Differential TCR signaling dynamics tune graded gene expression in early-activating CD8+ T cells

Michael P. Gallagher

University of Massachusetts Medical School

Let us know how access to this document benefits you.

Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss

Part of the Immunity Commons

Repository Citation

Creative Commons License
This work is licensed under a Creative Commons Attribution 4.0 License.

This material is brought to you by eScholarship@UMassChan. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMassChan. For more information, please contact Lisa.Palmer@umassmed.edu.
Differential TCR signaling dynamics tune graded gene expression in early-activating CD8+ T cells

A Dissertation Presented By:

MICHAEL P. GALLAGHER

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:

DOCTOR OF PHILOSOPHY

November 13, 2020

Program of Immunology and Microbiology
Dedication

This work is dedicated to my two little girls Paige and Molly.
Acknowledgements

I first would like to thank my mentor Leslie Berg, who invested in me early on in my graduate school career. Leslie's mentoring style is a perfect blend of inspiration, insight, independence, accessibility, and friendship. She allows all of her students to find their own way, but always offers help when needed. Her logic and reasoning regarding experimental design and problem solving will be with me forever as a scientist.

Thank you to the members of my thesis committee and dissertation committee, Joonsoo Kang, Neal Silverman, Kate Fitzgerald, and Manuel Garber, for providing expertise and guidance throughout my graduate career. Many thanks to my outside examiner Adam Courtney.

I would also like to thank the amazing Regina Whitehead, our self-proclaimed "Lab Mom," who made everything that happened in the Berg Lab easier, efficient, and was always most welcoming. Many, many thanks to Sharlene Hubbard, whose hard work with my mouse colony is evident throughout this dissertation and I cannot imagine having done it without her help.

As for members of the Berg lab, I’d like to thank Yves Falanga, who quickly transformed from a hard-driving rotation student mentor to my bench-mate and friend. I’d like to especially think Jim Conley, whose project
sparked questions that seeded the roots of my own project. In the early years of my thesis work, Jim was always kind, helpful, and willing to share all of his reagents and protocols that were coincidentally nearly 100% overlapping with my needs. All my lab mates in the Berg lab: Hyoung-Soo Cho, Beth Olesin, Nilima Kolli, always helped make a happy and welcoming Lab environment. Their company is, and will be, missed.

I need to thank my friend and collaborator Pranitha Vangala, who has an astonishing ability to take on more and more projects, including my own, and continue to produce amazing work. Pranitha’s mentorship was invaluable in my ability to obtain new skill sets in transcriptomics and genomics, and opened my eyes to a world of computational biology. I hope to continue to build on the foundation she provided me.

I owe immense gratitude to Andrea Reboldi and the members of his laboratory. When I found myself suddenly alone at UMass, he offered a bench and a friendly environment to allow me to complete my work. This made a difficult transition in my graduate experience not only possible, but much more amenable.

I would like to thank the University of Massachusetts Graduate School of Biomedical Sciences. Everyone within the administration and faculty all have been fantastic guides, teachers, and mentors. I’d like to thank everyone in the pathology department, students, postdocs, faculty, and administrators
who are all inspirational scientists and colleagues. Specifically, I'd thank Joan, Linda, Mallika, Rick, and Jill, who magically make the department run seamlessly and in the past 2 years were extremely gracious and patient in ensuring I could finish my research.

I would like to thank my parents, Pamela and Paul, who instilled in me a fierce love of science and creativity from an early age – and continue to be endlessly loving and supportive of my academic pursuits. I would like to thank my siblings Ryan and Katie, and my siblings in-law Lindsey and Luke for their love and lifelong friendships.

Lastly, but quite honestly first, I would like to thank my beautiful family who I love so much. You are what carry me through life's struggles and who I look forward to loving and growing with every single day. Thank you, Ashley, for being the most amazing person, supportive wife, and loving mother that you are. Strong and patient – believing in my dreamy dreams as much as I did – enough to move to Worcester while continuing to grow the minds of tomorrow in Natick. I am forever grateful. Paige and Molly - when I started this endeavor, you weren't here! But now you are, and someday I hope to somehow convey to you how you’ve already taught me so much and help me to be better. I am so proud, even though you are both so little. I see the world ahead of you, much like how I feel now about my own new beginnings at the end of this degree.
Abstract

The strength of peptide:MHC interactions with the T cell receptor (TCR) is correlated with the time to first cell division, the relative scale of the effector cell response, and the graded expression of activation-induced proteins. The TCR proximal tyrosine kinase ITK simultaneously influences many biochemically separate signaling cascades. T cells lacking ITK exhibit selective impairments in effector T cell responses after activation, but under the strongest signaling conditions ITK activity is dispensable. To gain insight into whether TCR signal strength and ITK activity tune observed graded gene expression through unequal activation of disparate signaling pathways, I examined NFAT, NF-κB and MAP kinase pathways during early activation of individual naïve OT-I CD8+ T cells using peptide-loaded antigen presenting cells. Utilizing both measurement of transcription factor translocation in single T cell nuclei and conventional phospho-flow cytometry, I observed digital activation of Erk-MAPK and NFAT1 at all peptide doses and avidities. However, NF-κB activation showed a graded response to variation in TCR signal strength and was more sensitive to treatment with an ITK inhibitor. Inhibitor-treated cells showed poor induction of AP-1 factors Fos and Fosb, NF-κB response gene transcripts, and survival factor Il2 transcripts. ATAC-seq analysis revealed genomic regions most sensitive to ITK inhibition are enriched for NF-κB and AP-1 motifs. Together, these data indicate a key role for ITK in orchestrating optimal activation of separate TCR downstream pathways, specifically aiding NF-κB activation. More broadly, I describe a mechanism by which variation in TCR signal strength can produce patterns of graded gene expression in activated T cells.
# Table of Contents

**Acknowledgements**

**Abstract**

**Table of Contents**

**List of Figures**

**Publications**

**List of Symbols, Abbreviations, or Nomenclature**

**Chapter I: Introduction**

Preface

Perspective and importance of CD8+ T cell research

CD8+ T cell activation kinetics

Differentiation of T effector and memory cells

TCR signal strength control tunes T cell activation and differentiation

Graded gene expression in response to graded TCR signals

TCR machinery and signal initiation

Signal transduction downstream of PLC-γ1

ITK structure, function, and control of TCR signaling

Thesis Objectives

**Chapter II: Methods**

Mice

Stimulation of CD8+ T cells

Nuclei isolation

Confocal imaging

Antibodies and flow cytometry

Cell Sorting

RNA-seq library preparation

Processing and analysis of RNA-seq reads

Gene ontology

ATAC-seq library preparation

Alignment and processing of ATAC-seq reads


### Chapter III: Differential TCR signaling within individual CD8+ T cells

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>50</td>
</tr>
<tr>
<td>Results</td>
<td>57</td>
</tr>
<tr>
<td>Nuclei flow cytometry assay optimization</td>
<td>57</td>
</tr>
<tr>
<td>TCR regulation of digital NFAT activation in single T cells</td>
<td>61</td>
</tr>
<tr>
<td>Graded NF-κB activation within digitally switched T cells</td>
<td>65</td>
</tr>
<tr>
<td>ITK activity tunes graded NF-κB signaling</td>
<td>69</td>
</tr>
<tr>
<td>Discussion</td>
<td>77</td>
</tr>
<tr>
<td>Digital calcium and NFAT signaling in activating T cells</td>
<td>79</td>
</tr>
<tr>
<td>Signal strength and T cell motility</td>
<td>82</td>
</tr>
<tr>
<td>Digital Erk1/2 signaling in naive T cells</td>
<td>84</td>
</tr>
<tr>
<td>TCR signal strength regulates graded NF-κB activation</td>
<td>87</td>
</tr>
<tr>
<td>Novel use of a nuclei isolation assay for flow cytometry</td>
<td>90</td>
</tr>
</tbody>
</table>

### Chapter IV: ITK control of early gene transcription in activating CD8+ T cells

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>93</td>
</tr>
<tr>
<td>Results</td>
<td>95</td>
</tr>
<tr>
<td>TCR signal strength and ITK control of early gene transcription</td>
<td>95</td>
</tr>
<tr>
<td>ITK regulation of early changes in DNA accessibility</td>
<td>106</td>
</tr>
<tr>
<td>NF-κB signaling enhances select graded gene expression</td>
<td>109</td>
</tr>
<tr>
<td>Discussion</td>
<td>112</td>
</tr>
<tr>
<td>Single T cell responses</td>
<td>114</td>
</tr>
<tr>
<td>Early gene kinetics and function</td>
<td>118</td>
</tr>
<tr>
<td>Cooperation of transcription factors</td>
<td>121</td>
</tr>
</tbody>
</table>

### Chapter V: Discussion

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interpretation of major findings: ITK function</td>
<td>125</td>
</tr>
<tr>
<td>How TCR signal strength scales the effector response</td>
<td>130</td>
</tr>
<tr>
<td>Significance of TCR signal strength and ITK activity in disease</td>
<td>136</td>
</tr>
</tbody>
</table>

### References

Page references range from 45 to 145.
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Summary of proximal TCR signaling.</td>
<td>28</td>
</tr>
<tr>
<td>1.2</td>
<td>Summary of TCR downstream signaling pathways.</td>
<td>32</td>
</tr>
<tr>
<td>3.1</td>
<td>Regulation of graded IRF4 expression in CD8⁺ T cells.</td>
<td>51</td>
</tr>
<tr>
<td>3.2</td>
<td>Hypothesis: ITK activity tunes graded gene expression through differential activation of Ca²⁺/NFAT signaling.</td>
<td>55</td>
</tr>
<tr>
<td>3.3</td>
<td>Overview of nuclei isolation techniques for flow cytometry analysis.</td>
<td>58</td>
</tr>
<tr>
<td>3.4</td>
<td>Nuclei isolation from APC and T cell co-cultures.</td>
<td>60</td>
</tr>
<tr>
<td>3.5</td>
<td>Digital NFAT1 translocation in OVA peptide stimulated naive OT-I cells.</td>
<td>61</td>
</tr>
<tr>
<td>3.6</td>
<td>NFAT1 export dynamics in OT-I nuclei.</td>
<td>62</td>
</tr>
<tr>
<td>3.7</td>
<td>TCR signal strength and ITK activity drives digital NFAT activation in CD8⁺ T cells.</td>
<td>66</td>
</tr>
<tr>
<td>3.8</td>
<td>Comparison of NFAT1, NF-κB (p65), and p-Erk activation behavior in naive OT-I cells.</td>
<td>68</td>
</tr>
<tr>
<td>3.9</td>
<td>ITK inhibitor PRN-694 selectively dampens NF-κB activation.</td>
<td>70</td>
</tr>
<tr>
<td>3.10</td>
<td>PMA and ionomycin supplementation during peptide stimulation reveals synergistic NF-κB signaling activation.</td>
<td>73</td>
</tr>
<tr>
<td>3.11</td>
<td>Data summary of TCR signal strength effects on activation of separate signal pathways</td>
<td>75</td>
</tr>
<tr>
<td>4.1</td>
<td>RNA-seq replicate correlation and principal component analysis</td>
<td>96</td>
</tr>
<tr>
<td>4.2</td>
<td>Inhibition of ITK dampens immediate TCR signaling-induced transcripts.</td>
<td>98</td>
</tr>
<tr>
<td>4.3</td>
<td>Graded TCR signaling induces a single transcriptional program.</td>
<td>100</td>
</tr>
<tr>
<td>4.4</td>
<td>Example patterns of detected early transcripts.</td>
<td>102</td>
</tr>
<tr>
<td>4.5</td>
<td>Immediate transcripts are differentially sensitive to PRN-694 treatment.</td>
<td>104</td>
</tr>
<tr>
<td>4.6</td>
<td>TCR signaling and ITK control graded accumulation of early gene product c-Fos.</td>
<td>105</td>
</tr>
<tr>
<td>4.7</td>
<td>Early ITK-dependent changes in DNA accessibility are enriched for NF-κB and AP-1 binding motifs.</td>
<td>107</td>
</tr>
<tr>
<td>4.8</td>
<td>Specific NF-κB inhibition differentially reduced c-Fos protein accumulation.</td>
<td>110</td>
</tr>
</tbody>
</table>
Publications


## List of Symbols, Abbreviations, or Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>APL</td>
<td>Altered peptide ligand</td>
</tr>
<tr>
<td>ATAC-seq</td>
<td>Assay for transposase-accessible chromatin</td>
</tr>
<tr>
<td>BATF</td>
<td>B-Cell Activating Transcription Factor</td>
</tr>
<tr>
<td>BCL10</td>
<td>B-Cell Lymphoma 10</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium</td>
</tr>
<tr>
<td>CARMA1</td>
<td>CARD-containing membrane-associated guanylate kinase protein-1</td>
</tr>
<tr>
<td></td>
<td>CCR5 C-C Chemokine Receptor 5</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium-release-activated Calcium Channels</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte-associated Protein 4</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular Signal-regulated Kinase</td>
</tr>
<tr>
<td>G4</td>
<td>SIIGFEKL APL</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate-early gene</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of NF-κB kinase</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol Triphosphate</td>
</tr>
<tr>
<td>IRF4</td>
<td>Interferon Regulatory Factor 4</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITK</td>
<td>(IL-2) Inducible T cell Kinase</td>
</tr>
<tr>
<td>IkBa</td>
<td>Nuclear factor of α light polypeptide gene enhancer in B cells</td>
</tr>
<tr>
<td></td>
<td>inhibitor α</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Killer cell lectin-like receptor subfamily G member 1</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for Activation of T cells</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis</td>
</tr>
<tr>
<td>MALT1</td>
<td>Mucosa-associated lymphoid tissue lymphoma translocation protein 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated Protein Kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Median Fluorescence Intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MPEC</td>
<td>Memory precursor cell</td>
</tr>
<tr>
<td>MSigDB</td>
<td>Molecular signatures database</td>
</tr>
<tr>
<td>N4</td>
<td>SIINFEKL native OVA Peptide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor of κ light polypeptide gene enhancer in B cells</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T cells</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed Cell Death Protein 1</td>
</tr>
<tr>
<td>PHTH</td>
<td>Pleckstrin homology Tec homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-4,5-Bisphosphate 3-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-Bisphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol-3,4,5-Triphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC-γ₁</td>
<td>PLCγ Phospholipase C gamma-1</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PRG</td>
<td>Primary response gene</td>
</tr>
<tr>
<td>RasGRP</td>
<td>Ras Guanyl-nucleotide releasing protein</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology domain 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology domain 3</td>
</tr>
<tr>
<td>SLEC</td>
<td>Short-lived effector cell</td>
</tr>
<tr>
<td>SLP76</td>
<td>SH2 domain-containing leukocyte protein 76kDa</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated calcium entry</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box Transcription Factor TBX21</td>
</tr>
<tr>
<td>T4</td>
<td>SIITFEKL APL</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell Receptor</td>
</tr>
<tr>
<td>Tn5</td>
<td>a transposase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>Zap70</td>
<td>Zeta-chain Associated Protein Kinase 70kDa</td>
</tr>
</tbody>
</table>
Chapter I: Introduction

Preface

Upon matriculating to the University of Massachusetts Medical School, I studied immunology during my undergraduate coursework and also worked in multiple immunology laboratories which were seeking a better understanding in basic lymphocyte biology. These experiences helped frame my goals for my graduate work but also grounded my perspective in the real work of a scientist. I entered the graduate program with a sincere interest in adaptive immune responses, particularly in the exact details in how mammals efficiently generate specific cellular responses to disease; both in the initial recognition of a threat and persistent protection due to immunological memory.

During my tenure as a graduate student, I had personal goals of gaining topical expertise, sharpening my critical thinking skills, and training for cross-discipline technical proficiency. The Berg Lab offered overlap in many of my academic interests and the mentoring culture within the lab encouraged students to explore different and emerging disciplines pertinent to current immunobiology. When our questions about CD8+ T cell activation were approaching the limit of our cellular-based experimental designs. Dr. Berg welcomed my pursuit for answers through computational and genomics
approaches, in which her lab lacked extensive experience. Thus, I pursued a productive collaboration with Dr. Garber’s laboratory to help me acquire new skill sets to diversify my education in immunobiology and intracellular signaling transduction with transcriptomics and genomics approaches. I am confident moving forward equipped with my diversified training in pursuing new endeavors of scientific discovery.

**Perspective and importance of CD8⁺ T cell research**

In the 1950s, we still knew almost nothing about the “small lymphocytes.” By 1970, immune cells with potent cytotoxic capability were identified and in 1975 attributed to Ly-2 (CD8α) and Ly-3 (CD8β) expressing lymphocytes (Masopust et al., 2007). In the mere 45 years since, we have amassed a wealth of appreciation for how cytotoxic CD8⁺ T cell responses provide protection during even the most threatening immune challenges. By the late 1990s, it was demonstrated CD8⁺ T cells can be persuaded to act as a powerful treatment to combat cancer cells. We also learned when dysfunctional, CD8⁺ responses significantly contribute to autoimmunity and to the persistence of chronic infections. Most of these discoveries were made possible through simultaneous revelation regarding the molecular workings of the T cell receptor (TCR) complex and how it directs the transcriptional reprogramming of activated T cells. But the story is still unfinished.
Immunologists today are charged with the task of fully unlocking the natural and therapeutic potential of CD8\(^+\) T cell responses. As I write in autumn of 2020, the world is amidst a global SARS-CoV-2 COVID-19 pandemic, and the need to study the basic biology of CD8\(^+\) T cell responses has never been more clear (Le Bert et al., 2020; Weiskopf et al., 2020). Ever-increasing access to faster computational methods and networking algorithms has allowed us to integrate the function of the TCR with its global effects on transcription, metabolism, and chromatin remodeling. I believe we are in the midst of a personalized medicine revolution with technologies like checkpoint blockade inhibitors and patient-derived T cell immunotherapies only marking the beginning of possible solutions to pursue that will undoubtedly improve the quality of life worldwide. Continued research in the fundamental biology of T cells is paramount to maximize the therapeutic potential.

**CD8\(^+\) T cell activation kinetics**

Since the discovery that the TCR recognizes linear peptides bound in the groove of major histocompatibility (MHC) molecules, immunologists have studied how the quality of this specificity dictates T cell responses (Bjorkman et al., 1987a, b; Masopust et al., 2007). In mice and humans, mature T cells circulate in the blood, continuously entering and exiting lymphoid organs
scanning activated APCs for their cognate antigen. During a model lymphocytic choriomeningitis virus (LCMV) infection in mice, it is estimated the likelihood an individual naive T cell will activate is about 1 in $1 \times 10^5$. In other words, a single mouse contains only about 100-200 cells that are capable of recognizing a specific viral peptide (Blattman et al., 2002; Obar et al., 2008). Yet only hours after contact and activation, single naive clones rapidly divide up to 14 times; an astonishing achievement of metabolic reprogramming (Blattman et al., 2002).

Single T cell clones are unlikely to be lone responders during a viral challenge. Many different individual T cells seed the polyclonal response within lymphoid organs, an environment where clones compete for APC contact. While many individual T cell clones may identify a cognate antigen and sufficiently activate, unequal distribution of surface peptide:MHC molecules, co-stimulatory molecules on APCs, cytokine production, and the strength of the interaction between individual clonal TCR molecules and peptide:MHC all encourage competition. Through this competitive nature, specific clones tend to dominate a polyclonal response - often those with highest TCR specificity. TCR signal strength regulates the the peak size of a responding T cell population, but does not seem to impact the cytolytic capacity of effector cells (Richard et al., 2018; Tscharke et al., 2015). Therefore smaller, “subdominant” simultaneously expanded CD8+ clonal populations are
also important in providing protection against a diverse array of other epitopes and may be helpful in preventing viral escape (Tscharke et al., 2015). Curiously, single identical T cell clones transferred into separate animals do not all expand to the same degree when challenged (Buchholz et al., 2013). This demonstrates the probabilistic nature of T cell activation following antigen interactions and robust *in vivo* CD8+ responses rely on the participation of many clones.

