid compartment either through immunization or infection rapidly accelerates the process to within 7 days (Behrens et al., 2004). The destruction of the β-islet cells rapidly leads to Type 1 diabetes (T1D) in the mice, which can be monitored by measuring glucose in the urine.

Here, we examined if *Itk*$^{-/-}$ T cells could migrate to the pancreas in the RIP-mOVA mice and induce T1D. King et al described a sharp affinity threshold in this model using multiple APLs of OT-I. The T4 peptide was at the threshold with ~25-50% of the mice getting T1D and the N4 and V4 (similar to G4) peptides having a response rate of 100 and 0% respectively. We hypothesized that the T4 affinity antigen would be sensitive to ITK activity since we knew that high affinity

TCR stimulation was largely independent of ITK. The low affinity ligands had no T1D phenotype and were not useful (King et al., 2012). To confirm that the WT and *Itk*$^{-/-}$ OT-Is were both activated after adoptive transfer, we labeled the T cells with CFSE and harvested the spleens 3 days after immunization with the T4 peptide. (Figure 4.5A-B) Both T cell populations in the spleen diluted the CFSE dye, indicating robust proliferation after immunization. We then determined the ability of WT and *Itk*$^{-/-}$ T cells to induce T1D in the mice after T4 immunization. In our hands, the T4 peptide at 50μg was consistently above activation threshold in WT OT-Is with all of the mice in the group getting T1D within 5 days, suggesting that the sharp threshold is likely sensitive to small changes in experimental conditions or environment. (Figure 4.5C) However, we did observe a threshold effect in the *Itk*$^{-/-}$ OT-I transfer with 50% of the mice getting T1D within 8 days. The other 50% did not succumb to disease out to 3 weeks. There was also a delay in T1D induction in the mice that did show pathology from 5 to 8 days. This was confirmed by histological staining of the pancreas at day 5 with mice receiving WT OT-I T cells showing islet cell destruction consistent with T1D, which was not observed in the mice receiving *Itk*$^{-/-}$ OT-Is. (Figure 4.5D) This delay in onset is consistent with the delay in signaling kinetics observed in vitro (Chapter 3) and also delay in CD8^{+} effector cell expansion in *Itk*$^{-/-}$ mice infected with LCMV(Atherly et al., 2006a).

Figure 4.5. ITK activity lowers the signaling threshold for autoreactive T cells to cause T1D in adoptive transfer model.

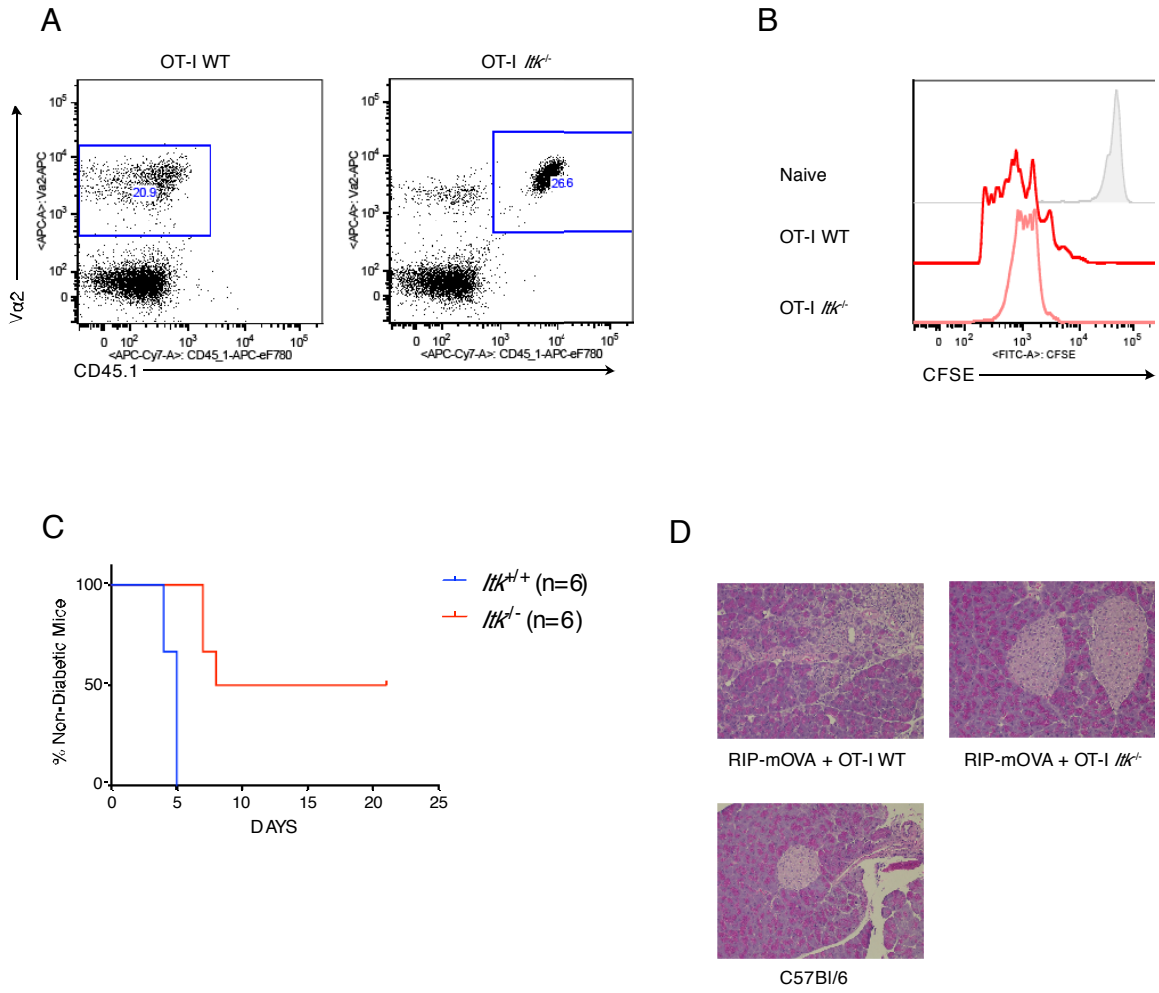


Figure 4.5. ITK activity lowers the signaling threshold for autoreactive T cells to cause T1D in adoptive transfer model.

(**A-B**) 5.0 x 10^6 OT-I T cells (WT and *Itk*$^{-/-}$ CD45.1$^+$) were labeled with CFSE and adoptively transferred into RIP-mOVA mice i.v., then immunized with 50μg of T4 peptide and 25μg of LPS i.p. 24h later. 3d after stimulation, spleens were harvested and stained for flow cytometry. (B) Representative histogram plots of CFSE dilution of WT and *Itk*$^{-/-}$ OT-Is.

