A Novel Role for NF-κB in Proximal T Cell Signaling

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A Novel Role for NF-κB in Proximal T Cell Signaling

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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DEDICATION

This work is dedicated to Douglas Allen Gordon and Ryan Matthew MacCaull; two amazing men whose lives were cut short by cancer.
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ABSTRACT

The interrogation of T cell signaling over the past fifty years has led to the discovery of amazingly intricate cascade networks and elaborate descriptions of individual proteins’ domains and functions. A complex landscape has been rendered in which proteins relay messages from the extracellular ligation of the TCR by a cognate peptide loaded MHC via changes in sub-cellular location, phosphorylation, and binding affinities and partners to enact nuclear localization of three key transcription factors required for cellular effector function and proliferation: AP-1, NF-AT, and NF-κB. Dogma has favored activation of each of these transcription regulating elements to be a linear and parallel activity, thus very little interaction between pathways has been highlighted by previous findings in the molecular immunology community. The focus of this dissertation explores the role of NF-κB in T cell signaling with emphasis on subunits p50, cRel, IkBα, and IKKβ, and with respect to NF-κB’s ability to modulate calcium and NF-AT signaling, proximal TCR phosphorylation, and CRAC and purinergic calcium channel proteins.

The role of NF-κB in T cells can be a difficult thing to establish, as this thirteen member family innervates almost every cellular process from homeostasis to activation, and even functions in the opposing processes of survival and apoptosis. To convolute the investigation further, many family members also fulfill redundant tasks, as a result of their high evolutionarily conserved sequence homology. To this end, we discovered the
best way to evaluate the function of NF-κB in the activation of T cells was to knockdown two family members: p50 and cRel. In doing this, we rendered mice that were viable (unlike knockdown of RelA) and fertile, but possessed T cells that were highly unresponsive to strong stimulation (anti CD3/CD28) or foreign antigen (OVA) presented to mice bearing the correct transgenic TCRs (OT-1) by professional antigen presenting cells (APC).

Through in vitro assays, we discovered that in addition to the specific defects in NF-κB activation, NF-AT signaling was also greatly disrupted in these cells, sequela to retarded calcium influx and signaling. This was of great interest, as while several studies have shown that calcium signaling has the ability to amplify and fine tune NF-κB activation, there is a dearth of studies and publications highlighting the effect of an activated NF-κB pathway on calcium influx and signaling leading to the activation of NF-AT. Another fascinating discovery, that explicated the calcium reduction and NF-AT inhibition, was that ablation of p50 and cRel led to decreases in mRNA and protein levels of two additional NF-κB family members: IKKβ and IKKγ. The results presented here suggest that it is the reduction in IKKβ and IKKγ that leads to impaired phosphorylation of the key TCR proximal proteins: Zap70 and PLCγ1, and it is the decrease in activated PLCγ1 that renders less IP₃, and ultimately abrogates calcium signaling.

Overall, this thesis highlights the ability of IKKβ to enhance general proximal TCR protein phosphorylation (and specifically Zap70) leading to a greater influx of calcium (perhaps aided by IKKβ also augmenting the function of the CRAC protein, STIM1)
which leads to superior activation of NF-AT, and amplifies downstream cellular effector functions such as IL-2 production and proliferation. Moreover, this work demonstrates that NF-κB subunits likely form supermolecular clusters, and ablation of certain subunits (i.e. p50 and cRel) can lead to instability and decreased levels of other family members (i.e. IKKβ and IKKγ.)
CHAPTER 1

LITERATURE REVIEW OF T CELL SIGNALING

Signaling through the T Cell Receptor (TCR) to enact activation of a T cell is one of the most eloquent and precise biological mechanisms as yet discovered. The way each protein interacts with others in a tightly temporal and spatially relevant manner is more beautiful and strategically choreographed than any Capoeira [1]. All transduction begins with the ligation of a cell surface receptor, and in the case of CD8 T cells, this would be the “lock and key” effect of perfectly aligning the TCR with a Major Histocompatibility Complex (MHC) molecule loaded with a linear cognate peptide. If the binding affinity is high, this is all that is required to completely alter the landscape of the T cell, and to enact thousands of biochemical processes culminating in specific gene transcription, cellular proliferation, and asymmetrical cellular division [2].