Clonal expansion of CD8+ T cell populations during a response scales according to multiple factors including the availability of antigen (pathogen load and level of expression on APCs) and quality of TCR–peptide:MHC interactions (Mayya and Dustin, 2016). One study compared the dose of a pathogen, ovalbumin-expressing *Listeria monocytogenes* (LM-OVA), in mice to the precursor frequency of adoptively transferred barcoded OVA-specific TCR transgenic (OT-I) naive cells or “fixed” OT-I TCRβ, “free” TCRα T cells (van Heijst et al., 2009). Under either condition, they reported a consistent and “near complete” recruitment of antigen-specific T cells to the spleen regardless of the CFU of LM-OVA at inoculation. However, the maximum number of expanded clones was directly dependent on the dose of the inoculum. Similar results were observed when testing OT-I responses in mice infected with strains of *Listeria* expressing altered variations of OVA peptide (Zehn et al., 2009). Mice inoculated with weaker-affinity LM-OVA strains
recruited a similar number of OT-I cells as those inoculated with high-affinity strains. Both low and high-affinity peptides recruited a similar number of OT-I cells at early time points, but clonal expansion of OT-I cells scaled with the strength of the OVA peptide variant. Some contradictory studies analyzing more diverse T cell pools report not all antigen-specific precursors are recruited (especially lowest affinity clones), but there is consensus that the magnitude of clonal expansion is linked to the intensity of T cell stimulation (Tscharke et al., 2015).

**Differentiation of T effector and memory cells**

Expanded clonal CD8\(^+\) T cell populations are short-lived. During acute LCMV-Armstrong infection in mice, responding T cell populations typically peak at day 8, but quickly apoptose and contract to \(~10\%\) of maximum numbers as the virus is cleared. The remaining T cells persist as memory T cells, which have capability to quickly reactivate upon viral rechallenge (Kaech and Cui, 2012). Understanding how quiescent naive cells rapidly expand into cytotoxic effector phenotype cells and simultaneously generate a memory phenotype pool is an area of high research interest where a large amount of progress has been made in recent decades.

Effectors and memory CD8\(^+\) T cells are transcriptionally and epigenetically distinct; representing two separate cell fates derived from a
common precursor (Chen et al., 2018; Kaech and Cui, 2012). Recent review of effector and memory differentiation classifies previous hypotheses into two competing models; an “asymmetric” model and a “progressive” model of differentiation (Chen et al., 2018). The asymmetric model of differentiation describes that as early as the first division, an activated naive T cell asymmetrically segregates cell structures and transcription factors as it divides to yield one memory-like daughter cell and one effector-like daughter cell (Chang et al., 2007; Chen et al., 2018; Kaech and Cui, 2012). The effector precursor is thought to derive from the APC proximal side of the parent cell which is receiving strong TCR signaling. The progressive model of differentiation proposes a linear path towards final differentiated effector and memory states, citing important events such as repeated contact with APCs and continued stimulation gradually pushing activated cells through memory-like epigenetic states into functional memory and effector cells after multiple generations (Chen et al., 2018).

Diversification of clonal T cell progeny into cells with memory and effector phenotypes starts quickly after activation. As soon as 4 days after LCMV infection, memory precursor cells (MPECs) can be identified by high expression of surface IL-7Rα and low expression of KLRG1. Conversely, terminally differentiated, short-lived effector cells (SLECs) with high cytotoxic capacity are KLRG1 high and IL7Rα low (Joshi et al., 2007; Kaech
and Cui, 2012; Kaech et al., 2003). Both subsets are characterized by specific transcription factors that are important in defining their separate phenotypes. SLECs are high in T-box transcription factor (Tbet), B-lymphocyte-induced maturation protein 1 (Blimp1), interferon regulatory factor 4 (IRF4), and signal transducer and activator of transcription (STAT4) expression, all important in programming effector expansion and function. This includes production of granzymes, interferon-gamma (IFN-γ), interleukin (IL)-12, and IL-2. MPEC phenotypes include increased expression of B cell lymphoma (Bcl)6, specific timing of eomesodermin (eomes) expression, as well as persistence of “naive”-associated transcription factors like T cell factor 1 (TCF1).

Further transcriptional and epigenetic studies focused on the differentiation of effector and memory subsets revealed that memory cells resemble a naive cell phenotype. SLECs however, have closed chromatin near gene regions that regulate the program of homeostatic proliferation and quiescence found in naive and memory cells (Chen et al., 2018; Henning et al., 2018). Soon after activation, changes in DNA methylation and activating histone modifications such as H3K4me3 and H3K29ac help open the chromatin near effector-associated gene regulatory regions (e.g. Tbx21, Prdm1, Gzma, Gzmb, Ifng, Klrj1) in both MPECs and SLECs (Henning et al., 2018). By definition, SLECs more efficiently express the gene products
encoded by effector gene regions, perhaps due to stronger TCR signals. However, SLECs also simultaneously accumulate repressive H3K27ac H3Kme3 histone modifications around naive and memory-associated gene regions (e.g. Tcf7, Foxo1, Klf2), which permanently closes this chromatin and prescribes terminal differentiation.

MPECs transition to memory cell states after infection and many of the naive-like gene regions remain open. However, they differ from naive cells in that many effector-associated gene regions remain open. This has a major advantage upon reinfection, where activated memory cells can quickly start transcribing effector genes without extensive chromatin remodeling (Chen et al., 2018; Kersh et al., 2006). For example, naive CD8+ T cells express a high amount of Dnmt1, a methyltransferase that helps maintain methylation and suppression of the Ifng locus. Memory cells have lower expression of Dnmt1 and Ifng is more easily transcribed upon reactivation (Chen et al., 2018; Kersh et al., 2006; Lee et al., 2001).

**TCR signal strength control tunes T cell activation and differentiation**

A primary area of research interest in our lab is to understand how TCR signal strength directs different outcomes of the effector and memory differentiation during activation. As discussed above, the amount of the TCR signaling experienced early during activation scales the magnitude of the
CD8+ T cell response. Early studies suggested that weak TCR signals produce a small memory pool that has poor performance upon rechallenge (Daniels and Teixeiro, 2015). This may be true if very immunodominant epitopes are present during infection, because high-affinity clones outcompete low-affinity clones for antigen and during recall immunodominant clones again usually expand more greatly which further narrows the protective TCR repertoire (Busch et al., 1998; Daniels and Teixeiro, 2015). However, later studies examining only low-affinity epitopes revealed clones that poorly expand terminal effectors during the primary response produce capable and protective memory cells. To test this, Bevan and colleagues inoculated OT-I-transferred mice with altered OVA peptide-expressing Listeria strains (representing a gradient of peptide affinity for the OT-I TCR) (Zehn et al., 2009). As expected, low-affinity Listeria strains induced both smaller effector and memory pools. 138 days later they re-challenged the same mice with Vesicular Stomatitis Virus (VSV) expressing native OVA (high-affinity) peptide. The few memory cells derived from the low-affinity primary response expanded comparably to memory cells derived from a high-affinity primary response, indicating low-affinity interactions can produce functional and protective memory responses.

While TCR signal strength regulates the overall scale of clonal expansion, the Berg lab and others have explored how it also modulates the
ratio of terminal effectors to memory precursors (Kaech and Cui, 2012). Generally, stronger TCR signals increase the percentage of SLECs relative to MPECs (Joshi et al., 2007). TCR control of the SLEC to MPEC ratio is not absolute as other factors such as the amount cytokine signaling (IFN-γ, IL-12) also contribute, but effector cytokine expression is also proportional to the amount of initial TCR signal (Cui et al., 2009; Obar et al., 2008).

**Graded gene expression in response to graded TCR signals.**

The Berg lab and others have demonstrated that IRF4, an important effector-associated transcription factor, is expressed in a graded manner in response to graded TCR signaling (Man et al., 2013; Nayar et al., 2014; Raczkowski et al., 2013; Yao et al., 2013). In a 2014 publication, the Berg lab modulated TCR stimulation strength using different affinity peptides in a P14 transgenic mouse model and reported graded levels of IRF4 (Nayar et al., 2014). In the same study, to engineer graded levels of IRF4 within T cells, our lab used Irf4 sufficient, heterozygous floxed (Irf4+/fl), or homozygous floxed (Irf4fl/fl) mice. After LCMV infection, the number of effector cells expanded during infection scaled according to the “dose” of IRF4 expressed in each condition. Others, continuing to explore the role of IRF4, revealed that IRF4 activity is key in transcribing metabolic reprogramming in activating T cells (Man et al. 2013). These revelations about IRF4 helped put it at the center of
our focus connecting graded TCR signaling inputs to the previously observed transcriptional effects regulating the scale of clonal expansion.

In response to these studies, our lab was then focused on uncovering the TCR signaling mechanisms that coordinate graded IRF4 expression. My colleague James Conley designed experiments that revealed differential gene expression in response to graded TCR signaling. Using an OT-I mouse model and altered OVA-peptides defined by Bevan and colleagues (Zehn et al., 2009), preliminary data suggested that the activation marker CD69 had a sharp threshold of activation and its response was “all-or-none” or digital in nature. Under the same conditions, IRF4 consistently appeared graded.

Weaker TCR stimulation, achieved by using lower concentration of peptide or by using lower affinity peptide, consistently reduced the positive fraction of T cells digitally expressing CD69, but dampened the amount of IRF4 expressed within each CD69+ T cell. Under certain stimulation conditions, CD69 expression was the same as those cells stimulated with high-affinity peptide, yet IRF4 expression was almost halved. These experiments have recently been published (Conley et al., 2020). Our interpretation of this phenomenon has changed since initial observations, but at the time it caused us to hypothesize about how nuances in TCR downstream signaling pathways could produce the graded IRF4 expression we observed in otherwise digitally activated cells.
TCR machinery and signal initiation

A key feature of TCR signaling is its ability to discriminate between minute differences in peptide:MHC quality and density. The antigen specificity of the TCR is derived from alpha and beta chain pairs. The variable (V), diversity (D) and joining (J) gene loci in these chains are rearranged during development in the thymus to produce T cell clones with unique TCR regions that interact with peptide-loaded MHC molecules. TCR alpha and beta dimers associate with CD3 molecules, transmembrane proteins with important cytoplasmic signaling domains, to form the TCR complex. Also co-expressed at the mature T cell surface is either the CD8 or CD4 co-receptor, which stabilizes contacts with MHCI or MHCII molecules respectively.

The “affinity” of a given TCR clone for a particular peptide:MHC complex is manifested in the frequency at which contacts are made and unmade. The “on-rate” ($k_{on}$) and the “off-rate” ($k_{off}$) of TCR and peptide:MHC interactions measure an equilibrium that spans many orders of magnitude in possible values. A reductionist viewpoint could be that TCR “signal strength,” or at least the external factors that contribute to it, is attributed to a biochemical equilibrium predominantly defined in the off-rate. Peptides that elicit higher functional avidity in CD8+ T cells (more IFN-γ, IL-12, etc.) consistently have properties of long off-rates. Long measured $k_{off}$ has been shown to be the primary contributor to affinity and an excellent predictor for
CD8+ protective capacity in human immunotherapies (Nauerth et al., 2013). TCRs on immature T cells undergoing selection in the thymus with lower $k_{off}$ are also more likely destined for T regulatory cell (Treg) differentiation (Li and Rudensky, 2016). The highest-affinity TCR interactions may require only a single peptide loaded MHC molecule on an APC cell surface to initiate signaling and activate a single T cell (Altan-Bonnet and Germain, 2005; Huang et al., 2013). But while the spectrum of TCR off-rates include differences in several orders of magnitude, the mechanisms that actually permit sensing of discrete differences in TCR binding are thought to be found in the rate-limiting biochemistry within the cell.

The kinetic proofreading model of TCR signaling initiation provides a mathematical framework to explain how T cells discriminate between small differences in agonists (Chakraborty and Weiss, 2014; Rosette et al., 2001). This model is rooted in the idea that multiple, reversible biochemical intermediates are downstream of TCR ligation. Within T cells, ATP-driven phosphorylation and de-phosphorylation of these signaling intermediates provide rate-limiting forward progression of signal. This effectively exaggerates the small differences between off-rates of peptide:MHC and the TCR. Multiple TCR molecules can participate to add to the rate of the productive signal, thus higher concentration of low-affinity peptides can also yield productive signals. Kinetic proofreading is a way to link the spectrum of
Figure 1.1. Summary of proximal TCR signaling.

The TCR complex, comprised of TCR α and β chains, and CD3 molecules (ε, γ, δ) interacts with antigen peptide presented on MHC class I molecules on the surface of APCs. Co-receptor (CD8 or CD4) stabilizes these interactions by directly contacting MHC molecules. After antigen contact, conformational changes promote phosphorylation of CD3 cytoplasmic tails by active Lck. Further phosphorylation of LAT by Lck and Zap70 allow for scaffolding by adapter molecules such as SLP76 and GADs. This allows recruitment of active ITK, which can catalyze phosphorylation of PLC-γ1. Active PLC-γ1 digests stores of membrane bound PIP2 molecules into two second messenger molecules: DAG and IP3, which are released to influence downstream signaling. PRN-694 is a small covalent inhibitor of ITK kinase activity. Adapted from Li and Rudensky, 2016.

potential TCR signaling strength with precise regulation of downstream signal propagation within T cells.

The rate-limiting signaling intermediates that are proximal to the TCR are bathed in an environment of kinases and phosphatases battling for equilibrium. Simply, TCR ligation creates an environment that promotes kinase activity and makes phosphatase activity unfavorable. A deeper understanding about how this is achieved has required years of kinetic
modeling and examination of the TCR proximal biochemical players, with much of the focus to include three important events: activation of Lck, Zap70, and LAT.

In response to TCR ligation, one of the main roles of the Src kinase Lck is to phosphorylate cytosolic immunoreceptor tyrosine-based activation motifs (ITAMs) on the tails of CD3 class molecules. Evidence suggests that up to 40% of Lck is constitutively active in resting T cells, yet under these conditions CD3 ITAMs do not maintain active phosphorylation states. Further, the phosphatase CD45 can de-phosphorylate ITAMs up to 20 times faster than Lck can phosphorylate it (Hui and Vale, 2014). Thus, many studies have explored how TCR ligation promotes an environment for enhanced Lck efficiency. First, CD3 tails are normally hidden in the plasma membrane at steady state and ITAMs are less available for phosphorylation by Lck. TCR ligation with peptide:MHC mechanically pulls at the TCR, creating shear forces that cause conformational changes releasing CD3 tails from the plasma membrane into the cytosol. Second, Lck associates with T cell coreceptors CD8 or CD4. When a T cell finds an APC presenting a cognate peptide, TCRs and coreceptors form microclusters, thus Lck is placed at close proximity CD3. Third, TCR ligation and microcluster formation at the synapse pulls the opposing APC cell membrane close to the T cell membrane.
which forces segregation of bulky phosphatases (such as CD45) away from
the vicinity of CD3, allowing for kinase activity of Lck to predominate.
Lck is regulated by two tyrosine phosphorylation sites; Y394 and Y505. Lck auto-phosphorylation of residue Y394 activates the kinase domain. The
inhibitory residue Y505 is primarily phosphorylated by the tyrosine kinase
Csk, which then locks the protein conformation in an inactive form. Removal
of Y505 phosphorylation is catalyzed by phosphatases like CD45 and allows
for an open, primed conformation. However, CD45 can also dephosphorylate
the activating Y394 residue, thus can be seen to have dual regulatory
function. Antagonistic regulation of Lck phosphorylation states are found in
equilibrium, where TCR stimulation ultimately tips the balance of the Lck
pool toward active states (Chakraborty and Weiss, 2014; Gaud et al., 2018).

Lck phosphorylation of CD3 ITAMs recruits the kinase Zap70, which is
subsequently phosphorylated also by Lck and other active Zap70 molecules.
An important role of active Zap70 is to phosphorylate tyrosine residues on
linker for activation of T cells (LAT), a membrane-bound protein that extends
into the cytosol. Both LAT and other proteins like Slp76 provide scaffolding
for many other important adaptor molecules (e.g. Grb2, GADS, Vav1). LAT and
Slp76 also recruit important signaling molecules IL-2 inducible T cell Kinase
(ITK) for phosphorylation by Lck and the substrate for ITK, phospholipase-C-
gamma-1 (PLC-$\gamma$1) (Fig. 1.1).
Signal transduction downstream of PLC-γ1

Activation of PLC-γ1 triggers numerous downstream pathways and is recognized as an important node in T cell activation and signal propagation (Fig. 1.2). PLC-γ1 digests molecules of phosphatidylinositol 4,5-bisphosphate (PIP₂) lipids in the plasma membrane into two “second messenger” signaling products: diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) (Smith-Garvin et al., 2009). DAG production influences multiple separate downstream signaling cascades including mitogen-activated protein kinase (MAPK) and nuclear factor-kappa (NF-κ)B. Generation of IP3 initiates a process called store-operated calcium signaling (SOCE) within T cells. IP₃ binds to the IP₃ receptor (IP₃R) on the membrane of the endoplasmic reticulum, which causes ER release of calcium (Ca²⁺) into the cytosol. STIM molecules in the ER membrane sense this drop of luminal Ca²⁺ and then associate and open calcium (CRAC) channels in the T cell plasma membrane, causing a massive influx of extracellular Ca²⁺ into the cytoplasm, leading to calcineurin activation and ultimately nuclear factor of activated T cells (NFAT) activation (Smith-Garvin et al., 2009; Trebak and Kinet, 2019).

Two NFAT (NFAT1 & NFAT4) proteins are constitutively expressed in resting naive T cells and restricted to localization in the cytoplasm. Upon Ca²⁺ flux during active signaling, calcineurin phosphatase activity removes numerous inhibitory phosphates on NFAT proteins and allows nuclear import
Figure 1.2. Summary of TCR downstream signaling pathways.

After produced by PLC-γ1, DAG and IP₃ influence activation of numerous transcription factor pathways. The three major pathways examined here are NF-κB, MAPK, and NFAT. To activate NF-κB, DAG activation of PKC-δ influences assembly of the CARMA1-BCL-10-MALT1 (CBM) complex, which can activate the IKK complex. Active IKK allows for ubiquitylation and degradation of inhibitory IκB, releasing NF-κB dimers (e.g. p65-p50) for translocation to the nucleus. DAG also aids in MAPK pathway activation by activating RasGRP which contributes to the pool of active Ras, setting off a signaling cascade including Raf, MEK, and Erk1/2. The MAPK pathway terminates with many functions, which includes activation of AP-1 transcription factors. The other second messenger generated by PLC-γ1 activation, IP₃, causes intracellular Ca²⁺ flux via store-operated calcium entry (SOCE). Downstream of Ca²⁺ entry, calcineurin rapidly de-phosphorylates sequestered NFAT molecules, which then is permitted to translocate to the nucleus. Adapted from Li and Rudensky, 2016.

and subsequent NFAT-dependent gene transcription (Macian, 2005; Trebak and Kinet, 2019). Within seconds after TCR stimulation induces Ca²⁺ flux, NFAT dephosphorylation and translocation behavior is digital - rapid and near complete (Marangoni et al., 2013; Podtschaske et al., 2007). Nuclear export of NFAT is much slower, on the order of minutes (Marangoni et al., 2013). Some of the experiments I present here in this dissertation further explore NFAT1 behavior in single cells, specifically highlighting how peptide
affinity and concentration regulate the kinetics of the responding population (Gallagher et al., 2018).

One of primary effects of PLC-ɣ1 dependent DAG production is activation of the MAPK signaling cascade. Extracellular signal-related kinases 1 and 2 (Erk1/2), exhibit strong digital activation in response to TCR ligation (Altan-Bonnet and Germain, 2005). A pivotal study in 2009 revealed digital Erk activation is made possible through a positive feedback signal mechanism (Das et al., 2009). DAG production from PLC-ɣ1 was important in seeding active Ras protein, but they showed son-of-sevenless (SOS)-mediated Ras activation was the trigger for the digital switch.

Canonical NF-κB activation is mediated by DAG signaling to PKC-Θ, which activates the CBM complex, then the IKK complex, and finally degradation of inhibitor of κB (IκB)alpha to release NF-κB proteins for translocation in the nucleus for transcriptional activity (Lucas et al., 2004; Smith-Garvin et al., 2009). A 2010 study reported that NF-κB activation in naive T cells exhibited digital switching (Kingeter et al., 2010). However, as discussed in Chapter III of this dissertation, this conclusion is incomplete, and NF-κB activation behavior in T cells is more nuanced. Previous studies have revealed that NF-κB also is regulated by Ca^{2+} levels (Dolmetsch et al., 1997; Ishiguro et al., 2006; Podtschaske et al., 2007). Further, more recent
investigations into the nuance of PMA and ionomycin stimulation of T cells shows synergistic effects on NF-κB transcriptional activity (Brignall et al., 2017). Together, it seems that the NF-κB pathway is not solely dependent on DAG as it is often depicted.