(**C**) 5.0 x 10^6 OT-I T cells (WT and *Itk*$^{-/-}$ CD45.1$^+$) were adoptively transferred into RIP-mOVA mice i.v., then immunized with 50μg of T4 peptide and 25μg of LPS i.p. 24h later. Mice were monitored daily for urine glucose levels (>1000 mg/dL was considered diabetic) out to 3 weeks. N=6 per group of mice.

(**D**) Histology of pancreas tissue from RIP-mOVA mice adoptively transferred with OT-I cells (same protocol as in (**C**)) harvested at day 5 compared with a WT mouse for H&E staining. 20X images.

Data are representative of two to three experiments.

Discussion

The mechanisms controlling autoreactive T cell extravasation, through transendothelial migration, are not completely understood. Here, we use a combination of assays to look at changes in selectin binding in single activated $CD8^+$ T cells. Consistent with results previously seen in $CD4^+$ T cells (Cho et al., 2015), reduced TCR signaling through ITK inhibition resulted in impaired selectin binding. We hypothesize that this is due to blocking glycosylation of PSGL1, likely sensitive to changes in metabolism, a process regulated by the ITK-dependent transcription factor IRF4 in early activated $CD8^+$ T cells (Man et al., 2013). However, other possible selectin ligands, CD44 and CD226 were also sensitive to the strength of TCR signaling. CD44 expression was unique in that it had both a binary and graded response to antigen dose and ITK activity. With antigen dose, cells transitioned from $CD44^-$ to $CD44^+$. However with PRN694 treatment, the cells formed two populations: $CD44^-$ and $CD44^{lo}$. The cells that were able to produce the glycoprotein made considerably less with higher variability in expression observed among the cells. The reduction in maximum expression and increased variability is consistent with our observations of ITK-mediated IRF4 and CD25 expression.

With the striking migratory defect observed in *Ctla4*$^{-/-}$*Itk*$^{-/-}$ mice (Jain et al., 2013), we next examined if CD28 costimulation contributed to this defect in selectin binding. Using bulk splenocytes from *Cd80*$^{-/-}$*Cd86*$^{-/-}$ ($B7^{-/-}$) mice as antigen presenting cells (APCs), we found that selectin binding depended on both TCR signaling and CD28 costimulation for maximum effect. This also

correlated with expression of CD44 and CD226. We also found that genes sensitive to changes in TCR affinity and ITK activity, IRF4 and CD25, were diminished in the absence of CD28. This isn't entirely surprising due to the codependence of both signaling pathways, specifically through PI3K activation ensuring PIP_3-mediated recruitment of the LAT signaling complex to the plasma membrane (Esensten et al., 2016). These results suggest that there is likely an overall amplification of signaling that preferentially modulates genes that are sensitive to changes in TCR affinity. We hypothesize that the kinetics of gene upregulation are modulated by CD28 in a similar fashion as the effect seen on IRF4 through ITK inhibition.

We next turned to a mouse model of autoreactive $CD8^+$ T cell trafficking to test the effect of low TCR signaling on TEM in vivo. Using the RIP-mOVA adoptive transfer model, we compared mice that were introduced with OT-I T cells with and without ITK. After immunization with the low affinity T4 peptide, we found that mice receiving OT-I *Itk*$^{-/-}$ T cells were 50% less susceptible to T1D. The mice also had a delay in T1D induction from 5 to 8 days. The variation of T1D in the OT-I *Itk*$^{-/-}$ group is consistent with a sharp TCR signaling threshold previously observed in this model (King et al., 2012). We hypothesize that the strength of stimulation of the T4 peptide is at this threshold and genes controlling TEM are more sensitive to ITK activity at this dose and affinity of antigen. We predict that high affinity PSGL1 is dependent on this strength of signaling and without proper post-translational modification is unable to effectively bind to the endothelial cell wall and start the process of extravasation. This sensitivity is

likely only observed in the context of autoreactive T cells without an inflammatory microenvironment to drive TEM by antigen-independent means. Further research is needed to determine the subset of genes that could be controlling autoreactive T cell trafficking. Identifying all of the key players involved would further the development of treatments in autoimmune diseases like multiple sclerosis, a pathology mediated by autoreactive T cell infiltration into tissues.

CHAPTER V: DISCUSSION

Unraveling the complexities of TCR signaling mechanisms and how they drive T cell function is an area of considerable research in immunology. Highly dynamic T cell functions: clonal expansion, memory formation, metabolic reprogramming, and migration all depend on the quality of contact between the TCR and its cognate antigen. Here, we attempt to further understand how changes in TCR signaling effect the transcription of early response genes in $CD8^+$ T cells, focusing on CD69, Nur77, CD25, and IRF4.

We further explored the relationship between TCR signal strength and the Tec kinase ITK. A protein tyrosine kinase downstream of the TCR, ITK is unique in that it is not required for TCR signal transduction nor is it required for certain T cell functions. The *Itk*$^{-/-}$ mouse is able to mount an effective immune response to multiple pathogens including those that require a robust $CD8^+$ T cell response, LCMV and Vaccinia virus, although with slower kinetics of clonal expansion (Atherly et al., 2006a). However, the knockout T cells are not completely normal and are defective in early calcium influx. Soon researchers discovered defects in $CD4^+$ T cell function, specifically in T_H9 and T_H17 polarization and cytokine production (Gomez-Rodriguez et al., 2016; 2014). The transcription factor IRF4 was a gene discovered to be sensitive to ITK activity and its function then linked to $CD8^+$ T cell clonal expansion, with expression levels proportional to the magnitude of proliferation (Nayar et al., 2015; 2014; 2012). Here we describe a signaling mechanism connecting TCR signal strength and ITK activity to IRF4 expression, a signaling process that was previously unknown.

Using the OT-I TCR transgenic model, we were able to carefully titrate both the dose and affinity of antigen and determine that some genes (e.g. *Cd69* and *Nr4a1* (Nur77)) are mainly driven by antigen dose, while others, (e.g. *Irf4* and *Il2ra* (CD25)) are sensitive to both dose and affinity of the TCR:pMHC interaction. For example, cells stimulated with high doses of the low-affinity G4 peptide were able to compensate for CD69 and Nur77 but were unable to produce the same levels of IRF4 and CD25 that their higher affinity N4 and T4 clones could.

We then explored the contribution of ITK on TCR signal strength using OT-I *Itk*$^{-/-}$ mice and the small molecule inhibitor PRN694. Similar to the effects seen with low affinity TCR activation, ITK inhibition specifically blunted the ability of the T cells to make maximum amounts of IRF4 and CD25. Similarly, CD69 and Nur77 were largely unaffected by the loss of ITK. Using the small molecules PMA and Ionomycin, we identified the calcium signaling pathway was a possible mediator of maximum IRF4 expression, because of the linear relationship observed between IRF4 expression and Ionomycin concentration.