1.1. Immune Synapse

The landscape change is due to the formation of an Immune Synapse (IS). The IS can be broken down into three regions: the central Super Molecular Activating Complex (c-SMAC), the peripheral SMAC (p-SMAC) and the distal SMAC (d-SMAC). TCR recognition of its cognate peptide on a MHC molecule, presented by a dendritic cell (DC), causes microclusters of signaling components to form in the p and d-SMAC [3]. Much work has been done by the Shaw lab to illustrate the importance and antigen
requirements of signaling and receptor down regulation / destruction in the c-SMAC. They were able to conclusively demonstrate that the c-SMAC is, in fact, an area of enhanced signaling by measuring the amount of sites phosphorylated on the TCRζ chain. In the p-SMAC, where the microclusters nucleated, an average of four sites were phosphorylated, by the time the cluster had migrated to the c-SMAC, all six sites were tagged with the addition of phosphate groups. Moreover, there is an accumulation of Phosphatidylinositol 4,5-bisphosphate (PIP2) (the precursor of downstream effectors diacylglycerol (DAG) and inositol triphosphate (IP3), whose roles in signal transduction will be elucidated further in proceeding sections) only in the c-SMAC. Additionally, they were able to show that this enhancement of signaling, due to clustering of receptors, was necessary to elicit T cell activation from weak agonistic peptides; weak agonists need the c-SMAC for concentrated signaling; strong agonists do not [4]. This leads to a situation where the c-SMAC can be either activating or degrading based upon the TCR’s affinity for the peptide presented in the MHC cleft. The Shaw lab also nicely fit the opposing models of the c-SMAC being a site of enhanced signaling while also being the site of enhanced receptor degradation, as one begets the other. Clustered signaling brings in more proteins whose natural downstream targets will eventually draw in ubiquitinases and additional negative signaling components, thus terminating the signal post effective propagation.

Arguably, the single most important factor in the IS is the actin cytoskeleton which begins shuttling these microclusters into the center, and it is through size exclusion that different molecules collect in distinctive areas of the IS. Larger molecules, such as CD45 and CD43, remain in the d-SMAC, LFA and Talin are the
main components of the p-SMAC, and in a robust IS, TCR – MHC, CD28, Linker of Activated T cells (LAT), and Protein Kinase C θ (PKCθ) will cluster in the c-SMAC [4]. Michael Dustin’s lab further showed that CD28-CD80 clusters were responsible for bringing PKCθ to the IS, and that would explain their co-segregation in the c-SMAC [3]. Chemokine receptors (CCR5 and CXCR4) are also brought into the IS; it is believed that this switches the receptors from Gi to Gq, and thus they lose their ability to signal for chemotaxis, thus contributing to the more stable interaction between the T-cell and APC [5].

Another function of actin reorganization during the formation of the IS, as shown by the Hoth lab, is to reposition mitochondria at the border of the c and p-SMAC [6]. This allows the mitochondria to act as calcium sinks, maintaining a low level of cytoplasmic calcium, and allowing the cell to maintain a favorable ionic polarity; thus promoting the constant influx of calcium across the cytoplasmic membrane. This flow is required for calcium to act as an operational second messenger, another topic that will be explored in further sections.

It was another major contribution by the Dustin lab that exposed the role of ubiquitin and TSG101 (a critical component of the ubiquitin recognizing, ERCRT-1 family) in the formation of the c-SMAC. They were able to show that in the presence of MG132 (a proteosome inhibitor – that ultimately leads to a severe increase in poly-ubiquitinated proteins and a corresponding decrease in the free ubiquitin pool) TCR-MHC microclusters, although fully phosphorylated and active, could not translocate to the c-SMAC. Once ubiquitin was proven necessary for c-SMAC formation, they extended their inquiry to TSG101 [7], and found that this protein was also essential for
homing TCR-MHC microclusters to the c-SMAC, and ultimately for TCR down regulation. Moreover, they implicate TSG101 as the sorting complex that causes the segregation of PKC-CD28 containing microclusters from MHC-TCR microclusters once they have arrived in the c-SMAC, bundled from the p-SMAC [8].