NFAT, MAPK, and NF-κB signaling make up a core group of critical signaling transduction pathways in T cells (Smith-Garvin et al., 2009). To date, few studies have examined all of these pathways simultaneously in activating cells (Dolmetsch et al., 1997; Podtschaske et al., 2007). Moreover, fewer have stimulated T cells with peptide antigen, but rather use chemical or anti-CD3 stimulation. The experiments in this study used OT-I and OVA peptides to do exactly that (Zehn et al., 2009). In addition, as ITK catalyses PLC-γ1, I explored the role plays ITK in tuning all of these pathways.

**ITK structure, function, and control of TCR signaling.**

Since its discovery, understanding the role of the ITK has been a principal focus in the Berg Lab (Gibson et al., 1993; Heyeck and Berg, 1993; Siliciano et al., 1992; Tanaka et al., 1993; Yamada et al., 1993). ITK is the predominant TEC kinase expressed in T cells (Berg et al., 2005). Early knockout experiments demonstrated that ITK is important for robust T cell activation after TCR stimulation (Andreotti et al., 2018; Andreotti et al., 2010;
Liao and Littman, 1995; Schaeffer et al., 1999). Littman and colleagues showed that Itk−/− mice have marked decrease in thymic development of single positive CD4 T cells (Liao and Littman, 1995). In the same study, Itk−/− both CD4+ and CD8+ T cells with transgenic TCRs had defects in thymic development and proliferation after TCR stimulation, but not after PMA and ionomycin chemical stimulation, indicating ITK activity is important in proximal TCR signaling.

To identify the biochemical role ITK and the other TEC kinase expressed in T cells, Rlk, play in proximal TCR signaling, Schwartzberg and colleagues compared TCR stimulation of Itk−/−, Rlk−/−, and Itk−/− Rlk−/− mice (Schaeffer et al., 1999). After anti-CD3 stimulation, all mutant mice displayed defects in proliferation, expression of activation markers such as CD25, and low secretion of IFN-γ compared to WT. Comparing Itk−/− and Rlk−/− mice clearly demonstrated a greater importance of ITK activity over Rlk activity, as many Rlk−/− phenotypes were minimal. Significant defects in survival of Itk−/− mice after infection with the intracellular parasite Toxoplasma gondii, highlighted the in vivo relevance of ITK activity. Importantly, measurement of downstream Ca2+ signaling and MAPK signaling events in Itk−/− Rlk−/− T cells provided evidence that Tec kinases phosphorylate PLC-γ1.

Similar to other Tec kinases, ITK protein contains a kinase domain, a Src homology (SH)2 domain, a SH3 domain, and a pleckstrin homology-Tec
homology (PHTH) domain. The SH2–SH3 cassette allows for association with phosphorylated SLP76 molecules. The PHTH domain recognizes phosphatidylinositol (3,4,5)-triphosphate (PIP_3) lipids, helping recruit ITK to the plasma membrane. In signaling T cells, active PI3 kinase (PI3K) produces membrane PIP_3 and increased phosphorylation state of SLP76 draws ITK molecules close to the kinase–dominant TCR proximal environment and the LAT signalosome, where PLC-γ1 can associate.

Exactly how TCR stimulation produces a dynamic range of responses defined by distinct gene expression patterns is still not completely understood; however, it is clear that the three main signaling pathways leading to transcriptional activation downstream of the TCR: MAPK, NF-κB, and Ca^{2+}/NFAT – are critical for much of this regulation.

**Thesis Objectives**

The projects presented in this dissertation were initially rooted in the need to better describe the function of ITK. ITK affects the production DAG and IP_3 in equimolar amounts (Andreotti et al., 2010; Smith-Garvin et al., 2009). Within the Tec kinase family, ITK performance in kinase assays is not very impressive and activation is more or less dispensable under strong stimulation conditions (Andreotti et al., 2010; Joseph et al., 2013). Yet ITK activity was proving critical in giving CD8 T cells the ability to optimally tune
graded gene expression, such as IRF4, at 24 hours (Conley et al., 2020). I reasoned that close examination of the major signaling pathways directly under the influence of ITK, specifically Ca\textsuperscript{2+}/NFAT, NF-κB, and MAPK, may not all respond similarly to changes in TCR stimulation or ITK activity. The primary goal of my thesis was to link ITK activity and TCR signal strength with the regulation of early gene transcription.

**Chapter III: Differential TCR signaling within individual CD8+ T cells**

This chapter tested the hypothesis that ITK activity specifically tunes Ca\textsuperscript{2+}/NFAT signaling. To do this, I optimized a flow cytometry assay to measure transcription factor translocation in single, peptide stimulated cells with flow cytometry. I then measured NFAT, NF-κB, and pErk1/2 activation after stimulation with APCs presenting variable affinity peptides or during treatment with ITK inhibitor PRN-694. The key observation was that ITK regulates the digital activation kinetics of NFAT, but NF-κB signal intensity was very sensitive to PRN-694 treatment.

**Chapter IV: ITK control of early gene transcription in activating CD8+ T cells**

Developed alongside experiments in Chapter III, the experiments in this chapter tested the hypothesis that differential signaling conditions produced by weakened ITK activity would have immediate effects on graded
gene expression. To do this, I stimulated OT-I cells with peptide antigen with or without PRN-694, and performed RNA-seq and ATAC-seq. Transcriptional differences that I observed were then validated for differences in protein expression in single cells by flow cytometry. I also used an NF-κB inhibitor to test the extent that NF-κB signaling regulated graded early gene expression.
Chapter II: Methods

Mice

Mice were bred and housed in a specific pathogen-free facility at the University of Massachusetts Medical School (Worcester, MA) in accordance with Institutional Animal Care and Use Committee guidelines. OT-I transgenic Rag1-/- mice (B6.129S7-Rag1tm1Mom Tg(TcraTcrb)1100Mjb N9+N1) and C57BL/6 wildtype mice were purchased from Taconic Biosciences. CD45.1+ (B6.SJL-PtprcaPep3b/BoyJ) mice were purchased from The Jackson Laboratory. Unless otherwise noted, experimental cohorts consisted of age and sex-matched littermates aged 6-12 weeks.

Stimulation of CD8+ T cells

Freshly harvested OT-I Rag1-/- mouse splenocytes were pooled, RBC lysed, and enriched for CD8+ cells with an EasySep™ negatively selective magnetic isolation kit (STEMCELL Technologies). OT-I cells prepared for use in nuclei isolation experiments were then treated with CellTrace™ Violet reactive dye (Invitrogen) for 20 minutes to fluorescently label cells (including nuclei). OT-I cells were cultured at 2 x 10^5 cells per well (unless otherwise noted) and incubated with or without 50 nM (or otherwise noted) ITK/RLK inhibitor PRN-694 (Principia Biopharma) for 30 minutes 37°C. In some experiments,
OT-I cells were incubated with or without IKK inhibitor IKK-16 (Sigma) or MEK inhibitor PD325901 (Tocris Bioscience). For antigen-presenting cells, RBC-lysed splenocytes harvested from wildtype mice were cultured at 4 x 10⁵ per well and incubated with indicated concentrations of OVA “N4” peptide (SIINFEKL), altered OVA “T4” peptide (SIITFEKL), or altered OVA “G4” peptide (SIITFEKL) (21st Century Biochemicals) for 60 minutes at 37°C. OT-I cells and peptide loaded splenocytes were then combined and incubated at 37°C for indicated stimulation times. For cell preparations used for molecular analyses (e.g. RNA-seq and ATAC-seq analyses), splenocytes from CD45.1+ wildtype mice were used as peptide presenting cells for easy exclusion from CD45.2+ OT-I cells via cell sorting.

**Nuclei isolation**

To measure translocation of nuclear proteins, I isolated cell nuclei after stimulation for fixation and subsequent flow cytometry analysis. To do this, I utilized a sucrose buffer-based protocol that I and others have published (Gallagher et al., 2018; Podtschaske et al., 2007). To summarize, stimulated cells were pelleted and washed with 200 µL of ice cold "Buffer A" containing 320 mM sucrose, 10 mM HEPES (Life Technologies), 8 mM MgCl₂, 13 EDTA-free cOmplete Protease Inhibitor (Roche), and 0.1% (v/v) Triton X-100 (Sigma-Aldrich). After 15 min on ice, the plate was spun at 2000 x g and 4°C
for 10 min. This was followed by 2X 200 µL washes with “Buffer B” (Buffer A without Triton X-100) at 2000 x g and 4°C.

**Confocal imaging**

For imaging, whole cells and nuclei were fixed and stained with fluorescent Abs similarly to flow cytometry preparation. After staining, pellets were resuspended in ~10 µl of PBS, pipetted onto glass slides, and mounted with ProLong Gold Antifade Reagent with DAPI (#8961; Cell Signaling Technology). Images were collected on a Leica SP5 confocal microscope with a 63X oil objective using Leica LAS AF software. Images were processed and merged using ImageJ software (National Institutes of Health).

**Antibodies and flow cytometry**

Stimulated cells and isolated cell nuclei were fixed and permeabilized with the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience), except cells used for anti-p-Erk1/2 analysis, which were fixed with 4% paraformaldehyde (Electron Microscopy Services) and permeabilized with 90% ice-cold methanol (FisherSci). Fluorescently labeled flow cytometry antibodies against IRF4 (3E4), CD69 (H1.2F3), and Egr2 (erongr2) were purchased from eBioscience. Antibodies against CD8a (53-6.7), CD8b (53-5.8),
CD25 (3C7), CD90.2 (53-2.1), pErk1/2 (4B11B69) were purchased from BioLegend. Antibodies against NFAT1 (D43B1), NF-κB p65 (D14E12), c-Fos (9F6), and c-Myc (D84C12) were purchased from Cell Signaling Technology. Anti-CD45.1 (A20) was purchased from BD Pharmingen. PE-conjugated F(ab')2-goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody was purchased from Invitrogen.

**Cell Sorting**

Samples of stimulated CD45.2+ OT-I cells and CD45.1+ wildtype splenocytes mixtures were stained with CD8a and CD45.2 antibodies and 7-AAD and OT-I cells were sorted (BD FACSaria) into 100% FBS and pelleted.

**RNA-seq library preparation**

Total RNA from ~300,000 OT-I cells per sample was collected with the RNeasy micro kit (Qiagen) with a 15-minute on-column DNase digestion (Qiagen) to remove genomic DNA. Total RNA quality and quantity was determined with fragment analysis (University of Massachusetts Medical School Molecular Biology Core Lab) and Qubit Fluorometer (Invitrogen) analysis. cDNA libraries were generated following a modified paired-end SMART-seq protocol (Kriegsman et al., 2019; Ramsköld et al., 2012). Briefly, at least 20 ng of input RNA was used for reverse transcription with
SMARTscribe reverse transcriptase (Clontech). Whole transcriptome amplification (WTA) was performed with Advantage 2 polymerase (Takara Bio). WTA reactions were monitored with qPCR to determine optimal cycle number. WTA libraries were then sized-selected with AMPure XP DNA SPRI beads (Beckman Coulter), tagmented with Tn5 transposases (Illumina Nextera XT), barcoded, and amplified with cycle number determined via qPCR monitoring. Final libraries were further size-selected with SPRI beads to an average size of 300-500 bp and quality was assessed with fragment analysis and Qubit analysis. Libraries were pooled and sequenced on a NextSeq 500 sequencer (Illumina).

**Processing and analysis of RNA-seq reads**

Adapter sequences were trimmed from quality raw sequencing reads with Trimmomatic-0.38 (Bolger et al., 2014) and then aligned to mouse ribosomal RNA with Bowtie2 v2.3.2 (Langmead and Salzberg, 2012). Unaligned reads were retained, and gene expression was estimated (transcripts per million and expected counts) with RSEM v1.2.29 (Li and Dewey, 2011) configured to align to a mm10 RefSeq transcriptome with Bowtie2 v2.3.2. Samples were filtered to retain expressed genes (expected counts > 200) and batch effects between replicates were corrected with limma v3.42.2 (Ritchie et al., 2015). Differential expression analysis was performed with DESeq2 v1.26.0 (Love et
al., 2014) to identify induced genes (stimulated conditions vs. unstimulated controls) or condition-specific changes in expression (untreated vs. PRN-694 or N4 vs T4 OVA peptides). Hierarchical clustering and k-means clustering of differentially expressed genes was performed within R v3.5. Heatmap visualizations of gene clusters were drawn with ComplexHeatmap (Gu et al.).

**Gene ontology**

I utilized the R package msigdb v7.0.1 to compare clusters of differentially expressed genes with the Molecular Signatures Database (MSigDB) Hallmark (H) and Immunologic (C7) gene sets (Godec et al., 2016; Liberzon et al., 2015; Subramanian et al., 2005). The top five terms with FDR <0.05 were displayed in the Results.

**ATAC-seq library preparation**

Precisely 50,000 stimulated OT-I T cells were FACS sorted at the same time as RNA-seq samples and pelleted in 100% FBS. ATAC-seq libraries were generated similarly as described in Buenrostro et al., 2013 (Buenrostro et al., 2013). Briefly, cell nuclei were isolated and transposed with 8 µL Tn5 (Illumina Nextera) at 37°C for 60 minutes. DNA fragments were isolated with a Clean and Concentrator Kit (Zymo) and then Illumina barcoded and amplified with NEBNext High-Fidelity 2x PCR Master Mix (New England Biolabs). A portion
of the reaction was performed as a separate qPCR reaction to determine ideal cycle number. Samples were then size selected with SPRI beads to include fragments up to 450 bp, ensuring to maintain small (<200 bp), nucleosome-free fragments. Quality of final ATAC-seq libraries was assessed with fragment analysis and Qubit analysis. Libraries were then pooled and sequenced on an Illumina NextSeq 500.

Alignment and processing of ATAC-seq reads

Adapter sequences were trimmed from raw sequencing reads with Cutadapt v1.3 (Martin, 2011) and then aligned to the mouse genome (mm10) with Bowtie2 v2.1.0 with the parameter -X 2000. PCR duplicates were removed with Picard's markDuplicates v2.17.8 and aligned reads were sorted and filtered with SAMtools v1.4.1 (Li et al., 2009). For visualization of fragment coverage TDFs were generated with IGVTools v2.3.31 (Robinson et al., 2011; Thorvaldsdóttir et al., 2013).

Peak calling

Aligned ATAC-seq reads were trimmed to 29 bases closest to the Tn5 cut site with bedtools v2.26.0 (Quinlan and Hall, 2010) and then peaks were called with MACS2 v2.1.1 (Zhang et al., 2008) using parameters --bw 29 --nomodel -q 0.0001. Summits of called peaks across all samples were merged and
“slopped” ±100bp to create a master peak reference file using bedtools v2.26.0. Peaks were annotated with names of closest genes using a mouse OT-I TSS BED file and bedtools v2.26.0 (closest -D ref -t all). Peaks with summits within 500 bp from TSSs were labeled “promoter” peaks and summits further than 500bp were labeled “enhancer” peaks. Peak coverage for each ATAC-seq sample was calculated using bedtools v2.26.0 (intersect; coverage).

**Differential Peak Analysis**

Calculated peak coverage values for two ATAC-seq replicate experiments were used as input for differential analysis using DESeq2 v1.26.0. All differential peaks (compared to unstimulated controls) were clustered using either hierarchical or k-means clustering methods within R. Heatmaps were generated with ComplexHeatmap.

**Motif enrichment.**

Each cluster of annotated differentially accessible peak regions was tested for de novo motif enrichment using HOMER v4.10.3 (findMotifsGenome.pl -size 200) with background comprised of peak regions found in all other clusters (Heinz et al., 2010).
Genomic Visualizations

Genomic tracks were created by plotting BigWig files with help of Gviz (Hahne and Ivanek, 2016) PMA and ionomycin ATAC-seq tracks and NF-κB ChIP-seq tracks were extracted from publicly available sources (Mognol et al., 2017; Oh et al., 2017).
Chapter III:
Differential TCR signaling within individual CD8+ T cells

Preface

This chapter encompasses the principal findings presented in the following publications:


As well as experiments in the prepared manuscript:

Summary

In this chapter I present experiments that measured NFAT, NF-κB, and Erk1/2 responses in single CD8+ T cells. In an attempt to connect observed graded gene expression induced at 24 in response to graded TCR stimuli, I hypothesized that Ca2+/NFAT signaling was differentially tunable by signal strength and ITK activity. Single cell flow cytometry data measuring individual pathway activation did not support this hypothesis. Instead, the results suggest naive CD8+ T cells largely “switch on” downstream signaling pathways digitally, as measured with NFAT1 translocation and pErk1/2 activation. The rate of digital activation is dependent on the strength of stimulus, with weaker TCR inputs or ITK inhibition reducing the frequency that single T cells would active within a clonal pool. However NF-κB was an exception to these observations, requiring strong TCR stimulation to elicit robust activation, and was specifically sensitive to inhibition of ITK activity. These results imply separate downstream signal pathways activate independently from one another at different thresholds of TCR stimulation. Additionally they highlight a supportive role for ITK: amplifying the strength of TCR signaling within T cells to robustly activate all divergent pathways under its influence.
Introduction

When I began in the Berg Lab, preliminary data (now published) had demonstrated that TCR signal strength carefully tuned the peak amount of IRF4 expressed in activated T cells after 24 hours (Conley et al., 2020). Under our in vivo testing conditions, IRF4 induction had a temporal window of peak expression; weaker-stimulated cells did not accumulate additional IRF4 protein at later time points. Regulating the concentration of specific OVA peptide variants presented on APC cell surfaces could modulate the amplitude of IRF4 expression. These experiments demonstrated the quality of early TCR signaling had considerable effects in regulating the effector programming within newly-activated cells.

At the same time, another series of experiments utilizing OT-I Rag1-/-Itk-/- mice was teaching us how ITK activity also tuned IRF4 expression. However, the role of ITK activity was nuanced, as it was more beneficial during moderate or weaker signaling conditions. When stimulated with moderate or low-affinity altered OVA peptides (T4 or G4), activated OT-I Itk-/- cells (based on digital CD69 expression) accumulated notably less IRF4 protein than WT OT-I cells (Fig. 3.1A-B). OT-I Itk-/- cells stimulated with strong, native (N4) OVA peptide had a lessened, but consistent defect in IRF4 expression. Treating WT OT-I cells with different doses an ITK inhibitor
modulated IRF4 expression gradually within T cells that were digitally CD69 positive (not shown). Increasing the dose of a peptide presented to Itk−/− T cells could not fully recover the defect in IRF4 expression compared to WT (Fig. 3.1B). Similarly, high concentration of weaker T4 or G4 peptides could not reach the same maximum IRF4 expression achieved with high affinity N4 peptide. However, increased peptide concentration could consistently
recover deficiency in CD69 expression due to lack of ITK or stimulation with weak affinity peptide (Fig. 3.1A). From these experiments my colleagues concluded that TCR input and ITK activity modulated graded gene expression within cells that switched on digitally. Signal transduction beyond proximal TCR triggering required further examination to better understand the mechanism that achieves these expression patterns.

The preliminary results above supported previously developed concepts that ITK activity does not steadily amplify all TCR signals, but rather its contribution is more beneficial during weaker signaling conditions (Andreotti et al., 2010). It was unclear whether the additive signal contribution of ITK activity is limited or ITK activity is biochemically regulated, or both. Further, gene expression in activating CD8+ T cells displayed differential sensitivity to ITK activity (and broadly, signal strength), as demonstrated by the disparate patterns in expression when comparing genes like CD69 and IRF4.

There is evidence that connects TCR ligation with digital T cell activation. Increasing the dose of peptide reliably increases the proportion of responding T cells as measured by IL-2, IFN-γ cytokine, or CD69 and Myc expression (Altan-Bonnet and Germain, 2005; Conley et al., 2016; Das et al., 2009; Huang et al., 2013; Preston et al., 2015). However, the amount of protein measured within single cells is similar. I observed CD69 reliably switched on
digitally in individual cells, but IRF4 expression was malleable and dependent on the strength of signal or ITK activity (**Fig. 3.1A-B**).

The question of how digitally switched T cells regulate graded gene expression remained unanswered. Does the complex orchestration of diverse intermediate downstream signaling pathways reflect both the digital switching behavior of the proximal TCR and analog tuning capability? Does ITK play a role in such differential patterns of signaling?

Because the primary substrate for ITK is PLC-\(\gamma\)1, I focused my efforts on characterizing major signaling pathways downstream of PLC-\(\gamma\)1 simultaneously in activating cells. This included pathways terminating with NFAT (Ca\(^{2+}\)) and NF-\(\kappa\)B transcription factors, and the Erk/MAPK cascade. Digital signaling in individual cells has been best characterized by digital phosphorylation of Erk, a phenomenon achieved through both positive and negative feedback mechanisms (Altan-Bonnet and Germain, 2005; Das et al., 2009). Digital activation of both the Ca\(^{2+}\) pathway and the NF-\(\kappa\)B pathway have also been described under some conditions (Dura et al., 2016; Kingeter et al., 2010; Podtschaske et al., 2007).