The calcium signaling pathway then lead us to focus on the calcium-sensitive transcription factor NFAT and its likely relationship to IRF4 induction. Using a nuclear isolation assay adapted for flow cytometry, we discovered that NFAT activation was bimodal with the percentage of T cells with NFAT in the nucleus proportional to both dose and affinity of antigen. The all-or-nothing response was also sensitive to changes in ITK activity. However, this binary response to signaling was not similar to CD69 and Nur77. The maximum

percentage of cells with activated NFAT was driven by peptide affinity not dose. The percentage of activated NFAT was also sensitive to ITK activity, consistent with IRF4 data. By examining activated NFAT after the first hour of APC:T cell contact, we discovered that antigen affinity was also regulating the kinetics of NFAT activation. Even larger doses of the T4 peptide could not alter the time of activation. These findings led us to hypothesize that this delay in NFAT activation could explain the defect seen in IRF4 expression.

To look at the early kinetics of IRF4 upregulation, we used a flow cytometry method of mRNA detection. An adaptation of fluorescence in situ hybridization techniques, these assays allowed us to examine *Irf4* and *Cd69* mRNA transcription in early activated T cells stimulated with peptide-pulsed APCs. We modulated signal strength by inhibiting ITK. Consistent with the NFAT data, we found that the early kinetics of *Irf4,* and not *Cd69*, mRNA upregulation was sensitive to TCR signal strength. These kinetic findings allowed us to develop a model of IRF4 regulation where a delay in mRNA upregulation is driven by a delay in NFAT signaling. This delay is proportional to both antigen affinity and ITK activity and places the lower affinity clonal population at a disadvantage for IRF4 production.

This handicap in IRF4 production is connected with the effector response of $CD8^+$ T cells to infection (Nayar et al., 2015; 2014). We also examined the effect of TCR signal strength in the context of autoimmunity, specifically in the context of autoreactive T cell migration. We found that TCR signaling and CD28 costimulation cooperated to regulate selectin binding, a critical early step in

transendothelial migration. This defect was mediated by ITK and correlated with the impaired ability of *Itk*$^{-/-}$ CD8^{+} T cells to lead to T cell-mediated pathology in an autoimmune adoptive transfer model. These data suggest that genes involved in migration, e.g. selectin binding, are also regulated by TCR signal strength. We posit that these genes are likely regulated by delayed mRNA kinetics in a similar manner as *Irf4*. In the following sections, we outline some of the caveats to these interpretations and propose additional experiments to address them. We suggest additional model systems to look at other T cell functions that could be modulated by these mechanisms.

New Insights into TCR Signal Strength and the CD8^{+} T Cell Transcriptome

Much of the research on graded TCR signaling regulation of the transcriptome has been done in CD4^{+} T cells. Antigen dose and affinity are known to have an effect on CD4^{+} T cell differentiation with dose linked to T_H1 over T_H2 polarization (Constant 1995, Hosken 1995). Two recent papers have used different genomics techniques to look at changes in CD4^{+} T cell transcription in response to different strength TCR stimulation. Iwata et al. used varying doses of αCD3 stimulation in T_H2 cells (Iwata et al., 2017). They were able to link BATF and IRF4 expression to antigen dose and then, performing BATF ChIP-Seq experiments, characterized distinct subsets of cells that were dependent on BATF-IRF4 and sensitive to the strength of TCR stimulation, expanding on previous work linking IRF4 and BATF to T cell transcription regulation (Kurachi et al., 2014). Going further, the authors then mapped enhancer regions of the genome that had varied occupancy of the BATF-IRF4

complex, discovering a new motif, AICE2, which was only bound in cells stimulated with high affinity antigen. This work suggested that graded IRF4 expression levels in T cells could drive T cell function through altered binding dynamics to different enhancer regions, implying that enhancer regions with lower affinity for the BATF-IRF4 complex require maximum expression of the transcription factor for occupancy.

Allison et al looked in AND CD4 TCR transgenic T cells stimulated with different doses and affinities of cognate peptides (Allison et al., 2016). They were then able to map areas of the genome that were sensitive to antigen dose and affinity, specifically areas that were enriched in histone acetylation, by performing H3K27Ac ChIP-Seq experiments. Consistent with our findings that *Irf4* and *Il2ra* are highly sensitive to TCR signal strength, they found these genes highly enriched in enhancer elements. These regions also bound AP-1 and NF-κB further linking these pathways to the early response genes (Allison et al., 2016).

Comprehensive genomic studies of $CD8^+$ T cells involving graded TCR stimulation have recently been published (Richard et al., 2018). Here, Richard et al use the cutting-edge techniques of single-cell RNA sequencing (scRNA-seq) and mass cytometry (Using the CyTOF system) to look at changes in the transcriptome as well as proteome in activated OT-I T cells stimulated with different APLs. Using these single cell techniques, the authors added the dimension of time to their experiments and determined that graded TCR signaling drove the rate at which each cell initiated transcriptional activation, consistent with our observations with delayed NFAT and *Irf4* mRNA activation.

However, this delay did not effect the cytolytic capacity of the activated CD8 T cells (Richard et al., 2018). We speculate that there could be a differential effect of genes acting on the fitness of the cell itself and those driving the fitness of the clonal population.

These genomic studies have expanded our knowledge of TCR signaling dynamics and its modulation of transcription in T cells, further solidifying the role of IRF4 as an early response gene that is sensitive to changes in signal strength. We hypothesize that there are genes like *Cd69* and *Grzb* that are not dependent on the kinetics of transcriptional activation while other genes like *Irf4* and *Il2ra* may be more effected by the delay and in turn drive the diminished clonal expansion seen in vivo with low TCR stimulation. Using techniques used in these studies, it would be interesting to see which genes are sensitive to ITK inhibition, likely the genes most dependent on strong TCR stimulation. These techniques can also help to tease apart the contributions of transcriptional activation kinetics on the single T cell and the clonal population of T cells, identifying which genes are effected by the heterogeneity of signaling dynamics. This could then answer key questions regarding the transcriptional control of memory and effector differentiation in T cells, one model, which posits that TCR signal strength is the main driver of cells into the TEC pool. (Daniels and Teixeiro, 2015; Kaech and Cui, 2012) We hypothesize that there is a subset of migration genes that are also sensitive to TCR signal strength. Identification of this gene subset could be further researched using these genomics methods, specifically in the context of autoreactive T cells.