Finally, the Swat lab was able to show a role for Vav (a Ras–Guanine Nucleotide Exchange Factor (GEF)) in the homing of TCR-MHC microclusters towards the c-SMAC that was independent of its catalytic activity. They elegantly demonstrated that VavNULL (missing all three isoforms) T cells could not relocate, fully phosphorylate, and activate TCR clusters from the periphery to the c-SMAC, and they were able to attribute this to Vav as an adaptor protein tethering TCR to F-actin [9]. Dubiously, this effect was rescued by using a GEF-inactive mutant of Vav, demonstrating a role for Vav1 as an adaptor protein promoting the formation of an efficient IS, not just a Guanine Nucleotide Exchange Factor protein that will ultimately lead to the activation of Ras. However, this was actually an additional layer of cytoskeletal reorganization orchestration by Vav, as the Takesono lab had already shown that Vav was responsible for creating active Cdc42 and Rac1 (through the catalytic activity of Vav’s GEF domain). Cdc42 and Rac1 then activate WASp and WAVE2, respectively; the activation of these two proteins is essential for actin polymerization leading to cytoskeletal reorganization [10], thus allowing the formation of a proper IS that will link the T cell and APC for the duration of stimulation.
1.2. Signal Transduction

The proteins, and motifs contained within them, used to transduce the signals from the TCR, are relatively simplistic, few, and conserved across a multitude of eukaryotic biological processes. The proteins include protein kinases, protein phosphatases, ubiquitin ligases, and protein adaptors (while proteases play necessary roles, they are not characterized as signal transducers [11]). The motifs of signal transduction break down into four categories that mirror the protein classes: phosphorylation, dephosphorylation, ubiquitylation, and protein-protein interactions (with the fourth motif usually requiring modifications of one or all interacting proteins, by one of the other 3 motifs to create a conformation change or docking site.)

Undoubtedly, the most important motif for cellular transduction appears to be phosphorylation, as 90% of signal transduction is modulated by the addition of a phosphate group to an acceptor amino acid [12]. Phosphorylating a protein by either a Serine/Threonine or a Tyrosine Kinase, can lead to: 1.) a conformation change of the protein, perchance unveiling its catalytic domain 2.) creation of specific docking sites for other proteins, especially those containing SH2 domains [13] 3.) a change in binding affinities of peptides 4.) restriction of protein movement or aid in protein localization to other compartments 5.) activation of enzymes 6.) deactivation of enzymes. Ironically, while Tyrosine phosphorylation only comprises 4% of global, cellular phosphorylation [14], it is unquestionably the most abundant and necessary for T cell activation [15]. In fact, elegant biochemical assays have revealed that the TCR (which lacks a cytoplasmic signaling domain, but is non-covalently attached to six CD3 chains (3 distinct dimers of
CD3δε, CD3γε, and CD3ζζ)) is activated through tyrosine phosphorylation of these ITAMs, thus beginning T cell signaling [16].

1.2.1. The Regulation and Role of Lck

There are two main tyrosine kinases that are involved in the initiation of signal transduction from successful binding of a peptide loaded MHC – TCR: Lck, a Src family member, and Zap70, a Syk family member [17]. Lck is a membrane bound protein containing both myristolated and palmitylated sites, that is non-covalently linked to CD4 or CD8 (as shown in Figure 1.1) depending on lymphocyte subset [18]. Lck is regulated by two main proteins: C-terminal Src kinase (Csk) (a tyrosine kinase that phosphorylates Lck at a Tyr 505, rendering it inactive) and CD45 (a phosphatase that strips the phosphate from Tyr 505 allowing for conversion of Lck into its catalytically active form.) The Weiss lab has contributed much of the known body of work on the interaction between these three molecules, and ruled that there is dynamic interplay (a constant phosphorylation/dephosphorylation of Tyr505) in resting T cells. As shown in Figure 1.1 (far left side), this balance only needs to be slightly perturbed during the first moments of TCR-pMHC interaction, to favor sequestration of Csk, and activation of Lck [19]. Notably, the Winkler lab showed a role for serine phosphorylation as an additional negative regulator of Lck activity. Moreover, this phosphorylation event is most likely committed by PKCθ, a serine/threonine protein kinase which is activated several interactions downstream of activated Lck, making this part of an activation induced negative feedback loop, not a method of blocking signal initiation [20] (Figure 1.1 – right side.)
Regardless, once Lck is activated, its target for phosphorylation is the TCRζ and CD3 associated chain, where depending on strength of signal, up two three paired sites (for a total of six) will be phosphorylated [21]. These phosphorylated sites on the TCRζ chain become the docking site for the Syk family member Zap70 via its tandem SH2 domains, as shown in Figure 1.1. Once docked, Zap70 will sequentially auto-
phosphorylate, change conformation, and phosphorylate the protein adaptor LAT and the protein adaptor, and fellow kinase and Syk family member, SLP-76 [22].