I posited that separate pathways do not always respond similarly to a TCR stimulus. For example, what is the relative amount of Ca\(^{2+}\) activation within single cells during TCR conditions that digitally trigger pErk? Pathways may require different thresholds of upstream TCR stimulation or
ITK activity, creating conditions that could impart imbalance in activation of NFAT, NF-κB, and MAPK. I reasoned factors not acting in concert may have implications for differential regulation of activation-associated transcription, which I had already observed. I believed close examination of simultaneous pathway activation would better link proximal TCR signaling mechanics with graded gene expression.

Preliminary data (now published) using altered OVA peptide-stimulated OT-I cells hinted that graded IRF4 regulation may be more dependent on Ca$^{2+}$ arm of PLC-γ1 dependent signaling (Conley et al., 2020). Titrated amounts of ionomycin, which increases intracellular Ca$^{2+}$, induced precise, graded IRF4 expression (Fig. 3.1C). PMA addition however, which mimics DAG, induced a small amount of IRF4 in a digital manner. This information, combined with the previous knowledge that Itk$^{-/-}$ T cells display significant defects in Ca$^{2+}$ signaling, I hypothesized that the Ca$^{2+}$ (NFAT) pathway would be more incrementally sensitive to TCR signal strength and ITK activity, while other pathways (e.g. NF-κB, MAPK) digitally respond to a threshold amount of TCR signaling (Fig. 3.2)(Atherly et al., 2006; Donnadieu et al., 2001; Liu et al., 1998; Miller et al., 2004; Schaeffer et al., 1999). I sought to measure each pathway simultaneously in OT-I cells to determine their relative activation.
I chose to utilize a TCR transgenic mouse model that would allow us to stimulate and analyze pools of naive, clonal CD8$^+$ T cells. The well-described OT-I TCR transgenic model offered CD8$^+$ cells that elicited robust TCR mediated T cell activation when stimulated with native ovalbumin peptide, but also had available a spectrum of altered OVA peptides of variable $k_{\text{off}}$ values and with varied measured functional avidities (Hogquist et al., 1994; Zehn et al., 2009). Splenic T cells isolated from OT-I Rag1--/-- mice cannot

Figure 3.2. Hypothesis: ITK activity tunes graded gene expression through differential activation of Ca$^{2+}$/NFAT signaling.

While the expression of genes like CD69 is not directly proportional to the signaling input at 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 duration of TCR binding, increasing the stability of activated 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. I hypothesized that the calcium signaling pathway is uniquely sensitive to ITK and can provide a mechanism for graded IRF4 expression. Adapted from Conley et al., 2016.
rearrange an endogenous receptor and only express an OT-I TCR. Thus all isolated CD8+ T cells were clonal and had not experienced antigen (all naive), which I could confirm by surface staining for activation markers such as CD44.

A tool that proved indispensable for my analyses was PRN-694, a covalent inhibitor of both ITK and RLK (Zhong et al., 2015). Naive CD8+ T cells express both ITK and RLK, but ITK predominates (Andreotti et al., 2010; Berg et al., 2005; Zhong et al., 2015). Splenic T cells from germ line Itk-/- mice develop in the thymus without the benefit of ITK, thus experience different selection conditions. Mature 'naive' Itk-/- cells present a 'memory like' phenotype in the periphery and may exhibit compensatory transcriptional programming, resulting in different expression of other Tec kinases (e.g. RLK). This makes comparisons between peripheral WT cells and Itk-/- cells less than ideal. Treating mature naive WT OT-I cells with PRN-694 allowed us to carefully inhibit the ITK-dependent PLC-γ1 phosphorylation without Itk-/- phenotypic effects.
Results

Nuclei flow cytometry assay optimization

To observe activation of separate TCR signaling pathways in individual cells, I optimized a flow cytometry assay that measures transcription factor protein content in ex vivo mouse naive T cell nuclei. Factors such as NFAT and NF-κB are normally sequestered in the cytoplasm and translocate to the nucleus following TCR engagement (Gaud et al., 2018; Smith-Garvin et al., 2009; Trebak and Kinet, 2019). A colleague in the department initially recommended nuclear fractionalization followed by flow cytometry analysis, with which they were familiar in applications for mouse dendritic cells.

Consulting already developed procedures for immune cell nuclear fractionalization, I first tested gradient separation methods (Poglitsch et al., 2011). This utilized iodixanol, an osmotically neutral density gradient medium, which has the benefit for carefully tuning lysis conditions to separate live organelles. In brief, live stimulated cells were centrifuged through gradient layers of iodixanol, including one detergent layer to lyse the plasma membrane. Nuclei were extracted from the middle layers, then washed and stained for flow cytometry (Fig. 3.3A).

The gradient-based procedure generated clean OT-I nuclei free of cytoplasmic tubulin proteins, mitochondrial COX IV membrane proteins, but
contained histones and stained positive for DNA intercalating propidium iodide (Fig. 3.3B). One drawback to this procedure was poor throughput. Low nuclei yield and the unwieldy layering of one gradient tube per sample.
limited the number of experimental conditions that could be simultaneously tested.

I turned to a sucrose solution-based nuclei isolation technique that had the advantage of being performed in a 96-well plate. A similar procedure had also been previously utilized for mouse T cell nuclei flow cytometry (Podtschaske et al., 2007). To perform this method, stimulated cells or cell cultures were centrifuged and briefly washed “on plate” with sucrose detergent buffers for lysis. Nuclei were fixed and stained for flow cytometry (Fig. 3.3C). The sucrose-based assay produced viable nuclei for analysis similar to the density gradient procedure, as evident by smaller forward and side scatter size, β-tubulin staining and binding of propidium iodide, but was more scalable in practice (Fig. 3.3D-E). Validation with confocal imaging confirmed NFAT1 was sequestered to the cytoplasm in naive cells, but 30 minutes after adding the Ca\(^{2+}\) ionophore ionomycin NFAT1 localized nearly entirely in nuclei (Fig. 3.3F). Importantly, nuclei isolated from unstimulated OT-I cells lacked NFAT1 signal.

In order to measure more physiological NFAT, NF-κB and Erk responses and to compare with patterns of graded IRF4 expression, I wanted to stimulate naive OT-I cells with APCs presenting peptide antigens. To analyze isolated OT-I nuclei from mixed cultures, I stained the pool of naive OT-I cells with cell proliferation dye CellTrace Violet prior to co-culture with
peptide-loaded APCs (Fig. 3.4A-B). Because CellTrace is a succinimidyl ester dye that binds all free amines, CD8$^+$ T cell nuclei retained fluorescence after isolation and could be distinguishable from unlabeled APC nuclei during flow cytometry analysis (Fig. 3.4C) (Filby et al., 2015). This allowed close examination of transcription factor localization specifically within OT-I
nuclei immediately after stimulation by splenocytes presenting cognate peptide (Fig. 3.4D).

**TCR regulation of digital NFAT activation in single T cells.**

To test the hypothesis that TCR signal strength could tune Ca\(^{2+}\)/NFAT signaling in CD8\(^+\) T cells, I used flow cytometry to characterize the behavior of NFAT1 localization in OT-I nuclei during variable peptide antigen stimulation conditions. Time course experiments revealed native OVA peptide stimulation induced digital NFAT1 translocation that persisted for at...
least 4 hours (Fig. 3.5A-B). Decreasing the concentration of OVA peptide presented to OT-I cells reduced both the maximum percentage of NFAT1 responding cells and the rapidity of converting a NFAT1 positive population (Fig. 3.5A,C). Importantly, these experiments revealed weaker TCR stimulation did not produce appreciable differences in fluorescence intensity of NFAT1 within individual cells. This indicated NFAT1 activation was probabilistic in nature within single cells, where TCR stimulus controlled how likely a cell would cross a signaling threshold to digitally switch on NFAT1. My initial observations of digital NFAT1 pathway activation mirrored digital

Figure 3.6. NFAT1 export dynamics in OT-I nuclei.

(A) Line plots depicting median fluorescence intensity (m.f.i.) of NFAT1 in isolated OT-I nuclei stimulated similarly to Fig. 3.5. After 30 min of stimulation with OVA peptide, either 5 mM EDTA, 5 mM EGTA, or 100 nM FK506 was added to culture media where indicated. Samples were collected 0, 15, 30, 60, and 90 min after adding inhibitors. (B) Representative histograms of three experiments performed showing EGTA effects on NFAT1 exit from OT-I nuclei. OT-I cells were cocultured with bulk splenocytes pulsed with 100 pM of OVA peptide. After stimulation for 30 min to allow for nuclear translocation of NFAT1, 5 mM EGTA was added to culture, and samples were harvested after 0, 30, 60, or 90 min.
behavior of proximal TCR triggering, and did not support the notion that Ca\textsuperscript{2+} (NFAT) could be finely tuned in a graded manner (Altan-Bonnet and Germain, 2005; Das et al., 2009).

To test whether sustained NFAT1 nuclear localization was due to continued, productive TCR signals or a prolonged effect after the initial contact, cell contacts were disrupted with chelating agents ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) or ethylenediaminetetraacetic acid (EDTA), or a calcineurin inhibitor FK506. When added 30 minutes after stimulation with N4 peptide, which would allow for maximum NFAT1 import, all treatment conditions exhibited a similar pattern in retreat of nuclear NFAT1 signal (Fig. 3.6A). While strong N4 OVA peptide stimulation imported NFAT1 in rapidly in digitally switched cells, the NFAT1 export rate due to each agent appeared to be consistently slower, taking about 60 minutes to return to baseline. NFAT1 export also does not appear to be regulated digitally, as T cells treated with EGTA have intermediate intensity of NFAT1 fluorescence (Fig. 3.6B). Observations of rapid NFAT import and slower export has been described previously (Marangoni et al., 2013). It was likely that continued productive TCR signals maintained persistent Ca\textsuperscript{2+} flux and NFAT1 nuclear localization (DISC). These experiments also do not support the possibility that stable proportions of
NFAT1 responders within a stimulated OT-I population represent an equilibrium of NFAT1 import and export (shuttling).

To characterize the behavior of NFAT activation during weaker affinity TCR stimulation, naive OT-I cells were stimulated with splenocytes loaded with SIITFEKL (T4) altered OVA peptide (Zehn et al., 2009). T4 peptide stimulated cells also displayed digital NFAT1 activation, as a positive fraction of cells emerged over time in a bimodal distribution (Fig. 3.7A). Varying concentrations of either N4 (native) OVA peptide or T4 and weaker SIIGFEKL (G4) altered peptides demonstrated that higher concentrations of weaker peptides could not achieve the same maximum percentage of NFAT1 responders that was observed after native N4 peptide stimulation (Fig. 3.7B). This pattern looked similar to peptide affinity control of maximum IRF4 expression at 24 hours; higher doses of weaker peptides simply cannot achieve N4 equivalent maximum IRF4 expression (Fig. 3.1B). One difference in NFAT1 activation due to peptide affinity was a delay in the onset of the first NFAT1 positive cells. Higher dose of T4 peptide could achieve a similar maximum percentage of NFAT1 responding cells as a lower dose of high affinity N4 peptide (e.g. 100 nM T4 peptide and 1 nM N4 both elicit ~80% NFAT1 responders) (Fig. 3.7C). However, NFAT1 positive nuclei were not detected until 30 minutes after stimulation and did not reach maximum until 60 minutes. N4 stimulated cells however, began digitally activating NFAT1 as
early as five minutes and the peak response was before 30 minutes. Thus, conditions exist where a weaker affinity peptides can elicit a similar percentage of NFAT1 responding OT-I cells as high affinity peptide, but over a longer duration of activation. Peptide affinity and concentration are therefore not precisely interchangeable.

To test whether ITK activity tunes Ca$^{2+}$/NFAT activation within single cells, I stimulated OT-I T cells with splenocytes presenting T4 peptide, with and without ITK/RLK inhibitor PRN-694. ITK-inhibited T cells exhibited digital activation of NFAT1, but generated a smaller fraction of responders (Fig. 3.7D–E). This reduction of NFAT1 responders was consistent across all doses of T4 peptide tested (Fig. 3.7E). Constant digital NFAT1 behavior indicated ITK inhibition did not dampen the intensity of NFAT1 activation within single T cells, but instead regulated the threshold of activation, not supporting my initial hypothesis. Having a better understanding of NFAT signaling behavior in response to peptide dose, affinity, and ITK activity, I next turned to measure relative activation of other pathways downstream of PLC-γ1.

**Graded NF-κB activation within digitally switched T cells**

To directly compare other signaling pathways within cells that digitally switch on NFAT activation, I also measured simultaneous NF-κB (p65) nuclear
Figure 3.7. TCR signal strength and ITK activity drives digital NFAT activation in CD8\(^+\) T cells.

(A) Representative histograms of NFAT1 fluorescence in OT-I nuclei isolated after T cells were stimulated with WT splenocytes pulsed with indicated doses of T4 peptide for 30 min. (B) Line plots of the percentage of NFAT1\(^+\) nuclei after 30 min of stimulation with WT splenocytes pulsed with indicated doses of either N4, T4, and G4 altered OVA peptides. (C) Line plots of the percentage of NFAT1\(^+\) OT-I nuclei over a 60-min time course after cells were stimulated and WT splenocytes pulsed with varying doses of either N4 (left, blue) or T4 (right, red) peptides as indicated. (D) Representative histograms of NFAT1 fluorescence in OT-I nuclei after cells were stimulated for 30 min with WT splenocytes pulsed with 25 nM T4 peptide with or without 50 nM PRN-694 treatment. (E) Line plots of the percentage NFAT1\(^+\) values are shown for the T4 peptide dose response with and without 50 nM PRN-694 treatment. Nuclei were gated on CellTrace Violet\(^+\) events. Data are representative of three more experiments.
localization and phospho-Erk1/2 activation in stimulated OT-I cells.
Consistent with previous descriptions, Erk reliably activated digitally after stimulation with strong native N4 OVA peptide (Fig. 3.8A) (Altan-Bonnet and Germain, 2005; Das et al., 2009). Under the same strong signaling conditions, NF-κB (p65) translocated markedly in nearly all cells. However, weaker stimulation with T4 peptide revealed differences between the activation kinetics NFAT, NF-κB and Erk pathways. A T4-stimulated pool of naive OT-I cells continued to switch on NFAT1 and pErk1/2 signal in a digital manner, albeit at different rates and proportions (Fig. 3.8B). After 15 minutes of stimulation, half of the T4-stimulated population was NFAT1+ whereas only Erk1/2 signal was still minimal. This pointed to different signaling thresholds for digital activation of NFAT and MAPK pathways. NF-κB (p65) translocation during T4 stimulation did not occur in a bimodal manner, but gradually increased in fluorescence intensity over a period of 120 minutes and appeared to be independent of the NFAT1 and pErk1/2 activation state. This indicated that NF-κB activation may be tunable to TCR signal strength, even within cells that had surpassed stimulation thresholds for digital NFAT and Erk activation.

Further examination uncovered that the dynamics of p65 translocation is relatively limited during TCR stimulation in naive T cells, as it responded with increased intensity to alternative modes of activation. PMA and
ionomycin mimics the effects of DAG and Ca\(^{2+}\) respectively downstream of PLC-\(\gamma\) phosphorylation. PMA/ionomycin therefore induces activation by bypassing any normal signal regulation by the proximal TCR signaling machinery. Either N4 peptide stimulation or PMA/ionomycin treatment translocated NFAT1 in a similar proportion of OT-I cells and the fluorescence intensity appeared similar in either condition; consistent with digital signaling behavior (Fig. 3.8C). PMA/ionomycin treatment induced p65 with considerably increased fluorescence intensity when compared to N4 peptide-stimulated nuclei. This intermediate level of p65 activation indicated that even 'strong', near supraphysiological TCR signaling conditions (≥100nM
N4 peptide) incompletely induce p65 in single cells. Thus, T cells that switch on digital NFAT1 or Erk1/2 activation have a dynamic range of possible NF-κB activation states.

**ITK activity tunes graded NF-κB signaling**

To assess how ITK regulates simultaneous activation of NFAT, NF-κB, and Erk, OT-I cells were peptide-stimulated with or without addition PRN-694. I first tested the same range of concentrations of N4 (native), T4, or G4, peptides utilized in experiments that characterized IRF4 expression and digital NFAT1 translocation (Fig. 3.1A-B, Fig. 3.7B). When ITK was inhibited, the fraction of NFAT1 positive nuclei was reduced during all peptide stimulation conditions, but least so in cells stimulated with strong N4 peptide (Fig. 3.9A). NF-κB (p65) translocation was notably hindered by ITK inhibition at all doses and affinities of peptide, including N4 stimulation (Fig. 3.9B). These results supported a disparity between simultaneous NFAT1 and NF-κB responses within single cells.

Time course analysis during stimulation with single doses of either N4 or T4 peptide showed that NFAT1 and Erk activation patterns continued at least 4 hours after initial TCR contact and maintained digital activation behavior during PRN-694 treatment (Fig. 3.9C). NFAT1 translocation continued to be less sensitive to ITK inhibition during N4 stimulation (Fig.
Figure 3.9. ITK inhibitor PRN-694 selectively dampens NF-κB activation.

(A-B) Line plots depicting NFAT1 or NF-κB (p65) activation in response to 30 minute stimulation by WT splenocytes loaded with indicated doses of N4, T4, or G4 peptide, with or without treatment with 50 nM PRN-694. (C) Line plots depicting either NFAT1 or NF-κB fluorescence within OT-I nuclei or p-Erk fluorescence in OT-I cells over 2-hour stimulation with APCs plus 100 nM of indicated peptide with or without 50 nM PRN-694. Histograms to the right represent nuclear or whole cell fluorescence patterns after 60 minutes of stimulation. (D) Histograms depicting NF-κB (p65) fluorescence within NFAT1+ nuclei after 1-hour stimulation in conditions as in C. (E) Line plots of change in normalized NFAT1 MFI (%) or normalized NF-κB (p65) MFI (%) within NFAT1+ OT-I nuclei after 1-hour stimulation with APCs plus 100 nM N4 or T4 OVA peptide, in the presence of titrated concentrations of PRN-694. Histograms shown are representative of 3 or more independent experiments.
Interestingly, the proportion of Erk1/2 activation in OT-I cells appeared unaffected by treatment with PRN-694, but weaker T4 peptide stimulation reduced the responding fraction by almost half compared to N4 (from ~75% to ~40%), indicating digital MAPK activation relies less on ITK activity under these conditions.

Measurement of NF-κB (p65) activation over the same 4-hour time course revealed either stimulation with weaker T4 peptide or ITK inhibition dampened the magnitude of the NF-κB (p65) nuclear fluorescence by two thirds (from ~75% to ~25%) (Fig. 3.9C). The effect of PRN-694 treatment was most apparent during N4 stimulation conditions, mirroring the effect during different peptide concentrations (Fig. 3.9C,B). Importantly, while weakened peptide stimulation or PRN-694 treatment reduced the proportion of NFAT1 responding cells, NF-κB fluorescence was reduced within nuclei that were similarly NFAT1 positive (Fig. 3.9D). This indicated NF-κB activation was not directly dependent on the state of Ca^{2+}/NFAT activation. NFAT1 translocation exhibited a lower threshold for digital activation, while p65 relied on stronger TCR input and the aid of ITK, albeit with less consistent translocation patterns on a per cell basis.

Titration of PRN-694 during N4 or T4 peptide stimulation further supported NF-κB translocation is consistently sensitive to ITK activity.
Because PRN-694 reduces the proportion of activated responders, I compared the NFAT1 and NF-κB MFI within only NFAT1 positive nuclei. PRN-694 titration progressively dampened the MFI of NF-κB (p65) within single N4 to T4 stimulated nuclei (Fig. 3.9E,D). NFAT1 MFI within NFAT1 positive cells was more resistant to PRN-694 addition, especially during strong TCR stimulation. This was further evidence that ITK activity modulates the probability of all-or-none NFAT1 activation in single T cells (Fig. 3.9E).

While NFAT activation is directly influenced solely by activation of calcineurin, NF-κB proteins have multiple signaling entry points that can influence their activation (Lucas et al., 2004; Smith-Garvin et al., 2009). This includes DAG activation of IKK complex proteins as well as Ca\(^{2+}\) activation of CaM kinases (Ishiguro et al., 2006). As ITK activity is upstream of both DAG and Ca\(^{2+}\), it could possibly have multifactorial control over NF-κB. The experiments above show NF-κB activation is not directly dependent on NFAT1 activation state, however the highest NF-κB (p65) MFI for any stimulation condition was usually within NFAT1 positive cells, which may reflect additive Ca\(^{2+}\) and DAG influence as NF-κB inputs.