Looking at Graded TCR Signaling Dynamics In-Vivo

Looking at signaling dynamics in vivo presents a variety of challenges due to the rapid dynamics of TCR signaling. While reporter mice like Nur77-GFP have proven quite useful, measuring the kinetics of activation can be difficult because of the stability of GFP. T cells that express the reporter have been activated through their TCR, however it is impossible to know how long the signaling event lasted, only that it happened. Researchers have recently described a new technology of reporter that can add this extra dimension of kinetic analysis. This new tool, a timer of cell kinetics and activity, or Tocky (Toki means time in Japanese) uses a unique genetically encoded fluorophore that changes fluorescence as the protein degrades (Subach et al., 2009). Briefly, these researchers identified mutants of mCherry that had different stability dynamics. One mutant first encodes a fluorophore molecule that emits fluorescence in the blue spectrum. Then, after ~7h, the protein conformation changed facilitating an oxidation reaction and double bond formation at the fluorescent site and the fluorophore then emitted in the red spectrum, now with increased stability and a decay half life of ~20h (Subach et al., 2009). In short, this reporter can identify cells that recently turned on the Tocky gene ($Blue^+$), those that turned it on awhile ago (Red^+), and those that are continuously transcribing the gene ($Blue^+$ Red^+).

Bending et al did some elegant work using this technology to test new hypotheses in T cell biology (Bending et al., 2018). They made a reporter mouse by adding the *Nr4a3* gene promoter to the Tocky construct and looked at T cell

activation in vivo. *Nr4a3* is a relative of Nur77 and like its counterpart, is rapidly upregulated after TCR stimulation. Using *Nr4a3*-Tocky in OT-II cells as a proof of concept, they were able to capture the kinetics of the reporter with cells transitioning from blue to blue/red. Then with addition of anti-MHC II antibody to stop TCR signaling, the cells went from blue/red to just red. They then looked in thymic Treg precursors in these *Nr4a3*-Tocky mice at reporter expression in 7d neonates. Specifically, CD4SP thymocytes were gated on $FoxP3^+$ and $CD25^+$ expression. The $FoxP3^-CD25^+$ population was blue and the $FoxP3^+CD25^+$ population blue/red. The $FoxP3^+CD25^-$ population had low reporter expression. This suggested that newly activated thymocytes turned on CD25 first and then persistent signaling lead to activation of FoxP3, with the $FoxP3^+CD25^-$ population likely developing from weak TCR stimulation (Bending et al., 2018).

These reporter mice can be used to study the kinetics of graded TCR signaling ex-vivo and in-vivo. It is unclear how long TCR signaling persists in the context of low to medium affinity interactions and how ITK activity controls this persistence. By looking at the *Nr4a3*-Tocky reporter in OT-I T cells under various signaling conditions, we can test the hypothesis that ITK is critical in maintaining durability of signal. The LmOVA strains can be used to test this time dependence in-vivo. Generation of an *Irf4*-Tocky mouse would be interesting to bridge the research contained in Chapter 3 with these new questions.

A unique genetically encoded calcium biosensor has also been reported in T cells (Le Borgne et al., 2016). Using the mCameleon construct, a FRET based reporter that uses CFP-Calmodulin and YFP-M13 peptide, which binds itself

because of the calcium-dependent interaction between the protein and peptide, the authors looked at antigen avidity and calcium flux in single cells. They generated knockin mice with a Tet-inducible construct. They then tested it out in multiple TCR transgenic mice including OT-I. By looking at early naïve T cell interactions with APCs, they were able to measure the initial wave of calcium flux and characterized the amplitude and oscillation patterns. T cells were grouped by transient, sustained, and oscillating patterns. Interestingly, if you dilute the N4 peptide, the majority of the responding T cells have a sustained calcium flux. Only the percentage of responding cells is diminished. When the dose is held constant and the affinity is changed by adding the Q4 and V4 APLs, the ratio of responders is the same. However, the number of cells with oscillating calcium flux is slightly increased. These experiments point to the all-or-nothing response of calcium flux triggering as sensitive to antigen dose, while the oscillation patterns are controlled by the affinity of the TCR:pMHC. Our hypothesis would be that graded TCR signaling and ITK activity would drive this shift from oscillation to sustained pattern. It is still unclear if sustained calcium oscillation drives nuclear NFAT activation. Using this reporter, you could test this by finding the dose of antigen, which gave predominately-oscillating cells, likely a low dose of low affinity peptide, and test the cells for nuclear NFAT. These oscillation patterns can then be linked to IRF4 upregulation by measuring *Irf4* mRNA by flow.

Graded TCR Signaling and TCR Repertoire Diversity

This work has focused on defining the signaling mechanism connecting graded TCR signaling, IRF4 expression, and $CD8^+$ T effector cell expansion. We could use these methods to examine other aspects of T cell biology. One major hypothesis that has been under considerable investigation is that the strength of TCR signaling could be controlling the breadth of the TCR repertoire during infection, with high affinity interactions ensuring the greatest number of clones that survive and broadest spectrum of TCR sequences. Having a more diverse TCR repertoire, specifically in the memory pool, ensures that the organism can mount a robust recall response to reinfection with the same pathogen and possible mutant variants. This is usually best illustrated in the context of Influenza A infection where humans are yearly exposed to different variations of the virus. Indeed, the frequency of flu-specific $CD8^+$ T cells was found to be protective against H1N1 during the 2009 pandemic, emphasizing the importance of $CD8^+$ T cell memory in flu vaccination strategies (Sridhar et al., 2013).

While there is evidence that the avidity of the TCR for antigen drives clonal diverisity in $CD4^+$ and $CD8^+$ T cells in multiple infection models (Busch and Pamer, 1999; Cukalac et al., 2014; McHeyzer-Williams et al., 1999; Price et al., 2005), it remains unclear if ITK activity and IRF4 expression are driving repertoire diversity. This could be tested in WT, $Itk^{-/-}$, $Irf4^{+/fl}$, and $Irf4^{fl/fl}$ T cells. Different strategies could be used to look at repertoire diversity post-infection. In the *Listeria monocytogenes* model system you could use the APL variants (LmOVA) to look at how changes in TCR binding can alter clonal diversity and use the

variants to look at secondary reponses in the context of heterologous challenge (N4, T4, and V4) (Zehn et al., 2009).

To look at small changes in TCR clonal diversity, WT, *Itk*$^{-/-}$, *Irf4*$^{+/fl}$, and *Irf4*$^{fl/fl}$ mice could be crossed onto a transgenic line (e.g. Vβ5) that holds constant the beta chain, narrowing the repertoire and allowing sequencing of the CDR3 region of the alpha chain (Stadinski et al., 2011). Mice could be infected with LmOVA (N4, T4, and V4) strains followed by isolation of splenic T cells at the peak effector (D8) and memory (D45) time points. Then sorting of H2-K^{b}-SIINFEKL (N4, T4, or V4) specific CD8^{+} T cells followed by single-cell deep sequencing of the TCRα CDR3 gene should measure changes in diversity when comparing WT, *Itk*$^{-/-}$, *Irf4*$^{+/fl}$, and *Irf4*$^{fl/fl}$ T cells. One caveat for the *Itk*$^{-/-}$ mice is the germline knockout, a Cre-inducible system would be preferred, allowing the T cells to exit the thymus normally without the skewed innate cell phenotype (Atherly et al., 2006b). The hypothesis would be that without strong TCR signaling (*Itk*$^{-/-}$) and robust effector function (*Irf4*$^{+/fl}$ and *Irf4*$^{fl/fl}$), the pathogen-specific TCR repertoire would be narrower with only high affinity clones making up the effector and memory pools.