To separate DAG and Ca\(^{2+}\) components of NF-κB activation in OT-I cells, PMA or ionomycin was supplemented to wells of OT-I cultures during
N4 stimulation with or without PRN-694. Ionomycin addition to cultures during N4 stimulation did not modify the NFAT1+ fraction (which was already >90% responders) or modulate the NFAT1 fluorescence intensity, but did increase the proportion NFAT1 responders during T4 stimulation as expected (Fig. 3.10A-B). Ionomycin alone (in the absence of peptide stimulation) was sufficient to maximally translocate NFAT1, but did not induce appreciable NF-κB translocation (Fig. 3.10A). Ionomycin supplementation during N4 or T4 peptide stimulation increased NF-κB (p65) fluorescence intensity within individual nuclei, indicating the threshold of Ca^{2+} flux sufficient to translocate NFAT1 is not the same as the maximum contribution to NF-κB translocation. PMA supplementation maximized NF-κB (p65) translocation in
N4 or T4 stimulated cells and easily recovered the defect in NF-κB signaling due to ITK inhibition with PRN-694. These experiments demonstrated that under physiological peptide stimulation, separate downstream pathways are differently sensitive to the secondary messengers generated from the results of ITK activity. NF-κB activation relies more heavily on the amount of DAG, rather than Ca^{2+}, to tune translocation.

These supplementation experiments also highlighted the synergistic nature of Ca^{2+} and DAG signaling in NF-κB activation. Either ionomycin or PMA individually improved p65 translocation in N4 or T4 peptide-stimulated T cells to some degree (Fig. 3.10A-B), but neither ionomycin nor PMA supplementation alone could produce NF-κB (p65) translocation as impressively as simultaneous stimulation with both. Precisely how DAG and Ca^{2+} signaling branches converge to maximize NF-κB is unclear.

Collectively, the experiments in this chapter provided evidence that activation of the NF-κB pathway can be modulated gradually via TCR stimulus or ITK activity, even within cells that digitally switch on other pathways such as NFAT or Erk. This demonstrated differential downstream signaling patterns exist in individual activating T cells experiencing slightly different TCR stimulation.
A model of TCR downstream signaling can be arranged that summarizes the simultaneous and separate activation of NFAT, NF-κB, and MAPK in naive CD8+ T cells, including how ITK benefits each pathway differently (Fig. 3.11). MAPK and NFAT activation responded digitally at thresholded amounts of TCR signaling, represented by sharply transitioning activation curves (Fig. 3.11A). NF-κB activation was more gradual and
required stronger TCR stimulation to achieve robust activation. ITK activity provided least benefit to MAPK, shifting the threshold slightly, and a greater effect on NFAT, but the response of each remained digital. NF-κB activation benefited most from presence of ITK, as it enhanced the magnitude of activation.

Tracking hypothetical single cell trajectories over time depict TCR signal strength has control over the rapidity and fractional response of MAPK an NFAT activation in a stimulated clonal T cell pool (Fig. 3.11B). Strong signaling converts greater percentage of NFAT and MAPK responders in a shorter period of time, while weaker signaling converts a reduced fraction of responders over a longer period of time. Precision of when individual cell responses occur are less predictable during weaker signaling conditions. For NF-κB however, weaker signal strength has the effect of delaying activation and reducing the maximum intensity of the response, which is evidence of graded amounts of activation. Accompanying histograms provide hypothetical population ‘snapshots’ of the single cell trajectories drawn in the line plots, mirroring the real results recorded in the experiments in this chapter.
Discussion

The experiments presented in this chapter offer three main findings that provide new insight into the simultaneous orchestration of signaling pathways downstream of TCR triggering in naive T cells: First, they illustrate a more complete appreciation of the probabilistic nature of T cell activation. Within a stimulated clonal pool, extracellular constraints such as peptide dose and affinity, and activity of intracellular TCR proximal machinery including ITK, all independently regulate the proportion of digitally responding T cells. Second, within cells that digitally trigger the TCR, downstream pathways still can respond separately. Digital NFAT and Erk1/2 activation in OT-I cells are sensitive to different strength of TCR input, indicating they trigger at different thresholds. NF-κB activation consistently required stronger signal strength than NFAT1 to elicit robust activation and was most sensitive to ITK inhibition. Third, these experiments provide evidence for graded signaling patterns within activating cells, modulated in part due to ITK activity. Varying peptide affinity, dose, or treatment with ITK inhibitor modulated the fraction of digital NFAT1 responders, but dynamically controlled discrete amounts of NF-κB translocation. NFAT nuclear intensity was consistent under chemical stimulation with PMA/ionomycin, whereas those same conditions maximized NF-κB translocation, surpassing the limits
of peptide stimulation. Together, these data expand the understanding of a functional role for ITK in amplifying TCR signals: ITK activity lowers the TCR stimulation requirement for downstream pathways unequally, as separate pathways embody differing sensitivity to ITK-regulated messengers DAG and Ca\textsuperscript{2+} flux.

A primary goal for this research was to better understand the function of ITK. Since its discovery, great strides have been made in characterizing its expression and molecular regulation, as well as the discovery of its function in catalyzing PLC-\(\gamma\)1 activation (Andreotti et al., 2010). Yet mystery enshrouds ITK, as it is a relatively weak kinase within the Tec kinase family and its activity is not absolutely required for T cell activation (Andreotti et al., 2010; Joseph et al., 2013). Some of the earliest studies demonstrated that Itk\(^{-/-}\) mice can clear LCMV, vaccinia virus (VV), and vesicular stomatitis virus (VSV) similar to controls, albeit with slower effector T cell proliferation rate and weaker viral clearance kinetics (Atherly et al., 2006; Bachmann et al., 1997). Because primary Itk\(^{-/-}\) T cells exhibited dampened downstream Ca\textsuperscript{2+} and Erk signaling, it is thought ITK functions to amplify TCR intracellular signals and is not an all or none component to the TCR machinery. The data in this chapter adds to this concept. It is now clear that ITK does amplify downstream signals, but differentially lending aid to specific pathways. NF-\(\kappa\)B receives more benefit to ITK activity than NFAT or MAPK.
Digital calcium and NFAT signaling in activating T cells

My initial hypothesis proposed TCR signal strength and ITK tuned graded Ca\(^{2+}\)/NFAT signaling in a graded manner; implied from the ability of ionomycin to tune graded IRF4 expression. Examination of NFAT1 activation in peptide-stimulated nuclei did not support this hypothesis. I consistently measured clear, digital NFAT1 behavior switching on in OT-I cells. Peptide dose, affinity, or ITK inhibitor all modulated dynamics of the responder fraction of the OT-I pool. Digital NFAT1\(^+\) activation in primary mouse and human T cells has been reported previously (Podtschaske et al., 2007).

However, the experiments presented here utilized careful control of TCR signaling inputs to reveal NFAT1 behavior in clonal cells, as well as further simultaneous comparisons with other signaling pathways.

Store operated calcium entry (SOCE) can be thought of as an all or none event. Ca\(^{2+}\) depletion from the ER triggers STIM1/2 activation and rapid extracellular Ca\(^{2+}\) entry through ORAI channels in the plasma membrane (Trebak and Kinet, 2019). These events can occur in oscillations, made up of Ca\(^{2+}\) flux and refractory periods. Using an encoded calcium biosensor, Andrey Shaw and colleagues were able to measure patterns of Ca\(^{2+}\) flux in peptide-stimulated OT-I cells (Le Borgne et al., 2016). They observed transient, oscillatory, and sustained calcium flux patterns quickly after contact with APCs. Lowering peptide dose or affinity mildly decreased the percentage of
sustained Ca\textsuperscript{2+} responders and increased the percentage of oscillatory responders. NFAT1 proteins are rapidly de-phosphorylated by calcineurin and import to the nucleus quickly after the initial Ca\textsuperscript{2+} flux, but the NFAT nuclear export rate is slower, likely allowing NFAT to persist in the nucleus through refractory periods of Ca\textsuperscript{2+} oscillations, which can offer “signal memory” (Marangoni et al., 2013; Okamura et al., 2000). In the experiments in this chapter, NFAT1 persisted in the nuclei of responder cells for hours after initial contact. While the discrimination of cells based on sustained vs. oscillatory Ca\textsuperscript{2+} flux is possible, both states may induce similar patterns of NFAT activation. The maximum percentage of NFAT1 responders was dictated by TCR peptide affinity, dose, or ITK inhibition. This limit may have underlying correlation with the ratio of sustained/oscillatory and transient responders.

Conventional measurement of Ca\textsuperscript{2+} flux in T cells is performed by calculating the ratio of two calcium sensitive fluorescent dyes in a stimulated pool on a flow cytometer observed over time. This assay was critical in identifying initial Ca\textsuperscript{2+} signaling defects in ITK deficient mice (Atherly et al., 2006; Schaeffer et al., 1999). Because each cytometer event is a different cell, the results represent an average of the Ca\textsuperscript{2+} flux within the population. Representations of fractional Ca\textsuperscript{2+} responses within the population are lost. Utilizing flow cytometry of isolated stimulated cell nuclei, the experiments
presented here offer improved analyses which measure responder and non-responder Ca\textsuperscript{2+}/NFAT1 fractions within the stimulated pool. Weaker stimulated OT-I cells consistently exhibited slower NFAT1\textsuperscript{+} conversion than stronger stimulated cells, which may be an example of the probabilistic nature of NFAT activation behaving as a digital switch, or alternatively, may be indicative of inherent heterogeneity within naive T cells. Another study cleverly monitored single OT-I cell Ca\textsuperscript{2+} responses by tracking hundreds of individual peptide-loaded APCs and naive OT-I cell pairs in microfluidics chambers (Dura et al., 2015). Similar to NFAT1 results here, they demonstrated that OVA peptide concentration modulates the overall fraction of cells that spike Ca\textsuperscript{2+} and the rapidity of the population conversion. The benefit of single cell tracking made it clear strong TCR signals appeared to condense the period during which single responder cells spiked Ca\textsuperscript{2+}, appearing more synchronous. Weaker signaling delayed and dilated the period of response, with individual Ca\textsuperscript{2+} spikes appearing more scattered across the population. Importantly, individual cells that exhibited strong, rapid Ca\textsuperscript{2+} spike after peptide stimulation were more likely to exhibit similar patterns during repeat stimulation. This was an indication for heterogeneity of signaling capabilities inherent within a clonal OT-I pool. Whether fractional NFAT1 (and similar patterns that can apply to Erk1/2) responder
populations are due heterogeneity or are a manifestation of probabilistic effects of TCR triggering is still not clear.

**Signal strength and T cell motility**

Although not directly addressed here, another important factor linking TCR signal strength to the kinetics of T cell activation is the effect of cell motility. Previous in vivo imaging experiments tracked naive T cell interactions with dendritic cells within live mouse lymph nodes and discovered that early activation can be divided into three temporal phases (Mempel et al., 2004). T cells first scan, a period where they have many brief encounters with DCs, followed by a second period of stable interactions, and finally a third phase of rapid proliferation and motility. A subsequent study revealed that the duration the T cell scanning phase is largely controlled by the concentration of presented peptide on DCs (Henrickson et al., 2008). Weaker stimulated cells scanned for longer periods of time before transitioning to form stable DC contacts. Also, splenic CD8+ T cells that encountered weak affinity peptides during *Listeria* infection migrated to the blood sooner than T cells that encountered strong affinity peptides (Zehn et al., 2009). Thus weakest affinity clones are less expanded and often found at the site of infection first. Others have rationalized these mechanisms would
maximize the expansion of clones that experienced highest-affinity interactions.

Whether signal strength control of cell motility has a role in the activation kinetics of OT-1 cells in the ex vivo stimulation experiments presented here is not clear. Evidence for “three phase” T cell activation without lymph node structures or stromal cells in culture dishes is also lacking. There is evidence of a role for ITK in actin polymerization, which is critical in cytoskeletal rearrangement and cell polarization to appropriately form contacts with APCs (Comrie and Burkhardt, 2016; Dombroski et al., 2005). *Itk*−/− mice or reduced ITK expression in Jurkat cells exhibited disrupted actin polymerization, with decreased activation of Wiskott-Aldrich syndrome protein (WASP) and decreased recruitment of the guanine nucleotide exchange factor Vav (Dombroski et al., 2005; Labno et al., 2003). Rescue with a kinase-inactive form of ITK was sufficient to reestablish actin polymerization and Vav recruitment. This suggested that ITK had a kinase-independent scaffolding role during activation-associated actin reorganization. Studies of T cell cytolytic function suggest that *Itk*−/− T cells can form synapses with target cells normally; whether this extends to naive cell contacts with APCs is untested (Kapnick et al., 2017).

Experiments in this chapter utilized PRN-694, a small molecule covalent inhibitor of ITK kinase activity, so it is plausible that scaffolding
functions of ITK could remain and measured temporal defects in signal pathway activation within ITK-inhibited cells are not due to decreased cell motility or actin polymerization (Zhong et al., 2015). Broad motility defects in activated mature T cell and autoreactive T cell migration in the periphery due to ITK deficiency have been described, but whether this is due to cytoskeletal organizational defects or dysregulation of cell surface adhesion molecules and homing receptors is not clear (Cho et al., 2020; Jain et al., 2013). Imaging experiments which track OT-I and APC interactions could be performed to investigate the degree at which TCR signal strength or ITK activity regulate OT-I cell motility in vitro stimulations with APCs.

**Digital Erk1/2 signaling in naive T cells**

Early experiments revealed that Erk1/2 activation was dampened in Itk-/- T cells (Atherly et al., 2006; Schaeffer et al., 2000; Schaeffer et al., 1999). Therefore it was surprising to see that Erk1/2 phosphorylation was insensitive to treatment with ITK inhibitor PRN-694 under conditions that reduced NFAT1 responders and reduced the magnitude of NF-κB translocation. However, it is noted that ITK deficiency also has minimal effect on digital CD69 expression at 24 hours (Conley et al., 2020). Bimodal CD69 expression, which is utilized as an early surface marker for T cell activation, correlates strongly with digital Erk1/2 activation patterns (Das et al., 2009;
Roose et al., 2007). Thus, CD69 expression patterns in Itk-/- mice may reflect earlier Erk1/2 behavior, supported by ITK inhibitor experiments presented here.

Later studies and the experiments in this chapter, measured strong digital pErk1/2 behavior in OT-I cells following stimulation with peptide:MHC on APCs (Altan-Bonnet and Germain, 2005). This behavior was found to be regulated by a positive feedback mechanism involving LAT-associated Grb2-SOS (Das et al., 2009). Serial TCR interactions with antigen generate analog accumulation of active Ras proteins via RasGRP in the TCR proximal environment. When Ras surpasses a threshold, positive feedback of SOS is triggered, which leads to digital activation of the MAPK cascade including Erk1/2. While PLC-ɣ1 production of DAG helps enhance RasGRP activation prior to SOS activation, PMA stimulation alone (a DAG mimic) bypasses TCR ligation and does not recruit Grb2-SOS, and displayed analog Erk1/2 activation through direct DAG-PKC-RasGRP-Ras-Raf signaling (Das et al., 2009; Roose et al., 2007). It is possible that DAG is not limiting in triggering SOS and dampened amounts of DAG generated when ITK is inhibited remain sufficient to elicit digital activation of Erk1/2. This could account for a lack of an effect of PRN-694 addition in my experiments.

Most of the Erk1/2 defects measured in early ITK knockout studies were via immunoblotting whole cell lysates from anti-CD3 antibody-
stimulated mouse T cells or Jurkat cells (Atherly et al., 2006; Schaeffer et al., 2000; Schaeffer et al., 1999). Fundamental differences between anti-CD3 stimulation and peptide:MHC stimulation are possible. One study comparing antibody stimulation and peptide stimulation reported soluble anti-CD3/CD8+ antibody stimulation induced immediate and more intense p-Erk1/2 activation in OT-I cells when were normalized to activation or other TCR proximal molecules (e.g. Zap70, LAT), while stimulation with conjugated SIINFEKL (OVA) peptide:MHC multimers was relatively weaker and delayed (Wang et al., 2008). Perhaps only presentation of physiological MHC multimers can organize TCR proximal machinery, promote conformational changes in TCR/CD3 complexes, as well as segregate phosphatases to properly regulate kinetic proofreading and ligand discrimination. Likewise crosslinking the TCR with soluble anti-CD3 may bypass any nuance associated with kinetic proofreading mechanisms of ligand discrimination and signal cascades.

p-Erk1/2 measurements here were made from separate (often plate-copied) replicates, but unfortunately not measured within the same cells that were used for simultaneous NFAT1 and NF-κB measurements. A caveat of nuclear preparation of samples is loss of ability to measure cytosolic proteins, including phosphorylated Erk1/2. Further, the p-Erk1/2 measurements were made from methanol-permeabilized cells, while isolated
nuclei were permeabilized with detergent reagents. Therefore, simultaneous measurement of p-Erk, NFAT, and NF-κB activation was not achieved. Ideally it would have been advantageous to measure all pathways within the same cells. This could be accomplished with imaging flow cytometry approaches that observe nuclear NFAT and NF-κB translocation and phospho-Erk1/2 fluorescence while not disrupting cell membranes.

**TCR signal strength regulates graded NF-κB activation**

A previous investigation into the behavior of NF-κB in naive T cells concluded TCR ligation triggered digital activation (Kingeter et al., 2010). They observed phosphorylation and degradation of inhibitor of κB (IκB)α and phospho-RelA in CD4⁺ and CD8⁺ T cells after stimulation with anti-TCRβ antibodies. However, the data presented in this chapter suggest otherwise, as NF-κB activation was less digital and more analog, especially as peptide stimulation was weakened or ITK was inhibited. This may be due to differences in stimulation conditions (antibody mediated TCR cross-linking vs peptide:MHC stimulation). Under the strongest stimulation conditions tested here (native N4 OVA peptide), NF-κB (p65) translocation was relatively fast and complete, but no stimulation conditions produced bimodal responses. Another study inhibited calcineurin with cyclosporinA in primary mouse and human T cells and reported a decrease in digital NFAT signaling.
and a graded decrease in NF-κB activation (Podtschaske et al., 2007). This also highlighted the multiple signaling inputs, including DAG and Ca\(^{2+}\), that converge on NF-κB activation.

The canonical NF-κB pathway describes DAG activation of PKC-Θ, and subsequent activation of the CARMA1-Bcl10-MALT1 (CBM) complex, leading to IKK complex activation. Active IKK complex phosphorylates IκBα, marking it for ubiquitylation and degradation (Cheng et al., 2011; Gerondakis et al., 2014; Lucas et al., 2004). While DAG activation is critical for NF-κB, there is evidence that supports a calcium component to NF-κB signaling. One group found that following TCR stimulation, calcium-sensitive calmodulin-dependent protein kinase II (CaMKII) enhances activation of CARMA1 and subsequent NF-κB activation (Ishiguro et al., 2006). Another important, early study carefully modulated intracellular Ca\(^{2+}\) levels in primary B cells and revealed differential NFAT and NF-κB responses (Dolmetsch et al., 1997). They observed NF-κB responded to transient, large increases in Ca\(^{2+}\) and NFAT activation was controlled by less intense, but more sustained Ca\(^{2+}\) levels.

My data may support a role for calcium in NF-κB activation, as both ionomycin and PMA supplementation during peptide stimulation enhanced NF-κB (p65) translocation. PMA alone unsurprisingly elicited NF-κB activation, but ionomycin alone did not. This could be due to complete lack
of CARMA1 activation in Ca\(^{2+}\) only conditions. The results do suggest however, that DAG component to NF-κB activation is likely more important. There may be a dynamic amount of DAG required to robustly activate NF-κB, and ITK inhibition, or weaker TCR stimulation, may lower DAG abundance considerably. The second messengers DAG and IP3 are produced in equimolar amounts when PIP2 is digested by PLC-γ1, but the data here highlight that different pathways may have differing requirements of thresholds and sensitivity to each messenger for activation.