NFAT-Independent Regulation of IRF4 and NF-κB Signaling

While NFAT is likely playing a critical role in IRF4 regulation in CD8^{+} T cells, it is not likely the only transcription factor that is driving early expression. The PMA dose response experiment (Figure 3.4) suggests a calcium independent factor that could be playing a role, possibly NF-κB. NF-κB is one of the three main signaling pathways downstream of the TCR that regulate gene

expression in early-activated T cells. Like other signaling pathways, co-stimulation also plays a critical role with CD28 signaling driving PI3K activation, which recruits phosphoinositide-dependent kinase (PDK1) and AKT. Both PDK1 and DAG can activate protein kinase C (PKCθ), the kinase that sets the NF-κB signaling pathway in motion after TCR stimulation (Lee et al., 2005; Park et al., 2009).

Briefly, the signaling cascade starts with PKCθ phosphorylating the CARD domain of CARMA1 causing a conformational change, allowing it to bind to BCL10 and MALT1. This three-protein complex (CBM) then recruits other factors, TRAF6 and MIB2, which facilitate the polyubiquitination of BCL10 and MALT1. This polyubiquitination motif then recruits the IκB kinase (IKK) complex, allowing for the ubiquitination of IKKγ followed by the phosphorylation of IKKβ. This newly activated IKKβ then critically phosphorylates Inhibitor of κB (IκBα) the factor that sequesters the NF-κB heterodimers (p65 and p50) in the cytoplasm. After phosphorylation, IκBα is ubiquitinated and degraded, allowing for the release of NF-κB to translocate to the nucleus and bind DNA. (Paul and Schaefer, 2013)

While studies with αCD3 or TNFα stimulation of T cells suggest a digital regulation of the pathway, with the cell population shifting from p-IκBα negative to positive, clear data in the context of APC stimulated primary T cells is lacking (Kingeter et al., 2010; Tay et al., 2010). Further complicating the matter, there is much evidence that NF-κB regulation is also linked to calcium flux. Specifically, two calcium sensitive proteins, Calmodulin-dependent protein kinase (CaMKII) and the calcium dependent phosphatase Calcineurin, work to stabilize the

signaling cascade at the CBM complex with CaMKII phosphorylating CARMA and BCL10, and Calcineurin dephosphorylating other residues on BCL10, promoting complex stability and ubiquitination (Frischbutter et al., 2011; Ishiguro et al., 2007; Oruganti et al., 2011; Palkowitsch et al., 2011). The NF-κB family member c-Rel also binds to the *Irf4* promoter, however, c-Rel is not highly expressed in naïve T cells and is also sensitive to calcium levels (Antonsson et al., 2003; Grumont and Gerondakis, 2000), another connection between the two pathways. The crosstalk between calcium signaling and NF-κB activation create the possibility that both signaling pathways control IRF4 expression. (Figure 5.1)

We have some preliminary data suggesting an involvement of ITK in NF-κB pathway control. (Appendix I) In OT-I T cells treated with PRN694, there is a reduction in BclXL expression, a pro-survival protein important for the clonal expansion of TECs. BclXL is known to be driven by p65 (C. Chen et al., 2000). We also looked at *Irf4* mRNA expression in OT-I T cells treated with a small molecule inhibitor of NF-κB (BAY 11-7082) (Pierce et al., 1997). With pathway inhibition, there was also a delay in *Irf4* mRNA kinetics, while not as potent as FK506. More studies are needed to explore the relationship between graded TCR signaling and NF-κB activation. Using the nuclear and RNA flow assays, the activation of nuclear p65 and upregulation of *Bcl2l1* mRNA can be determined. These experiments would greatly add to our understanding of how the NF-κB pathway responds to graded TCR signaling in the context of TCR:pMHC stimulation.

Figure 5.1: Proposed model for NFAT and NF-κB dual control of early *Irf4* expression downstream of calcium signaling in activated T cells.

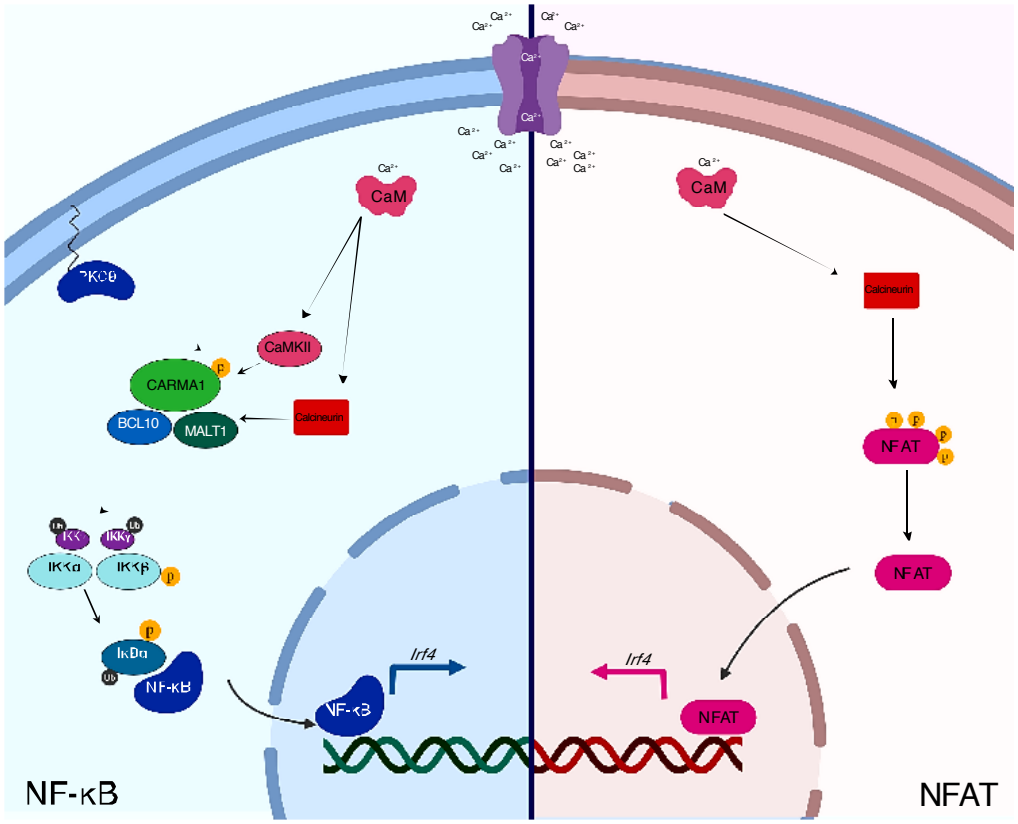


Figure 5.1: Proposed model for NFAT and NF-κB dual control of early *Irf4* expression downstream of calcium signaling in activated T cells.