Another insight is a new perspective on the limitations of signaling within naive cells. T cells interacting with APCs in a lymph node during a real infection experience varying intensity of co-stimulation that further amplifies activation states (Chen and Flies, 2013). The importance of “signal two” is well-described and paramount for a robust in vivo T cell response. NF-κB in particular is very sensitive to PKC-Θ and PI3K activation due to CD28 ligation and responds to activation of TNF family receptors such as 4-1BB and OX40 (Chen and Flies, 2013; Schmitz and Krappmann, 2006). CD28 enhanced NF-κB activity is key in regulating transcription at the Il2 promoter and inducing survival factor Bcl-xL, among numerous other roles (Boise et al., 1995; Marinari et al., 2004; Schmitz and Krappmann, 2006). In my experiments, PMA and ionomycin synergize to produce NF-κB activation far surpassing the strongest peptide stimulation conditions. These observations
underscore the need for co-stimulation and suggest graded NF-κB signaling in naive cells may act as a mechanism to tune the scale of functional, expanded T cell populations.

**Novel use of a nuclei isolation assay for flow cytometry**

Most of the data in this chapter were gathered using an innovative approach that measures translocation of transcription factors in isolated single T cell nuclei using conventional flow cytometry (Gallagher et al., 2018). Initially, I and others in my lab attempted to pursue imaging flow cytometry technology (FlowSight), with some success, but found limitations in analyzing small, naive primary mouse T cells. Conventional flow cytometry analysis of isolated T cell nuclei has the benefit of easy access to reagents, has high sample throughput, and greater fluorophore selections only limited by the cytometer. Combined with my methods for analysis of mixed primary cell stimulations using cell proliferation dyes, this assay is quickly adaptable and already proven to be a powerful tool to the Berg Lab and our collaborators. Some limitations exist however. One should be aware of the inability to stain surface proteins, due to removal of the plasma membrane. Additionally, many protein factors are not regulated via sequestration in the cytoplasm, thus
activation states may not be quantifiable by measured nuclear protein content.
Chapter IV:
ITK control of early gene transcription in activating CD8⁺ T cells

Preface

This chapter encompasses the principal findings presented in the following manuscript:


Summary

After 24 hours of activation, T cells express graded amounts of the transcription factor IRF4 dependent on the strength of TCR stimulus and level of ITK activity. To determine the extent TCR signal strength regulates the earliest gene transcription in naive cells, RNA-seq and ATAC-seq was performed on OT-I cells peptide-stimulated up to 2 hours, with or without treatment with ITK inhibitor PRN-694. A similar transcriptional profile was detected in all samples, independent of TCR signal strength, indicating a
single activation transcriptional program. Within the group of earliest genes detected (primary response genes) differential expression effects were observed. ITK inhibited cells had significant decrease in expression of nascent AP-1 transcription factor members, such as Fos, Fosb, and Fosl1, and NF-κB response genes. There was little difference in expression of Egr family members Egr2 and Egr3 and Nr4a family members. c-Fos protein accumulation was also limited in PRN-694 treated T cells. ATAC-seq experiments revealed early changes in chromatin sensitive to PRN-694 treatment were enriched for AP-1 and NF-κB motifs. These transcriptional patterns highlight how TCR signals can modulate accumulation of early gene products in cells and may be the consequences of differential TCR signaling patterns described in Chapter III.

Introduction

Prior to delayed effector-associated expression of genes like IRF4 that peak after 24 hours, naive T cells first undergo waves of transient, primary response gene (PRG) transcription that begin to change the metabolic and transcriptional state. These genes are the “first responders” to TCR-dependent transcription factor binding and activity (Fowler et al., 2011; Herschman, 1991; Tullai et al., 2007). Similar patterns of PRG expression are
found in many cell types where signaling causes exit from a quiescent state and entry to a metabolic state. Studies in receptor tyrosine kinase signaling revealed that first wave PRG transcripts such as Fos, Jun, and Myc do not accumulate appreciably, due to high AU-rich content marking them for degradation (Mayya and Dustin, 2016). While presence of PRG mRNA is short, there is evidence that T cells accumulate PRG products over longer durations as the result of sequential periods of TCR signaling (Clark et al., 2011).

While conducting signaling studies, it was becoming clear to me that most of the downstream signaling responses appeared digital with exception to NF-κB, which was specifically sensitive to weakened TCR stimulation or inhibition with ITK inhibitor PRN-694 (Chapter III). I hypothesized these differential signaling conditions have immediate effects on the balance of NF-κB-mediated PRG transcription transcription in stimulated naive OT-I T cells. If so, could these effects carry forward throughout later periods of activation?

A critical role for NF-κB in regulating T cell activation programming is clear. For example, NF-κB is important in upregulating survival factor BcL-xL (Boise et al., 1995). Early studies of the Il2 promoter indicated that NF-κB p65 had an active role in Il2 binding and early IL-2 expression (Jain et al., 1995). Induced NF-κB protein c-Rel also assists in IL-2 expression throughout T cell
activation. However, much of the literature is focused on combined TCR signaling and co-stimulation signaling via CD28 and TNF-family receptors that amplify NF-κB through intermediates like PI3K activity (Schmitz and Krappmann, 2006). It has been observed co-stimulation, cytokine signaling and continued TCR signaling all sum linearly to regulate T cell expansion late into the activation program (Marchingo et al., 2014). Thus, it was unclear whether NF-κB signaling plays a limited or active role in regulating the earliest waves of primary gene transcription.

Results

**TCR signal strength and ITK control of early gene transcription**

To evaluate the immediate transcriptional response during variable TCR signaling conditions, naive OT-I cells were stimulated in cultures with either N4 or T4 altered OVA-peptide loaded WT splenocytes, similar to the experimental conditions in Chapter III. To determine the extent of immediate transcriptional effects due to ITK activity, cells were also cultured with or without the ITK inhibitor PRN-694. cDNA libraries were then generated from sorted OT-I cells after 30, 60, or 120 minutes of stimulation. A distance matrix of variance-stabilized sequenced library results showed that sample replicates clustered similarly by stimulation condition (Fig. 4.1A). PCA analysis
revealed replicates grouped similarly, indicating largest variance components across samples were likely due to stimulation time and stimulation conditions (peptide and PRN-694 treatment) (Fig. 4.1B-D).

Figure 4.1. RNA-seq replicate correlation and principal component analysis
(A) Sample-to-sample correlation to compare RNA-seq replicates. (B-D) Principal component analysis of all RNA-seq replicates across all stimulation conditions.
Filtered, expressed transcripts were \( k \)-means clustered \((k=6)\) and groups were ordered according to temporal gene induction patterns (\textbf{Fig. 4.2A-B}). Clusters represented expression of immediate and delayed gene sets. The earliest gene groups (Clusters I and II) represented genes with transient abundance, with decreased expression by 120 minutes. Cluster I exhibited peak expression 30 minutes after TCR contact with peptide:MHC and was enriched for TCR signaling-related ontology terms. This included “AP-1 signaling,” “NF-\( \kappa B \) signaling,” and “NFAT signaling” (\textbf{Fig. 4.2C}). This indicated that TCR downstream signaling pathways immediately and directly regulate transcription of these early genes. TCR downstream pathway-related terms were also enriched in delayed gene clusters (Clusters II, III), suggestive of the effects of continued TCR signaling. Clusters of delayed genes first detectable at times greater than 60 minutes were enriched for terms linked to T cell effector function, “Myc targets” and cell cycle regulation (Clusters IV, V), and IL-2 signaling (Cluster III), representing secondary gene transcription beyond immediate TCR control and likely regulated by a mix of continuing TCR signaling and first wave gene transcription.

Clustered arrangement of the data also indicated decreased quality of TCR signaling induced similar patterns of transcription, but at lower abundance. Weaker TCR stimulation (T4 stimulation, PRN-694 treatment, or both) reduced transcript abundance within each cluster in a graded manner.
Figure 4.2. Inhibition of ITK dampens immediate TCR signaling-induced transcripts.

(A) Heatmap depicting mean variance-stabilized transformed (VST) normalized expression values of top 357 genes (Log2 fold-change > 2, mean expression > 1000, p-adj < 0.1) induced in OT-I CD8+ T cells at three timepoints (30, 60, and 120 minutes) after stimulation with APCs plus 100 nM N4 OVA or T4 altered OVA peptide with or without 50 nM PRN-694. Genes were grouped with k-means clustering into 6 clusters. (B) Line plots depicting Z-score of 2-hour expression time course of gene clusters identified in A. Scores for mean expression values for each cluster are grouped by conditions in A; N4 (blue), N4 + PRN-694 (red), T4 (orange), T4 + PRN964 (green). Total expression data for all conditions from three replicates are drawn in gray. (C) Enriched MSigDB signatures in gene clusters identified in A. Log$_{10}$ transformed adjusted p-values (FDR) of (up to) the top 5 terms (p-adj. ≤0.05) from both “Hallmark Gene Sets” and “Immunologic Signatures” MSigDB collections. Cluster VI did not contain gene set enrichments with these constraints. Data represent three separate biological replicate experiments each utilizing pooled splenocytes from three or more OT-I mice.
Whether an artifact of the limited resolution of pooled RNA-seq data or reflective of actual individual cell behavior, these results did not indicate any delay in gene induction during weaker stimulation conditions. For example, the earliest induced transcripts (Cluster I) peaked at 30 minutes, but this pattern was not shifted to a later time point during weaker stimulation. Based on data describing NFAT, NF-κB, and Erk signaling (Chapter III), delayed or asynchronous transcriptional activity would be predicted in weaker-stimulated cells.

These experiments allowed us to interrogate the immediate transcriptional response to TCR engagement and revealed that modulation of upstream TCR signal strength activates, overall, similar transcriptional programming. Because there was evidence that TCR signal strength and ITK regulates differential patterns in signal activation (Chapter III) and differential gene expression at 24 hours (Conley et al., 2020), it was possible this was connected to induction of a separate transcriptional program. However, weaker stimulation with T4 peptide or ITK inhibition with PRN-694 did not appear to induce a unique gene set. Differential expression analysis identified considerable overlap between significantly induced genes for each stimulation condition. While fewer genes were found to be significantly induced during weaker T4 stimulation \((n=284)\) or PRN-694 \((n=390)\) treatment, nearly all were also induced during N4 stimulation \((n=487)\) (Fig. 4.2B).
Figure 4.3. Graded TCR signaling induces a single transcriptional program.
(A-C) Indicated overlap of induced genes identified in each tested TCR signaling condition. N4 = blue (n=487), T4 = orange (n=284), N4 + PRN-694 = red (n=390), T4 + PRN-694 = green (n=122). (D-E) The effect of stimulation with weaker affinity peptide vs treatment with PRN-646. Linear correlation of Log2 fold-change values determined by differential expression analysis of either N4/N4+PRN-694 or N4/T4 comparisons at each time point.

4.3A-B). Similarly, nearly all of the induced genes during T4 with PRN-694 stimulation were also found within the T4 stimulated induced genes (Fig. 4.3C). Plotting Log2 fold-change values of differentially expressed transcripts
shared in weaker T4 peptide and PRN-694 treatment highlighted their similarities in transcriptional control (Fig. 4.3D-F). Whether modulating TCR stimulation or ITK activity, similar patterns emerged. This was further evidence that a determined initial transcription activation program is induced after TCR ligation.

Comparing select individual expressed genes demonstrated differential expression patterns. Some of the earliest genes detected included known immediate-early response genes, including AP-1 family proteins (Fos, Fosb) and Egr family genes (Egr2, Egr3), all found within Clusters I and II (Tullai et al., 2007). Peak Fos and Fosb transcription was dampened by either treatment with PRN-694 or weaker peptide stimulation (Fig. 4.4). Egr2 and Egr3 transcription appeared to be less sensitive, especially during PRN-694 treatment, where patterns of expression abundance appeared identical.

Nr4a family member transcripts (Nr4a1, Nr4a2, Nr4a3), also appeared less sensitive to PRN-694 treatment, especially during strong N4 peptide stimulation (Fig. 4.4). Recent work has discovered Nr4a family members (particularly Nr4a2, Nr4a3) are important in executing exhausted T cell programming and their regulation is largely attributed to NFAT activity (Chen et al., 2019; Jennings et al., 2020; Martinez et al., 2015; Mognol et al., 2017; Sen et al., 2016). As discussed in Chapter III, NFAT1 signaling was less affected by
Based on other previous experiments, I expected NF-κB signaling to be weakened during PRN-694 treatment. Consequently, transcripts responsive
to NF-κB signaling (Nfkbia, Nfkbid, Nfkbiz) were among the most sensitive to PRN-694.

Differential expression analysis at 30 minutes of supported these patterns in expression. PRN-694 treatment dampened induction of select transcripts including those encoding Fos, Fosb, and Fosl, as well as Myc, NF-κB response genes (Nfkbia, Nfkbid), but had less of an effect changing the relative expression of other induced transcripts like Egr family members (Egr1, Egr2, Egr3) and Cd69 (Fig. 4.5A). Similar patterns were found during T4 stimulation, but not completely, as PRN-694 treatment did not dampen Nfkbia and Nfkbiz, and did lower relative Egr2 abundance (Fig. 4.5B). This difference observed when weakening peptide stimulation conditions may be attributed to a fractional response of the stimulated population, reflecting slower digital activation kinetics as discussed in Chapter III.

PRN-694 treatment or weaker peptide stimulation also dampened expression of delayed genes that encode important effector functions and scale the effector response. Transcription of Il2, Irf4, Il2ra (encodes CD25), Ifng, and Tnf all decreased with weaker TCR signaling conditions (Fig. 4.4). This is expected based on TCR signaling and ITK-deficient phenotypes in graded protein expression observed at 24 hours and indicates some of these expression patterns may be determined early on during activation.
To determine whether immediate transcriptional changes due to TCR stimulation strength or ITK activity also lead to reduced early gene product accumulation during activation, I measured Egr2, c-Fos, c-Myc intracellular protein and CD69 surface expression in stimulated cells with flow cytometry (Fig. 4.6A-C). Both c-Fos and Egr2 proteins were detectable within 30 minutes of OVA stimulation and their expression continued to be detected for at least 6 hours, beyond the time of their peak RNA transcript abundance. Treatment with PRN-694 or stimulation with T4 peptide dampened the maximum amount of c-Fos expression compared to N4-stimulated OT-I cells measured out to 6 hours. Weakened signaling had less effect on maximum
Egr2, Myc, and CD69 expression. After 6 hours all 4 stimulation conditions were beginning to converge in protein expression, with exception to c-Fos.

Evident in the bimodal patterns of the flow cytometry histograms, Egr2, Myc, and CD69 appeared to activate digitally, while c-Fos expression was modulated in incremental amounts, appearing more graded (Fig. 4.6C).

Further, T cells that expressed simultaneous digital CD69 and Egr2, also
accumulated incremental amounts of c-Fos dependent on the TCR stimulation strength and ITK inhibition (Fig. 4.6D). These results confirmed that ITK signaling contributes to differential and graded gene expression in response to variations in TCR signal strength.

**ITK regulation of early changes in DNA accessibility**

To connect differential individual TCR signaling pathway behavior with control of immediate gene expression patterns, I measured immediate, genome-wide DNA accessibility changes with ATAC-seq and analyzed enrichment of transcription factor binding motifs using HOMER (Buenrostro et al., 2013; Heinz et al., 2010). Cells for ATAC-seq analysis were split and sorted from those used in RNA-seq experiments, to better directly compare accessibility changes with gene expression. Thus, samples were stimulated with either N4 or T4 peptide, with or without PRN-694 for 30, 60, or 120 minutes.

ATAC-seq replicates clustered together when plotted with PCA, indicating that variance was attributed to differences in stimulation. Similar to patterns found in transcriptome analysis, the strength of the TCR stimulation or ITK inhibition did not regulate an independent set of genomic regions, but rather regulated the intensity of a shared set of activation-associated pileups. Compared to naive, unstimulated OT-I cells, strong N4
stimulation induced the most significant differences in DNA accessibility,
most evident after 120 minutes (Fig. 4.7A). After only 30 minutes of N4 stimulation, about 10,000 DNA regions displayed differential accessibility compared to unstimulated cells (Fig. 4.7B). To evaluate differential accessibility due to ITK inhibition, N4 stimulated cells with and without ITK inhibition were compared. After 30 minutes of stimulation, the most significant changes in accessibility due to PRN-694 were found near many gene loci also identified in early transcriptional data including AP-1 factors (Fosb, Jun), NF-κB response genes (Nfkbia, Nfkb1), and genes encoding transcription factors important in regulating effector function (Rel, Nfatc1, Irf4) (Fig. 4.7C). This indicated that while many genomic regions change in accessibility similarly during activation, the few that were most sensitive to PRN-694 also were near early genes that echoed the most sensitive gene sets found in the transcriptional analysis.

k-means clustering of all 30 minute activation-induced DNA accessibility changes (differentially-accessible compared to unstimulated control) for all sample conditions revealed regulation of regions that had increased dependency on ITK activity (Fig. 4.7D). Regions that were less accessible after treatment with the ITK/RLK inhibitor were significantly enriched for AP-1 (Fral) motifs and NF-κB motifs. Regions that were less sensitive to PRN-694 treatment were significantly enriched for NFAT family motifs. The ATAC-seq results indicate that specific DNA regions were
differentially more reliant on ITK activity for optimal regulation. Based on the motif enrichment data, I interpret these accessibility changes to be due to, at least in part, decreased NF-κB signaling in ITK/RLK-inhibited cells.

**NF-κB signaling enhances select graded gene expression**

One of the strongest phenotypes observed when measuring signal pathway activation was greater defects NF-κB signaling in response to weakened TCR stimulus or PRN-694 treatment (Chapter III). PRN-694 treatment dampened transcription and protein expression of select early genes (e.g. Fos, Fosb, Nfkbia) (Fig. 4.4, 4.5A, 4.6A–C). To evaluate the extent NF-κB signaling controls these expression patterns, OT-I cells were stimulated in the presence of a potent inhibitor of IkB kinase (IKK), IKK-16. As a comparison, the MEK inhibitor PD325901 was also used to measure activation without MAPK cascade activation. A titration of IKK-16 demonstrated strong suppression of nuclear translocation of NF-κB (p65) with minimal effect on NFAT1 translocation or Erk1/2 phosphorylation after 1 hour stimulation with N4 peptide (Fig. 4.8A). While Erk1/2 is well characterized as exhibiting digital activation following TCR ligation (Altan-Bonnet and Germain, 2005; Das et al., 2009), titration of PD325901 gradually reduced p-Erk1/2 activation without affecting NF-κB (p65) or NFAT1.
Fig. 4.8. Specific NF-κB inhibition differentially reduces c-Fos protein accumulation.

(A-B) Histograms depicting effect of titration of IKK-16 (A) or PD325901 (B) on NFAT1, NF-κB, and p-Erk1/2 activation after 1-hour simulation with N4 peptide. (C) Histograms demonstrating the effect of 500 nM IKK-16 or 1000 nM PD325901 on c-Fos, Egr2, and CD69 protein expression in OT-I cells stimulated with N4 peptide for 2 hours. (D-E) Line plots of depicting 500 nM IKK or 1000 nM PD325901 treatment on c-Fos (D) and Egr2 (E) protein accumulation over 4 hours stimulation with N4 peptide. Compiled are three separate experiments, error bars are s.e.m.
translocation (Fig. 4.8B). This analog control of Erk1/2 phosphorylation is due to inhibition of MEK downstream of digital triggering events. Digital inhibition of Erk activation is achieved if a Src kinase inhibitor is utilized, which targets TCR proximal control of digital Ras activation (Vogel et al., 2016).

OT-I cells were then stimulated with WT splenocytes loaded with N4 peptide up to 4 hours, with or without IKK-16 or PD325901, and c-Fos, Egr2, and CD69 expression was measured with flow cytometry. Concentrations of IKK-16 and PD325901 were utilized that correspond to absent NF-κB and Erk activation respectively. During IKK-16 treatment, c-Fos expression displayed a greater defect in expression than Egr2 (Fig. 4.8C-E). This pattern was similar to the effect of inhibition of ITK with PRN-694 (Fig. 4.6). CD69 expression was also dampened by IKK-16 (Fig. 4.8C). Treatment with PD325901 however, greatly reduced c-Fos, Egr2, and CD69 expression (Fig. 4.8C-E). These results demonstrate a hierarchy in downstream TCR signaling pathway requirements for early gene expression. Specific genes are differentially sensitive to signaling conditions where NF-κB is absent, but others remain less affected. This suggests an auxiliary role for NF-κB during naive cell activation; amplifying gene expression in a graded manner. Under conditions where MAPK activation is limited, cells wholly fail to activate –
illustrating a dependency on all-or-none, digital MAPK activation under physiological signaling conditions.