The rapid rise in intracellular calcium ion concentration activates the calcium sensitive protein Calmodulin, which can then activate the phosphatase Calcineurin as well as the kinase CaMKII. Calcineurin removes the inhibitory phosphorylation groups on NFAT in the cytoplasm allowing the transcription factor to translocate into the nucleus. Calcineurin and CaMKII can also both stabilize the CARMA1-BCL10-MALT1 complex, which is critical for NF-κB signaling. We propose that since both transcription factors are sensitive to calcium concentration that they both could be playing a role in IRF4 regulation.

Using ITK Mutants to Understand ITK Activation and Delay of Signaling

While its biochemical function as a kinase has been known for some time (Berg et al., 2005), the roles of ITK's multiple subdomains on its activation state and function have recently been examined more closely (Andreotti et al., 2018). The Tec family of kinases have not been crystallized in their full-length form. Researchers have instead crystallized different subdomains and have used homology models between Lck, BTK, and ITK to gain better insight into the structural basis for activation.

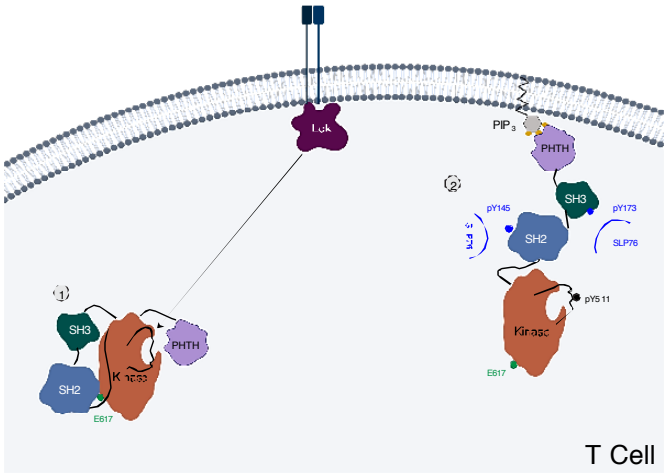
ITK consists of 4 main protein regions: the pleckstrin homology-Tec homology (PHTH), SH3, SH2, and kinase domains. Insights into Lck structure have lead to a proposed autoinhibited conformation of ITK (Joseph et al., 2013; 2007). In Lck, a tyrosine in the C-terminus (Y505) binds to its own SH2 domain, folding the protein together and masking the kinase domain activation loop. While ITK lacks this residue in its C-terminus, there is an acidic residue (E617) at the end of the kinase domain that can electrostatically interact with the basic residue (R265) in the SH2 domain, with mutants showing kinase activation (Joseph et al., 2017). This interaction is weaker than the Y505 interaction in Lck suggesting that the autoinhibited form of ITK could more easily unravel with SH2 binding to other proteins. ITK is additionally kept in its inhibited folded state through interactions between the PHTH and kinase domains (Devkota et al., 2017; Joseph et al., 2017). This inhibited state of ITK has more than one possible conformation with the weak inhibitory interactions and SH3 kinase linker binding to the PRR region promoting a less closed structure (Hao and August, 2002). (Figure 5.2A)

Multiple biochemical steps are required for the conformational change of ITK from its “closed” autoinhibited form and “open” activated form. One major interaction that is promoted by TCR and costimulatory signaling is the binding of the PHTH domain to PIP_3 at the plasma membrane (Yang et al., 2001). The phospholipid PIP_3 is the product of PI3K activity and is rapidly increased in concentration following T cell activation. Binding of the PHTH domain to PIP_3 is crucial for two main reasons: it relieves the inhibitory interactions between it and the kinase domain and it colocalizes ITK to the plasma membrane, placing it in proximity to the LAT/SLP76 signaling complex (Devkota et al., 2017; Joseph et al., 2017). The SH3 domain can bind to SLP-76, anchoring ITK to the signaling complex and promoting a more open state (Bunnell et al., 2000; 1996). The SH2 domain engages with the Y145 residue on SLP-76, releasing the autoinhibitory SH2-Kinase domain interaction. This then allows for autophosphorylation at Y180 when the SH3 domain interacts with the kinase active site. (Figure 5.2A)

Now that we know that ITK activity drives the kinetics of nuclear NFAT localization and *Irf4* mRNA upregulation, we can use these signaling readouts in T cells to test the functionality of these regulatory domains of ITK. These flow cytometry methods are excellent for single-cell and single molecule studies. By

Figure 5.2: Proposed model of ITK activation states and key residues to target in mutagenesis experiments to look at single-molecule dynamics of ITK in the activated T cell.

A



B

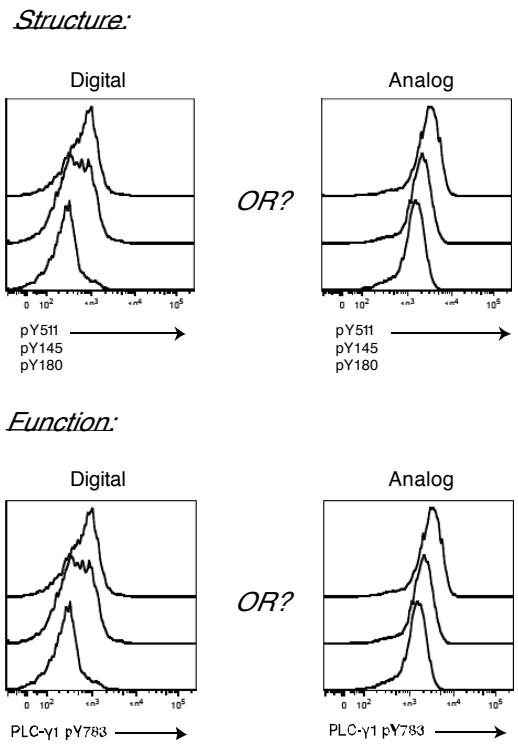


Figure 5.2: Proposed model of ITK activation states and key residues to target in mutagenesis experiments to look at single-molecule dynamics of ITK in the activated T cell.

(A) A model figure illustrating the autoinhibited conformation of ITK (on the left) and active conformation (on the right) as previously described (Andreotti et al., 2018). We propose experiments with mutants of the critical regulatory domain residues of ITK in primary T cells to study the molecular dynamics of the different conformation states in activated T cells, using single-cell methods. Key interacting residues are: E617 (Kinase domain) with R265 (SH2), Y180 (SH3) with SLP76, Y145 (SH2) with SLP76, K48 and R49 (PHTH) with Kinase domain, and KPLPPTP (Proline rich motif) with SH3.

(B) Using single-cell methods detecting phosphorylation sites of ITK and its target PLCγ, we propose examining the dynamics of ITK conformational change in activated T cells over time. We propose that each cell has varying pools of the different active states of ITK and that it is not an all-or-nothing response as seen with other kinases (PKD2) (Navarro et al., 2014). The histograms represent possible signaling outcomes.

creating mutants of the key residues that control ITK conformation and function, we can better understand the structure-function relationship. (Figure 5.2B) Using CRISPR technology to knockin these ITK mutants in primary T cells, we can answer a significant question regarding ITK biology: Does graded TCR signaling effect the fraction of ITK molecules in the open active conformation? Its possible that there are a range of conformation states present in the cell. Developing antibodies against these different phospho-tyrosine residues would also be critical for addressing these questions. Navarro et al used similar methods using mutants and phospho-flo to study protein kinase D2 (PKD2) in single T cells, where they determined that the entire pool of PKD2 inside a single cell was activated upon TCR stimulation (Navarro et al., 2014). One hypothesis is that ITK would not follow this binary model, rather the proportion of the pool of ITK molecules inside the cell would shift to greater numbers in the open states. Likely, this shift would be dependent on TCR signal strength since we know that ITK activity is required during weak TCR stimulation. This hypothesis could be addressed using mutants and phospho-flo in the OT-I TCR transgenic model.

This work can be further applied to study PLCγ activation. ITK phosphorylates PLCγ at Y783, however, the regulation of this process is highly complex and involves multiple protein-protein interactions at the LAT/SLP-76 signaling complex. (Figure 5.3) The C-terminal SH2 domain (SH2C) of PLCγ is normally bound to the SH3 domain, masking the Y783 residue. Multiple interactions between PLCγ, ITK, and SLP-76 facilitate the active state where ITK

Figure 5.3: Proposed model of PLC-γ1 activation states and key residues to target in mutagenesis experiments to look at single-molecule dynamics of PLC-γ1 in the activated T cell.

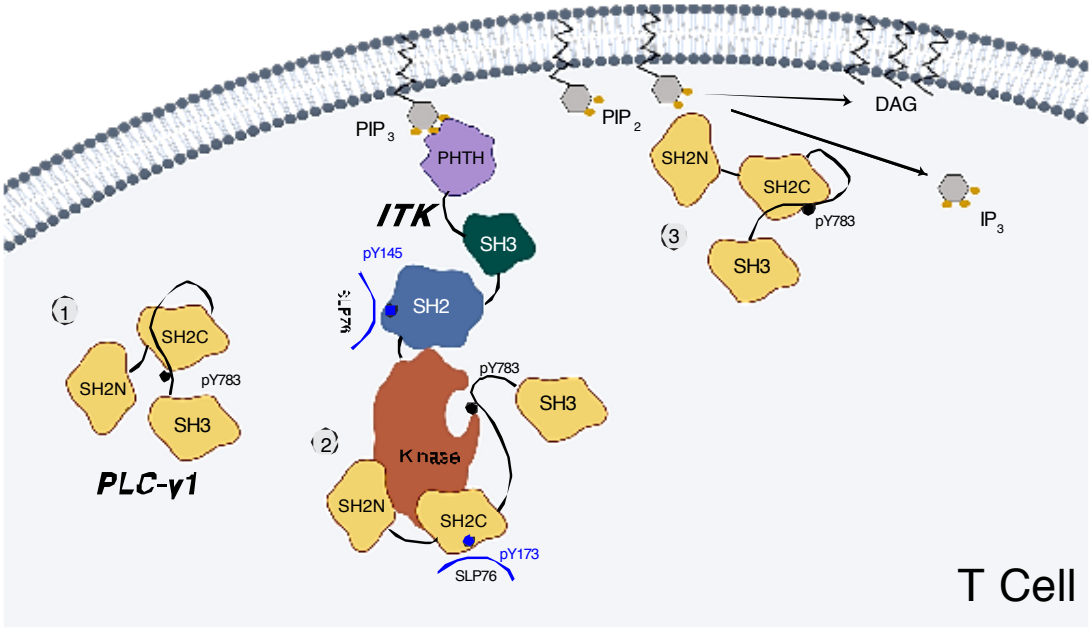


Figure 5.3: Proposed model of PLC-γ1 activation states and key residues to target in mutagenesis experiments to look at single-molecule dynamics of PLC-γ1 in the activated T cell.

A model figure illustrating the activation states of PLC-γ1 as previously described (Andreotti et al., 2018). We propose experiments with mutants of the critical regulatory domain residues of PLC-γ1 in primary T cells to study the molecular dynamics of the different conformation states in activated T cells, using single-cell methods. Key interacting residues are: Y173 (SLP76) with SH2C of PLC-γ1 and Y132 (LAT) with SH2N of PLC-γ1.

is able to phosphorylate Y783. The intramolecular pY783 and SH2C binding competes with the SLP-76 interaction with SH2C (Bunney et al., 2012; Devkota et al., 2015; Sela et al., 2011). (Figure 5.3) This can lead to the active enzyme falling off the LAT/SLP-76/ITK complex, creating room for another inactive molecule to take its place, amplifying the overall signal (Cruz-Orcutt et al., 2014; Devkota et al., 2015). It may be possible to use mutants of these different signaling molecules to study the effect of conformation states on TCR signal strength in primary T cells, using the OT-I model system and early NFAT activation or *Irf4* mRNA upregulation as single-cell readouts.

An additional level of proteasome-mediated PLCγ regulation was recently discovered. Cytokine-inducible SH2 containing protein (Cish), a member of the suppressor of cytokine signaling (SOCS) family, complexes with E3 ubiquitin ligase to target proteins for degradation. In T cells, Cish binds to PLCγ through its SH2 domain, facilitating PLCγ ubiquitination and degradation (Guittard et al., 2018; Palmer et al., 2015). It would be interesting to look at changes in Cish expression in the OT-I model and see if sustained signaling drives down expression, leading to a greater pool of PLCγ available for signaling.