Discussion

Understanding ITK control of graded IRF4 expression during T cell activation has been a prime focus in the Berg lab (Conley et al., 2020; Nayar et al., 2012; Nayar et al., 2014). My own experimental efforts expanded upon these observations and attempted to connect TCR inputs and ITK activity with the intriguing patterns of gene expression observed at 24 hours. The experiments in this chapter aimed to determine whether graded TCR signaling and variable ITK activity have tunable control over the earliest gene induction in newly-activating naive OT-I T cells. They were designed to describe the extent of graded early gene expression and identify unique gene sets during signaling conditions derived from Chapter III, known to induce differential patterns of TCR downstream signaling pathway activation and transcription factor activation.

The results in this chapter can be summarized as four major findings: First, TCR stimulation of naive OT-I cells induces a predictable program of activation-associated transcription. Modifying TCR stimulation with variable-affinity peptides or inhibition of ITK did not produce expression of unique gene sets. Instead, I observed waves of early and delayed gene sets consistent
across all qualities of stimulation. **Second**, ITK activity helps amplify the expression of specific groups of early transcripts. When ITK was inhibited during N4 stimulation, first-wave “immediate-early” genes such as Fos and Fosb, and NF-κB response genes displayed significantly decreased abundance during a short temporal window of expression. **Third**, ATAC-seq results suggest TCR signal strength control of early gene abundance is in part due to differences in AP-1 and NF-κB activity. This is because the DNA regions most dynamically regulated in response to ITK inhibition were enriched for AP-1 and NF-κB binding motifs. The ATAC-seq experiments also compliment the concept of a single activation transcriptional trajectory in CD8+ T cells, as varied amount of TCR signal strength modulated the intensity of changes in a consistent set of DNA regions and did not regulate unique gene regions. **Fourth**, Graded early NF-κB signaling in naive T cells amplifies the magnitude of select genes, but is not absolutely required for activation, unlike digital activation of the MAPK signaling cascade. This was demonstrated using inhibitors of IKK and MEK, which created conditions of OT-I activation that lacked either NF-κB or MAPK signals. c-Fos protein expression was consistently more sensitive to NF-κB inhibition than Egr2. Differential protein expression patterns mirrored stimulation conditions during ITK inhibition with PRN-694 (Chapter III), which also produced weakened NF-κB
activation. Overall, the experiments in this chapter contain novel observations about the nuance of graded TCR signaling and graded gene expression in early activating OT-I T cells. They also underscore a role for ITK in amplifying TCR signals to fine-tune the magnitude of select genes within a single, T cell activation transcriptional program.

**Single T cell responses**

RNA-seq and ATAC-seq experiments were designed concomitantly with experiments in Chapter III that measured activation of individual signal pathways. At the time, the control of TCR signal strength on the kinetics of fractional responses within a stimulated population were not yet fully appreciated to properly interpret the data. Initial hypotheses were more focused on whether ITK could tune individual signal pathways than the temporal and probabilistic frequency of individual T cells within the clonal pool switching on activation-associated transcription. Thus, to evaluate peptide antigen and ITK control of early transcription, I chose to design pooled RNA-seq and ATAC-seq experiments.

After looking at the data from the first RNA-seq biological replicate, it was clear that there were some temporal factors that were significant to consider in interpretation of the data. Based on the signal pathway phenotypes in Chapter III, I knew that T4 peptide stimulation did not fully
switch on NFAT1, with about 75% responders. PRN-694 treatment during T4 treatment reduced the NFAT1 responder fraction further to 50%. Therefore, when the same signaling conditions were used for pooled RNA-seq experiments, I should expect that much of the significant differential expression, especially between T4 and T4 with PRN-694 treatment, should be due to reduced fractional responses within the population, and less due to graded, differential signaling patterns. The ITK-specific effects during N4 treatment are more preserved, because based on NFAT responses discussed Chapter III, the fractional responses during PRN-694 treatment are similar. For example, PRN-694 treatment during N4 stimulation significantly reduced expression of Fos transcripts at 30 minutes, but did not reduce Egr2 transcript abundance. However, during T4 stimulations these kinds of differential relationships were lost, and PRN-694 treatment seemed to dampen all genes; likely due to a smaller proportion of activating cells. Therefore, I placed more weight on comparing N4 stimulation conditions than T4 stimulation conditions when interpreting the RNA-seq and ATAC-seq results discussed in this chapter. Yet I decided to include both N4 and T4 stimulation conditions for presentation of some data, as it demonstrated that T cells experiencing weaker signaling utilize the same transcriptional program.
To evaluate asynchronous activation of single cells and to counteract the effect of differing proportions of responding cells, single cell RNA-seq experiments were also considered. However, triplicate replicates of pooled RNA-seq and ATAC-seq experiments were completed. While single cell approaches excel in discerning unique transcriptional profiles within heterogeneous mixes of cells, it was not clear whether low copy number of nascent induced transcripts per cell would be sufficient to resolve minute differences in abundance within a similar set of genes induced by graded TCR stimuli. I noted that single-cell experiments would still be able to decipher fractional and asynchronous activation within populations.

In 2018, in an effort to evaluate how TCR signal strength regulates CD8+ effector cell fate and function, another group successfully tracked activation of single OT-I cells using multiple methods (scRNA-seq, conventional flow cytometry, mRNA flow cytometry, and mass cytometry) (Richard et al., 2018). They concluded that variable TCR stimulation conditions affect the proportion of T cell responders and the rate at which cells switch on, but the transcriptional “trajectory,” or progression through the activation program within each individual cell is similar. Weakly stimulated OT-I cells were less efficient in activation, but ultimately possessed the same full cytolytic capacity as strongly stimulated cells. My
experiments also supported the induction of a single activation-associated transcriptional program independent of TCR signal strength.

In the same single-cell study, the first-wave genes that were detected following strong N4 stimulation appeared similar to those reported from my dataset. Fosb, Nr4a1, Nr4a3, Egr1, Egr2, and Nfkbid are all among the highest abundance genes reported within 1 hour of stimulation. Curiously, they reported while weaker stimulation with T4 peptide reduced the fraction of responding cells that switched on, they did not detect notable graded abundance of those transcripts within single cells. These results agree with my experiments if the hidden fractional effects are considered. For instance Egr2 abundance is weakened by T4 stimulation, likely due to differences in smaller proportion of responders. My results however, test the role ITK during early activation, which primarily regulates second messenger IP3 and DAG production downstream of TCR proximal signaling regulation. During N4 stimulation, PRN-694 had little effect on reducing the fraction of NFAT1 or Erk1/2 responders, yet had significant specific effects on reducing Fos, Fosb, and NF-κB response genes. Therefore, some form of future single-cell analyses testing PRN-694 would be beneficial to assemble a more complete picture of ITK control of early transcriptional activity within CD8+ T cells. Using single-cell RNA-seq or mRNA flow approaches, I would predict PRN-694 treatment would produce graded expression of the same select
transcripts within individual OT-I cells. If T4 treatment did not induce graded expression of these genes, as reported in Richard et al., this would highlight an intriguing difference between proximal TCR signaling vs downstream regulation influenced by ITK.

**Early gene kinetics and function**

Immediate-early primary response gene transcription in response to ligand-receptor interactions have been documented for decades (Herschman, 1991). Following receptor ligation, multiple waves of primary response gene transcription occur that do not require prior new protein synthesis (Tullai et al., 2007). In naive CD8+ T cells, it is reasoned that primary response genes may be, in part, under direct control of TCR signal pathway activation. Kinetics of nascent immediate gene transcription is rapid and transient. For instance, induced AP-1 factors (e.g. Fos, Fosb, Fosl1) are detectable within the first few minutes after receptor ligation, but are of small abundance after an hour, as noted in the patterns in my data.

Many transcripts detected in my datasets encode transcription factors that represent conserved early activation programs in many cell types. This includes AP-1 factors (e.g. Fos, Fosb, Fosl1), Nr4a family members, and Egr members. I observed specific defects in some of these transcripts (e.g. Fos, Fosb) during weakened NF-κB signaling conditions. However, evidence for
direct NF-κB control of these genes is incomplete. A study of early and
delayed primary gene transcriptional kinetics and genomic architecture
found an over-representation of transcription factor binding sites upstream
of immediate-early gene promoters including SRF, API, CREB, KROX and NF-κB (Tullai et al., 2007). There may be an important role for NF-κB members in
regulation of some of these genes, but likely not solitary control. Aligning
ATAC-seq pileups with publicly available NF-κB (p65) ChIP-seq data indicated
that p65 may bind at differentially regulated regions near the promoter of
genes like Fosb. However, future ChIP-seq experiments, or low input "Cut-
and-run" experiments could more directly measure NF-κB binding and
evaluate the extent it is responsible for differential expression of genes like
Fos or Fosb in my datasets (Skene and Henikoff, 2017).

The accumulation rate of immediate-early genes during early
activation may be important in dictating the overall rapidity of the effector
response and contribute to fitness of a T cell to sequentially signal. In the
lymph node, T cells are able to scan activated APCs and make serial
interactions with intermittent TCR triggering (Marangoni et al., 2013; Mempel
et al., 2004). One research group compared c-Fos accumulation in stimulated
T cells and reported the protein was stable for many hours after its induction
and even after stimulus withdrawal (Clark et al., 2011). This echoes the patterns
in my data, where Fos transcript was detectable for only a brief 30-min
window after activation, but graded amounts c-Fos protein was detectable by FACS for at least 6 hours. They also reported T cells that briefly were stimulated prior to adoptive transfer into host mice had increased c-Fos expression and underwent more cell divisions after re-stimulation. Brief, sub-optimal TCR interactions may produce bursts of Fos transcript that lead to incremental accumulation of c-Fos protein. Thus, T cells can sum productive serial APC engagements to maximize activation potential.

Early studies in neurons determined TCF, serum response factor (SRF) and cAMP response element-binding protein (CREB) are important in regulating Fos transcription (Johnson et al., 1997). CREB activates in response to both PKC and Ca$^{2+}$ signaling via calcium-sensitive CaM kinase activity (Johnson et al., 1997; Sheng et al., 1990; Sheng et al., 1991). In Chapter III, NFAT1 translocation was digitally regulated and less sensitive to ITK inhibition. Increasing calcium flux with ionomycin treatment did not increase NFAT1 intensity. While NFAT1 may have a specific threshold of Ca$^{2+}$ levels that trigger digital activation, it is possible intracellular concentrations of Ca$^{2+}$ may rise higher. In fact, high concentration of OVA stimulation induces greater intensity of Ca$^{2+}$ flux as measured in single OT-I cells (Dura et al., 2015). Future studies could test whether graded activation of early genes like Fos may be driven by calcium-sensitive transcription factors, such as CREB, in an NFAT-independent manner.
For comparison, transcription of Egr2 and Egr3, two other immediate-early genes with peak abundance after 30 minutes of stimulation, were less sensitive to PRN-694 treatment than genes like Fos. Egr2 and Egr3 are important in regulation the scale of T cell proliferation (Miao et al., 2017). They are NFAT target genes, and there is evidence that NFAT alone can drive Egr2 transcription (Marangoni et al., 2013; Martinez et al., 2015; Rengarajan et al., 2000). Therefore, patterns of Egr2 and Egr3 transcription may be attributable to digital NFAT activation that is less dependent on ITK activity.

**Cooperation of transcription factors**

Regulation of activation-associated transcription involves the cooperation of many transcription factors. The Il2 promoter contains multiple sites for NFAT, NF-κB, and AP-1, which all bind to regulate transcription (Jain et al., 1995). Signal pathways that terminate with activation of different transcription factors may each activate independently, but many layers of redundancy and cooperation ensure only robust signaling conditions usher naive T cells from a quiescent state to a metabolically active effector state.

NFAT is known to associate with AP-1 proteins to cooperatively bind at NFAT/AP-1 binding sites to regulate gene transcription (Macian, 2005; Macian et al., 2001). Thus, genes that require NFAT:AP-1 interactions for
transcriptional activation, such as Il2, require sufficient, sustained TCR signaling conditions to activate the Ca$^{2+}$/NFAT pathway and induce abundant AP-1 factors like c-Fos and c-Jun. Further, NFAT proteins can promiscuously interact with other AP-1 dimers aside from Fos/Jun. As described in my transcriptional analyses, c-Fos and FosB are quickly transcribed within 30 min and are potent transactivators. Other less potent AP-1 family members are transcribed later, such as Fosl1 (Fra-1), and may replace c-Fos in AP-1 dimers to change the activating efficiency of NFAT:AP-1 and attenuating Il2 transcription (Jain et al., 1995).

A 2017 study was intent on dissecting the transcriptional effects of the Ca$^{2+}$ and DAG arms of TCR signaling. (Brignall et al., 2017). Using combined PMA and ionomycin stimulation of Jurkat cells, they discovered important synergistic effects. In reporter assays, the authors demonstrated that combined PMA and ionomycin treatment elicited far greater IL-2 expression and NF-κB activity than PMA or ionomycin alone, reflecting Il2 expression patterns kinetics I observed due to ITK inhibition, and capturing some of the nuances of NF-κB activation I observed in Chapter III. Combined PMA/ionomycin treatment was vastly more potent in inducing chromatin remodeling compared to either PMA or ionomycin alone. ATAC-seq pileups due to combined PMA and ionomycin were specifically enriched NFAT/AP-1 and NF-κB motifs, which were not found in regions accessible in PMA or
ionomycin alone. This is similar to my own results, where differentially accessible DNA regions during weakened TCR signaling via ITK inhibition were enriched for NF-κB and AP-1 motifs. This suggests strong TCR signaling conditions, which include robust NF-κB activation due to ITK assistance, optimally regulates activation-associated changes in chromatin. This study highlighted the importance of synergy among TCR signal pathways, which integrate at the chromatin level to regulate activation programming.

DNA binding sites within regulatory elements can also have variable affinity for transcription factors; another layer of control to tune graded transcriptional responses. In a recent study, researchers demonstrated groups of genes important in CD4+ T cell differentiation contain enhancers with high-affinity for IRF4-BATF transcription factor pairs (Gallagher and Berg, 2017; Iwata et al., 2017). Other groups of genes only contain low-affinity sites. Genes with high-affinity enhancers were induced under stronger TCR signal conditions, which generated greater graded amounts of IRF4 and BATF. Genes with low-affinity enhancers were expressed even under weak TCR signaling conditions. This demonstrated how graded TCR signal can differentially regulate transcription at the genome level. While TCR signals or ITK can regulate differential transcription factor activation in CD8+ T cells (Chapter III), individual patterns of activation-associated transcripts may be attributable to their specific genomic promoter and enhancer landscape.
There is evidence NFAT proteins also can bind DNA alone, without AP-1 binding partners. In a 2015 study, Rao and colleagues reported persistent NFAT signaling present during chronic infections drives “NFAT alone” regulation of T cell exhaustion-associated transcriptional states (Martinez et al., 2015). They engineered a constitutively-active form of NFAT1 that was unable to associate with AP-1 dimers and studied its DNA binding tendencies in mice. NFAT alone regulated increased expression of exhaustion-associated markers such as CTLA4, PD-1, TIM3, and LAG3. NFAT alone binding has also been shown to increase exhaustion-associated regulators Nr4a2 and Nr4a3 (Scott-Browne et al., 2016). Whether NFAT alone activity is contributing to patterns of Nr4a2 and Nr4a3 expression, or expression of other NFAT target genes Egr2 and Egr3 as described in my experiments, is not yet clear and could be focus of a future study (Rengarajan et al., 2000).
Chapter V: Discussion

Interpretation of major findings: ITK function

The conclusions about differential signaling pathway responses to TCR signal strength presented here are novel and have broad implications about how graded signals are converted to analog cellular responses. While not the first to compare relative activation of NFAT, NF-κB, and MAPK responses, this dissertation carefully dissected components of graded TCR signaling (peptide affinity and dose) and intracellular signal modulation due to ITK in single cells (Dolmetsch et al., 1997; Podtschaske et al., 2007). Moreover, these experiments characterize a specific supportive role for ITK in TCR signal transduction. ITK is known to not be absolutely required for signaling, but its activity helps amplify PLC-γ1 activation and enhance the fitness of T cell activation overall (Andreotti et al., 2010). Experiments here expose the complex behaviors among separate signaling pathways. Pathways can be ordered into a pseudo-hierarchy of ITK dependency: NF-κB signaling benefitting most, then NFAT, and lastly Erk1/2 (MAPK) activation. Flipping this hierarchy around, one can imagine that this represents a priority of early T cell activation requirements, where digital activation of MAPK and NFAT
are absolutely required and NF-κB is supplemented gradually to tune the response.

The experimental results in Chapter III support ideas permeating much of the current literature about T cell activation. They suggest that TCR triggering and much of downstream pathway activation is digital. Digital MAPK activation is well understood and bimodal patterns of NFAT translocation are clear (Das et al., 2009; Gallagher et al., 2018). When stimulating a pool of clonal cells like OT-I, fractional all-or-none responses represent individual cells switching on when passing a signaling threshold. For Erk1/2 activation it is a threshold of proximal TCR triggering and Ras activity that ignites positive feedback on SOS (Das et al., 2009). Opening of Ca\(^{2+}\) channels during SOCE rapidly spike intracellular Ca\(^{2+}\) levels and NFAT1 quickly responds (Trebak and Kinet, 2019).

In previous studies, NF-κB was reported to display digital activation behavior (Kingeter et al., 2010). Therefore the revelation that NF-κB displays analog behavior within digitally NFAT and Erk1/2 activated OT-I cells in response to graded TCR stimulation or ITK inhibition was unexpected. In my experiments, 100 nM of moderate affinity T4 peptide induced a maximum proportion of NFAT1 responders similar to N4 peptide, albeit at slower rate of conversion. NF-κB translocation was consistently inefficient under T4
stimulation conditions. Similar differential effects were observed when cells were treated with PRN-694.

The mechanism regulating graded NF-κB signaling is not fully explored here. Different from the Ca\(^{2+}/\text{NFAT}\) pathway and the cascade of MAPK signaling, NF-κB proteins are normally inhibited by IκB proteins during steady state (Cheng et al., 2011; Lucas et al., 2004). The rate of ubiquitinylation and degradation of IκBα may limit the rate of NF-κB release. The half-life of IκBα has been measured as long as 138 minutes in resting cells, but a 1.5 minutes in strongly stimulated cells (Henkel et al., 1993; Kanarek et al., 2010; Whiteside et al., 1997). In Jurkat cell lines, IκBα degradation can take up to 15 minutes following PMA/ionomycin stimulation. While rapid and bimodal IκBα degradation has been achieved in Jurkat cells and OT-II cells, the panel of stimulation conditions tested here have carefully revealed incomplete and graded states of early NF-κB signaling (Kingeter et al., 2010). Further, OT-I cells consistently displayed signs of NFAT1 activation almost immediately (less than 5 minutes), while NF-κB was more delayed, with weakly stimulated cells only achieving appreciable NF-κB nuclear protein content after 60 minutes. Whether weak peptide stimulation or ITK inhibition regulate the half-life of IκB could be tested in future studies.
NF-κB signaling was specifically sensitive to PRN-694 treatment. This indicated a role for ITK in boosting signals from weaker TCR inputs that could not sufficiently trigger NF-κB on their own, even under conditions that stimulate NFAT and Erk. Later during activation, after initial priming, combined TCR, CD28, and other co-stimulatory receptors become increasingly important in amplifying NF-κB signaling via PI3K (Chen and Flies, 2013). It may be advantageous for naive cells to ‘protect’ NF-κB, by making it more difficult to trigger with TCR stimulus alone. In a lymph node, instead of pools of clones stimulated with the same peptide, lone clones must scan activated APCs to compete for the best combination of antigen quality and concentration (Henrickson et al., 2008; Mempel et al., 2004). Slower NF-κB induction would make it more difficult for T cells to commit to the full activation transcriptional trajectory and allow for increased T cell scanning periods to accumulate early gene products such as c-Fos and c-Myc, which ultimately contribute to the scale of the effector response (Clark et al., 2011; Mayya and Dustin, 2016; Preston et al., 2015).

While ITK helps amplify PLC-γ1 production of DAG and IP$_3$ concentration to more effectively induce NF-κB, to combat unchecked NF-κB activation in naive cells, negative feedback dynamically regulates NF-κB efficiency as a transcription factor (Cheng et al., 2011; Smith-Garvin et al.,
2009). After N4 stimulation, I measured rapid induction of NF-κB inhibitor Nfkbia (IκBα) mRNA, which when translated can re-engage NF-κB and cause nuclear export. Also induced were Nfkbid, and Nfkbi, mediators of p65 transcriptional activity and Tnfaip3 (A20), a deubiquitinase known to inhibit NF-κB signaling and a target of NF-κB transcription (Annemann et al., 2016; Krikos et al., 1992; Wertz et al., 2004). Transcripts of all of these indicators of strong NF-κB signaling were significantly reduced in PRN-694 treated cells.

Based on the results in chapter III from PMA and ionomycin stimulation, it is clear that both DAG and IP₃ activation are required for optimal NF-κB translocation in naive cells. Strong N4 peptide sufficiently activated p-Erk1/2 and NFAT, but only partially activated NF-κB. RNA-seq and ATAC-seq evidence also suggested treatment with PRN-694 specifically dampened NF-κB associated early gene transcription. This highlights a unique role for ITK. My first hypotheses about how ITK regulated signaling included it tuning the Ca²⁺ effects of IP₃ signaling. However, this line of thinking is inaccurate. ITK sits upstream of PLC-γ₁, and its activity influences equimolar production of DAG and IP3; weaker TCR signaling and lower ITK activity reduce both DAG and IP₃ similarly. What my results demonstrate is that the nuance of ITK control of graded signaling is rooted in the fact that NFAT, Erk1/2, and NF-κB pathways each respond differently to changing
DAG and IP₃ concentrations. NF-κB proved to be most complex; sensitive to the synergistic effects of both DAG and the IP₃. This defines a role for ITK beyond lowering the digital signaling thresholds of NFAT and Erk1/2, but also improves stubborn NF-κB activation in naive cells.

**How TCR signal strength scales the effector response**

Two basic underlying questions were driving my research goals in understanding TCR control of CD8⁺ T cell activation: How do digital signals produce graded gene expression? And, to what extent do graded signals, such as those capable through NF-κB, contribute to graded gene expression?

The experiments presented here outline an interesting role for NF-κB, and indirectly ITK, as mechanisms to influence graded gene expression. Yet, evidence supporting graded NF-κB signaling behavior does not fit the same mold as other aspects of early TCR signaling. Numerous studies describe TCR proximal triggering and activation of the majority of downstream pathways as digital processes (Au-Yeung et al., 2014; Das et al., 2009; Huang et al., 2013; Kingeter et al., 2010; Podtschaske et al., 2007). This means that in a pool of clonal cells, a growing fraction of cells “switches on” over time and within each cell there are no intermediate states of signaling, similar to the behavior of NFAT and Erk1/2 that I observed. The importance of NF-κB transcriptional activity in regulating T cell activation programming is clear, but graded NF-
κB transcriptional activity is likely not the only or primary rheostat to scale the response.

Digital TCR triggering and digital signals are converted to graded gene expression within the first few hours of stimulation. TCR signaling and ITK-dependent expression of IRF4 within 24 hours is a well-studied example of a graded activation-associated gene (Conley et al., 2020; Huber and Lohoff, 2014; Man et al., 2013; Nayar et al., 2014). Much of the focus on IRF4 is because its transcriptional activity is critical in directing the further graded expression of important regulators of metabolic T cell reprogramming (Man et al., 2013). Incremental amounts of IRF4 protein can be detected as early as four hours after stimulation, and RNA-seq results here detected Irf4 transcripts within 2 hours. Yet other activation-associated gene products also are induced at graded amounts in response to graded TCR signaling. BATF, a frequent binding partner for IRF4, is induced at a similar time and has been found that its expression is also linked to TCR stimulation strength (Iwata et al., 2017; Kurachi et al., 2014). Expression of CD25, the IL-2R alpha-chain, is also proportional to peptide affinity when measured by FACS (Conley et al., 2020). In my own RNA-seq results, Il2ra, Il2, and Ifng transcript abundance (as well as most of the genes found in later induced clusters) were beginning to display graded patterns of expression reflecting variable TCR
signal conditions. Digital signal integration may occur during a temporal window of early gene product accumulation.

A major factor thought to convert digital biochemical processes to analog gene responses is the duration of TCR signaling (Mayya and Dustin, 2016; Tkach and Altan-Bonnet, 2013). In vivo, T cells first enter a scanning phase of making and breaking contacts with APCs (Mempel et al., 2004). Cells that encounter weaker affinity or lower concentration of antigen spend more time in the scanning phase (Henrickson et al., 2008; Mempel et al., 2004). Stronger TCR interactions lead to a shorter scanning phase. One study demonstrated T cells can sum antigen experiences from serial interactions with APCs. By intermittently interrupting T cell and APC contacts with a Src kinase inhibitor, they found IFN-gamma production was directly proportional to the total duration that T cells were engaged (Faroudi et al., 2003). To facilitate signal summation, a hysteresis period characteristic in MAPK activation and the relatively slow rate of NFAT export signaling behaviors that dampen the detrimental effects of antigen withdrawal and allow for signal “memory” (Das et al., 2009; Marangoni et al., 2013).

To explore graded induction mechanisms that lie beyond signal memory, others created molecular models to provide insights into how intermittent digital T cell signaling could produce slow accumulation of early gene products, like the AP-1 member c-Fos (Locasale, 2007). They
determined this behavior was plausible, and a later study showed c-Fos accumulation in vivo was able to reflect the summation of serial T cell stimulations (Clark et al., 2011). In the experiments presented here, ITK or NF-κB inhibition consistently reduced the accumulation of c-Fos protein within the first few hours after activation. The data do not indicate whether this reflects shorter duration of TCR engagement or some kind of throttled signaling (e.g. graded NF-κB) within individual cells. Because the in vitro stimulation methods tested here do not adequately simulate a lymph node environment, future experiments should test whether ITK activity dampens c-Fos accumulation due to reduced cell intrinsic signal intensity or due to producing longer, more intermittent signal duration. This may be possible using a combination of approaches that image live cell lymph nodes after OT-I adoptive transfer and peptide injection, as well as ex vivo FACS examination of T cells stimulated in vivo. These experiments would also compliment those proposed in the Chapter III Discussion, as it is still unclear how ITK activity regulates the duration of the T cell scanning period.

Accumulation of early gene products may linearly regulate later, secondary graded gene expression. Il2 transcripts were one of the most differentially expressed when OT-I cells were inhibited with PRN-694. The same signaling conditions produced deficient AP-1 activity (decreased Fos, Fosb) and decreased NF-κB activation. The Il2 promoter contains multiple
functional AP-1 and NF-κB sites, which could explain poor Il2 induction. (Jain et al., 1995; Jain et al., 1992). Increased dose of nuclear NF-κB (p65) in naive cells likely is a major activator of early Il2 expression (as a p50-p65 homodimer) (Jain et al., 1995). Further, later graded expression of induced NF-κB protein c-Rel responds to continued TCR signals and becomes increasing important in acting on the Il2 promoter and scaling IL-2 expression.

IL-2 is a potent cell survival cytokine during activation. IL-2 signals sum with TCR signaling inputs to regulate the number of cell divisions T cells undergo during expansion (Marchingo et al., 2014). IL-2 signaling was found to sustain the expression of the early induced transcription factor c-Myc (Heinzel et al., 2016; Preston et al., 2015). As observed by myself and others, c-Myc is induced quickly after TCR engagement and exhibits strong digital expression patterns. In my experiments, after 6 hours, regardless of peptide stimulation or ITK inhibition, all OT-I cells were converging on their c-Myc expression phenotype. IL-2 signaling prolonged the duration that c-Myc was maintained after digital induction. This correlated directly with number of cell divisions clones underwent (Heinzel et al., 2016). Cells that did not receive IL-2 supplementation lost degraded c-Myc more rapidly and ceased dividing sooner. This is an example of how early AP-1 and NF-κB activity,
which regulate IL-2, can lead to large differences in the scale of the T cell effector pool.

The Berg lab and others have utilized CD69 as a digital marker for T cell activation (Conley et al., 2020). CD69 has been directly associated with Erk1/2 activation, which is decidedly digital in behavior (Altan-Bonnet and Germain, 2005; Das et al., 2009). Yet, CD69 expression is not always indicative of a fully activated T cell. In fact, cells that are similarly CD69+ can display varying states of other markers of activation. A study of “suboptimal” signaling conditions demonstrated when Lck−/− OT-I cells were stimulated with N4 peptide, all cells switched on CD69 similarly, but were severely deficient in ribosome biogenesis, proliferative capacity, cell size, and had decreased expression of Il2ra, Bcl2l1 (Bcl-XL), Myc, and Ifng (Tan et al., 2017). c-Myc is a key regulator of the global transcription rate, thus important in scaling graded responses within individual cells (Dang, 2013). IL-2 supplementation during weak G4 peptide stimulation increased c-Myc expression present at 72 hours and increased cell proliferation rate and ribosome biogenesis (Tan et al., 2017). These signaling conditions test weak TCR interactions and highlight potentially hidden graded states within “activated” cells. Likely, graded expression of IRF4 is more representative of the global relative induction of transcripts within activating CD8+ T cells; gradual accumulation of early gene product that begins immediately after
TCR engagement has powerful influence over the scale of the CD8+ T cell response.

**Significance of TCR signal strength and ITK activity in disease**

The precise measurement of TCR signaling pathways and their immediate transcriptional effects described here have revealed important details about the relationship of NFAT, NF-κB and MAPK activation in naive CD8+ T cells. Specifically, robust NF-κB activation requires ample TCR signaling conditions and the assistance of ITK activity. This has implications in understanding human disease states and applications that require detailed control of TCR signals to achieve specific T cell functions, population expansion, and cytotoxic target cell killing. This includes response to pathogens, function and trafficking of autoimmune T cells, and anti-tumor T cell behavior. Tuning TCR signals and ITK activity to elicit specific control of NF-κB pathways during early activation and effector cell functions may be an important factor in optimizing the efficacy and success of T cell-mediated medicine.

Early experiments investigating the function of ITK suggested that it may have had an important role in permitting T cell responses to allergic diseases. Balb/c mice notably have CD4+ helper T cell responses skewed toward classic type 2 (Th2) profiles. However Balb/c Itk-/- mice did not
mount a Th2 response to *Leishmania major*, *Nippostrongylus brasiliensis*, or *Schistosoma mansoni* parasitic infections. Perhaps this was due to insufficient NFAT activation of IL-4 production to assist in Th2 differentiation (Andreotti et al., 2010; Fowell et al., 1999; Schaeffer et al., 2001). It is unclear however, whether low production of Th2 cells in Itk−/− mice was due to reduction of responding activating T cells or weakened tuned TCR signaling within activated cells which could alter patterns in function and differentiation. Based on the data presented here, it would be relevant to investigate whether tuned TCR signals, including graded NF-κB signaling, also play a role in ITK-dependent Th2 responses.

In the Balb/c Itk−/− parasitic infection models discussed above, CD4+ T cells were still able to differentiate towards Th1 fates, often sufficient enough to clear infection. Itk−/− T cells also displayed differential expression of another Tec family kinase, Rlk, which was higher in Th1 cells and likely contributed to signal amplification more readily than in Th2 (Miller et al., 2004). Supplemental ectopic Rlk expression in Itk−/− mice was sufficient to restore Th2 responses to *Schistosoma mansoni* eggs or airway allergy models (Sahu et al., 2008). This series of experiments exemplified the important redundancy of the Tec kinase family within T cell signaling machinery, but also that specific T cell subsets may rely more on ITK for signal amplification than others. Further, these experiments show germ line Itk−/− mice may
display phenotypes that are a result of different developmental conditions across different T cell types, rather than the specific contribution of ITK to graded signal transduction within single cells. The ITK inhibitor used in my experiments, PRN-694, inhibits the activity of both ITK and Rlk, thus I would expect it would have broader effects on both T_{h1} and T_{h2} responses during infection. Development of conditional Itk knockout mice, under control of a tamoxifen-inducible T cell-specific promoter (e.g. CD4), could more precisely evaluate ITK contribution to disease phenotypes.

There are specific examples of ITK-associated pathology in humans. In 2009, it was reported homozygous Itk deficiency in two sibling girls was fatal in the context of Epstein-Bar virus (EBV) infection (Huck et al., 2009). The patients exhibited high eomesodermin expression in CD8^{+} T cell populations, indicative of inefficient effector T cell expansion, and also lacked natural killer (NK)T cell populations. These phenotypes combined likely contributed to uncontrolled EBV replication. After years of evidence gathered from mouse models describing the importance of ITK in TCR signal transduction, this confirmed a critical role for ITK in signal amplification during CD8^{+} T cell responses in humans.

ITK is also important in regulating T cell function and trafficking during autoimmune disease. T cells lacking the co-inhibitory receptor CTLA-4, which counteracts the activating, signal-amplifying effects of CD28
co-stimulation, are unable to maintain tolerance to self and develop a fatal accumulation of self-specific T cells that infiltrate the periphery (Ise et al., 2010). In humans, polymorphisms in Ctl4 are linked to autoimmune disease states (Gough et al., 2005). Interestingly, Ctl4/− Itk/− double knockout mice do not develop disease, accumulating self-reactive T cells in lymphoid organs that are unable to migrate to the periphery (Jain et al., 2013). This phenomenon highlighted the important role for ITK balancing TCR and CD28 signal summation. It also suggested that signal amplification due to ITK activity is important in regulating cell migration machinery or programming.

In a separate study, ITK inhibition with PRN-694 reduced T cell infiltration and disease progression of a colitis model in mice. PRN-694 inhibition blocked T_{H}1 and T_{H}17 differentiation and also reduced their ability to migrate toward inflammatory cytokines in vitro (Cho et al., 2015). Itk/− mice also displayed defects in steady-state CD4+ and CD8+ T cell populations, with decreased homing-receptor expression, highlighting the role of ITK-mediated signaling in regulating T cell function in the periphery (Cho et al., 2020). Thus, beyond the role of amplifying TCR signals during naive CD8+ T cell priming as analyzed in this dissertation, ITK is a critical component of TCR-directed signaling in terminal cell states to appropriately balance T cell tolerance to self and responses to pathogenic challenges.
Precise control of TCR signals is an exciting focus to progress the development of successful therapeutics against human cancers. Tumor infiltrating CD8+ T cells make up a critical component of tumor infiltrating lymphocytes (TILs) that assist in control of malignancies and target tumor cell killing. However, a combination of immune evasion tactics by cancer cells and chronic activity of CD8+ T cell populations create significant obstacles that oppose efficient eradication of disease.

During both chronic viral infections and when challenged by malignancies, persistent exposure to antigen and inhibitory receptor stimulation pushes CD8+ T cell populations to progressively exhibit signs of T cell exhaustion (Wherry and Kurachi, 2015). High expression of PD-1 and CTLA-4 on the surface of CD8+ T cells, prescribed by specific induced T cell exhaustion transcriptional programming, work to dampen TCR signaling and inhibit the proliferative potential and cytokine production of T cell populations (Chen and Flies, 2013; Wherry and Kurachi, 2015). Cancer cells often express PD-1 ligands (PD-L1, PD-L2) which interact with PD-1-high T cells to inhibit function. CTLA-4 interacts with B7 molecules on tumor cells to inhibit T cell co-stimulation. Thus, CTLA-4 and PD-1 pathways were the initial core targets for checkpoint blockade immunotherapies.

Treatment with a combination of anti-CTLA-4 and anti-PD-1 antibodies block their inhibitory effects and rescue T cell anti-tumor
functions, which have been exceptionally successful in treating certain cancers, including some melanomas, non-small cell lung carcinomas (NSCLC), urothelial bladder cancers, and triple-negative breast cancers (TNBC) (Darvin et al., 2018; Pardoll, 2012). However, patient response to checkpoint inhibitors differs greatly. Individual tumor microenvironments can exhibit polarized degrees of immunogenicity, from highly inflammatory, which promotes T cell exhaustion phenotypes, to “immune deserts,” which present low amounts of antigen and co-stimulatory molecules, evading T cell priming and target killing, promoting tolerance of the tumor (van der Leun et al., 2020). Beyond checkpoint inhibitors, next generation of immunotherapies will have to consider a dynamic range of TCR signaling conditions; from persistent, exhaustion-promoting signals to suboptimal, anergy-promoting signals.

Chimeric antigen receptor (CAR) T cells are another promising therapeutic tool to target human cancer cells. CAR T cells express engineered TCRs made up of cytoplasmic signaling domain components of the TCR, such as CD3-ζ, fused with varied tumor epitope-specific extracellular domains such as antibody variable regions (Maldini et al., 2018). Therefore, new insights about how TCR signal strength and ITK activity balance downstream signal pathways are pertinent to help improve CAR T cell technologies. Patient-derived T cells transduced to express a CAR
specific for CD19 led to up to 90% remission in children with acute lymphoblastic leukemia (ALL) (Davila et al., 2014; Maude et al., 2014). While blood cancers respond well to CAR T cell therapeutics, exhaustion of CAR T cells presents greater challenges for effective killing of solid tumors.

Recent work revealed NR4A family transcription factors, which are regulated by persistent, prolonged NFAT signaling, actively regulate T cell exhaustion programming and limit NF-κB and AP-1 signals, (Martinez et al., 2015; Mognol et al., 2017; Scott-Browne et al., 2016). In an effort to increase the efficacy and reduce exhaustion of CAR T cells, one study infused human CD19-directed CAR T cells that lacked Nr4a family genes into mice with B16 tumors expressing human CD19 (Chen et al., 2019). Nr4a triple knockout human CD19 mouse CAR T cells promoted tumor regression, animal survival, and exhibited increased CAR T effector transcriptional profiles. Genomic accessibility assays showed Nr4a triple knockout CAR T cells were enriched for NF-κB and AP-1 motifs, while wild type CAR T cells were enriched for NFAT and Nr4a3 motifs. These results reiterate that effective CAR T cell target killing, and overcoming general phenotypes of T cell exhaustion, requires signaling conditions that balance steady NFAT signaling while also robustly engaging NF-κB and AP-1.

The importance of ITK signaling within tumor-specific T cells and T cell immunotherapies is not fully understood. As presented here, ITK activity
permits more robust activation of NF-κB and production of AP-1-associated transcription factors in naive cells. Precisely regulating ITK activity in the clinic will be challenging. A greater understanding about how to finely increase or decrease ITK activity may require novel approaches to tune the balance of TCR signaling needed to optimize the efficacy of patient natural TILs and ex vivo patient-derived CAR T cell therapeutics. High ITK activity and intense TCR signaling begins to stimulate cell death pathways. Selection of CAR designs should be uniquely chosen for individual cancers. Eradication of evasive “immune desert” tumors, which have low MHC/antigen and co-stimulatory molecules, may benefit from increased ITK activation to assist in TIL priming or to maximize CAR T expansion. Tumors that are particularly immunogenic may quickly promote exhausted tumor-specific T cells, thus it is necessary to test whether increased ITK activity could recover NF-κB and AP-1 signals within T cells that experience disproportionate NFAT signaling.

Conversely, use of ibrutinib, an inhibitor of both B cell-expressed Bruton’s tyrosine kinase (BTK) and ITK, has shown reducing ITK activity can improve T cell anti-tumor functions. In two separate studies, ibrutinib administration to chronic lymphocytic leukemia (CLL) patients provided a marked increase of CD4+ and CD8+ tumor-specific T cells and engrafted CAR T cells, with reduced expression of CTLA-4 and PD-1 (Fraietta et al., 2016; Long et al., 2017). Expansion of patient-derived CAR T cells also had increased
proliferation and exhibited fewer markers for exhaustion (e.g. PD-1, TIM-3, LAG-3) when expanded in the presence of ibrutinib (Fan et al., 2021). It is possible that weakened TCR signals may push fewer cells into towards cell death pathways. Use of a more potent specific inhibitor of ITK, such as PRN-694 may prove to further optimize expansion of CAR T cells.
References


Kinase and Calcium Signaling at the Level of Chromatin Underlies Inducible Gene Activation in T Cells. J Immunol 199, 2652-2667.


toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. Sci Transl Med 6, 224ra225.


CD8+ T Cells as Measured by Flow Cytometry of Isolated Cell Nuclei. Immunohorizons 2, 208-215.


Gu, Z., Eils, R., and Bioinformatics, M.S. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. academicoupcom.


Phase of T Cell Activation Using a Genetically Encoded Calcium Biosensor. The Journal of Immunology 196, 1471-1479.


Conformational Switch that Regulates Transcriptional Activity. Molecular Cell 6, 539-550.


versus extent of TCR occupancy on T cell activation: a revision of the kinetic proofreading model. Immunity 15, 59-70.


Tan, T.C.J., Knight, J., Sbarrato, T., Dudek, K., Willis, A.E., and Zamoyska, R. (2017). Suboptimal T-cell receptor signaling compromises protein translation,
ribosome biogenesis, and proliferation of mouse CD8 T cells. Proc Natl Acad Sci USA 114, E6117-E6126.