Strategies to Determine the Mechanism of CD28/ITK Control of Migration

The insights that were learned in Chapter 4 are only a starting point for a more in-depth look at the coordination between CD28 and ITK leading to the regulation of TEM in T cells. Since there is good evidence that ITK controls P-selectin binding, one of the first steps would be to determine if this is done through PSGL-1 or TIM-1. T cells from mice deficient in PSGL1 (*Selplg*$^{-/-}$) or Tim-1 (*Tim-1*$^{\Delta mucin}$)

could be treated with PRN694 and tested for P-selectin binding. These mice could also be crossed to *Itk*$^{-/-}$. These T cells deficient in PSGL-1 or Tim-1 can also be tested in-vivo using the 2-photon imaging technique used on precision cut lung slices (PCLS). (Jain et al., 2013) ITK regulation of these genes could also be tested in OT-I T cells activated with peptide and treated with PRN694 then stained for *Selplg* and *Tim1* by RNA-flo. The kinetics of mRNA upregulation will be critical to test because that is the major defect observed with *Irf4*. Since ITK is more required in low affinity TCR stimulation, these experiments will have to be done with the T4 and G4 peptides.

The RIP-mOVA / OT-I transfer model is also useful for testing a lot of these hypotheses. First, the RIP-mOVA mice can be crossed to B7$^{-/-}$ (*Cd80*$^{-/-}$ *Cd86*$^{-/-}$) in order to confirm the CD28 dependence in this autoreactive T cell model. Since the *Itk*-deficient OT-Is showed a variable induction of T1D in the mice, a careful dose response of T4 peptide should be tested to confirm the threshold of TCR signaling that is required for migration of these autoreactive T cells to the pancreas. These experiments can also be done using the LmOVA-SIITFEKL (T4) strain; testing the relationship between strength of TCR signaling on migration with or without an inflammatory environment. This model is highly adaptable and while it is non-physiological in its mechanism of T1D induction, it can be used to test small changes in TCR signaling on a relevant T cell function in-vivo.

Conclusions

The relationship between the quality of antigen and the strength of the T cell response is a central topic of interest in immunology. Using a reductionist approach, we were able to describe a signaling mechanism for IRF4 expression in $CD8^+$ T cells, which was driven by antigen dose and affinity as well as ITK activity. We further described a role of TCR signal strength on selectin binding and autoreactive T cell migration. We hypothesize that a subset of genes behaves in a similar way as IRF4. We also describe a critical function of ITK as an accelerator of signaling, explaining why it is important in some instances while not required for all T cell functions.

Understanding how changes in TCR signaling effect changes in gene transcription allows us to ask additional questions related to T cell functions. One of these processes is the generation of optimal T cell memory, which is not completely understood and is a central paradigm in the adaptive immune response. Further research in this area will only improve vaccine development and T cell therapies.

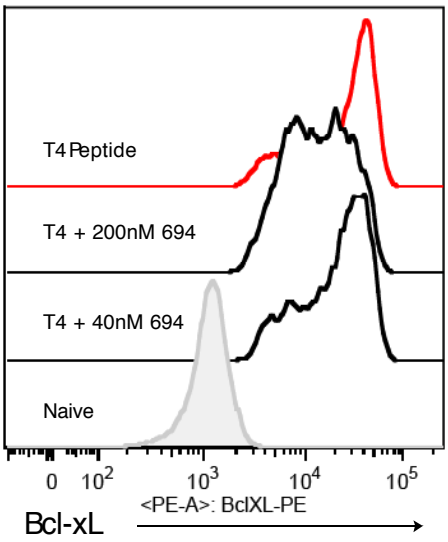
Appendix I

ITK activity contributes to NF-κB pathway activation and the NF-κB pathway inhibitor inhibits Irf4 mRNA expression.

Preliminary data in activated OT-I T cells shows that the NF-κB pathway could be playing a role in IRF4 regulation. In the first experiment, OT-I T cells were stimulated with the T4 peptide and stained for Bcl-xL expression after 24h. (Figure A.1A) We hypothesized that this survival protein, known for NF-κB dependence, specifically on RelA (p65) could be a good early response gene that is specifically dependent on the NF-κB pathway. We also tested the small molecule pathway inhibitor in the *Irf4* mRNA experiments where OT-I T cells were stimulated with T4 peptide and stained for *Irf4* mRNA early after activation. The inhibitor did delay the upregulation of the mRNA, consistent with the ITK inhibitor PRN694. (Figure A.1B) More studies are needed to determine the extent of NF-κB involvement on IRF4 expression. Specifically, we plan on using the nuclear flow cytometry method to look at early p65 activation in $CD8^+$ T cells.

Figure A.1: Bcl-xL expression is sensitive to ITK activity in activated OT-I T cells; NF-κB pathway inhibitor (BAY 11-7082) delays *Irf4* mRNA upregulation activated in OT-I T cells.

A



B

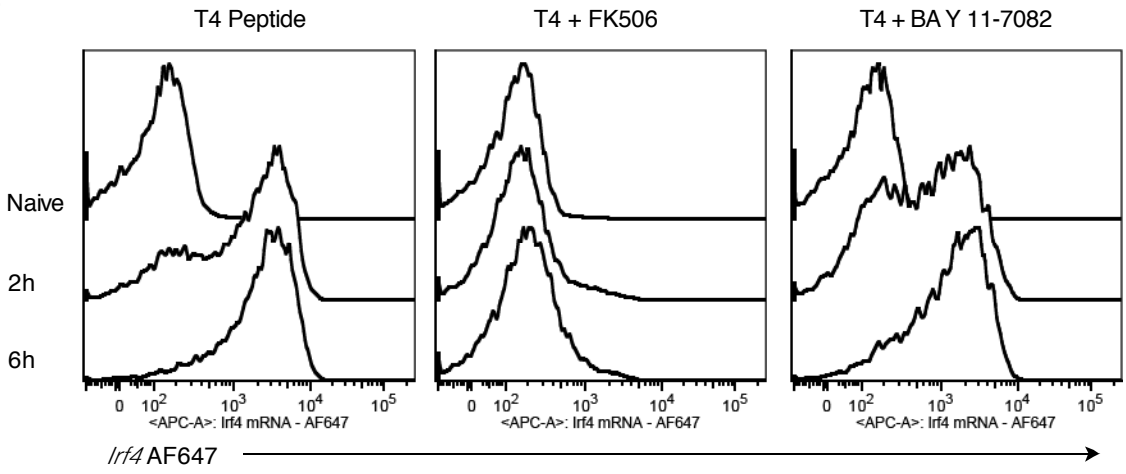


Figure A.1: Bcl-xL expression is sensitive to ITK activity in activated OT-I T cells; NF-κB pathway inhibitor (BAY 11-7082) delays *Irf4* mRNA upregulation activated in OT-I T cells.

(A) OT-I T cells were treated with 100nM T4 peptide for 24h either alone or in the presence of the ITK inhibitor PRN694 at doses of 200nM and 40nM. Representative histograms show Bcl-xL expression.

(B) OT-I T cells were treated with 100nM T4 peptide for 2 and 6h either alone or in the presence of 100nM FK506 or 1μM BAY 11-7082. Representative histogram plots show *Irf4* mRNA expression.

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