1.2.2. The Regulation and Role of Zap70

The Griffith lab has done extensive studies on the function of Zap70 during T cell activation, and one of their first findings was that formation of the Immunological Synapse is impaired, both structurally and constitutively in the absence of a catalytically active Zap70 [23]. Additional work by the Huttenlocher lab helped to map the defect in structure to the mis-localization of Talin, which is required to bring F-actin into the synapse [24], as shown in Figure 1.2. Unsurprisingly, defects in ERK, LAT and PLCγ1phosphorylation / activation were also noted in the Zap70 deficient Jurkat cells created by the Griffith lab. Thus showing a role for Zap70 in the regulation of the IS, however, it still best known for its role in TCR signal propagation.

Most of the protein tyrosine kinases in mammalian cells require that a tyrosine site, within their activation loop, be phosphorylated in order to ascertain a catalytic phenotype. Zap70 is not an exception to this rule, as there are multiple tyrosine and serine residues phosphorylated on activated Zap70. However, in the case of Zap70, it is an auto-phosphorylation in the activation loop. While phosphorylation of Y493 is necessary and sufficient to enact activation of Zap70, many events are precursor to this occurrence, as shown in Figure 1.2. One such event is phosphorylation at serine 520, which was discovered through the use of S to A mutant generation by the Couture lab [25]. The 520 region was chosen as it begins a well conserved motif, S_{520}DVWS_{524}, shared by many other protein serine/threonine kinases. The S_{520}A mutant led to an
almost complete lack of signaling from the TCR receptor. Interestingly, the mutation did not block the binding of Zap70 to the TCRζ chain (as was previously thought to be imperative for Zap70 activation); it actually stopped Zap70 from migrating to the plasma membrane. Elegant experiments involving addition of a myristolation site to the mutant, fully recovered activation of Zap70 and TCR signaling, as measured by robust transcription of IL-2, showing that phosphorylation of S520, and the resulting localization to the plasma membrane are required for Zap70 activation.

Because T cell activation must be a tightly controlled process, protein regulation falls into many classes, from localization, to activation, to inactivation, and finally degradation. Additionally, other proteins, such as the TCRζ chain, are actually recycled to propagate the signal of activation, especially in models of low antigen quantity, where the recycling is based upon serine phosphorylation of TCRζ by PKCθ [24]. During periods of high antigen quantity, by tyrosine phosphorylation enacted through Lck and Fyn, the TCRζ chain is internalized and degraded, and the Valitutti lab has demonstrated that other proteins associated with the TCRζ chain, notably Zap70, suffer a similar fate [26]. Through multiple methods, they were able to demonstrate that degradation of Zap70 and TCRζ have identical kinetics; although the timing is synchronized for degradation of these linked proteins, the mechanism of destruction appears to differ greatly. The first piece of evidence to support this theory is that use of bafilomycin A1 (known to block lysosome based degradation of TCRζ [27]) did not rescue Zap70 from demise. However, when calpeptin (a specific inhibitor of the calcium-dependent neutral protease calpain) was used, Zap70 degradation was ablated. Interestingly, this also blocked degradation of the TCRζ chain, evincing a role
for the destruction of associated Zap70 in marking TCRζ for degradation [28]. Moreover, they were able to show that, in vitro, calcium activated calpain was able to directly cleave Zap70, solidifying an activated calcium pathway as one of the main players in down regulation of TCR signaling. This phenomenon is demonstrated in Figure 1.2, from the top middle to left hand side.

**Figure 1.2. The Regulation and Role of Zap70 in T Cell Signaling.** Beginning on the right side of the diagram we show that an, as to yet be identified, protein phosphorylates Zap70 which causes Zap70 to auto-phosphorylate and change conformation. From there, it will bind the phosphorylated ITAMs on the TCRζ chain and phosphorylate LAT and Slp76, enabling the formation of the LAT signalosome. Zap70 also has the ability to inhibit the effects of negative regulators Dok1 and Crk-L, and to bind Talin, anchoring Zap70 to the cytoskeleton. However the phosphatase HPK1 will eventually strip both the TCR ITAMs and Zap70 of their phosphorylation, leading to their down regulation through calpain (Zap70) and lysosomal (TCR) based degradation. Illustration depicts that Zap70 degradation is a precursor to TCR degradation.
As Zap70 affects and amplifies the signaling to several downstream targets, it is of little surprise that it is tightly regulated at multiple levels. One unlikely candidate, LAT, believed by most in the field to be a positive regulator (or at worst a benign cornerstone) was shown to contribute to the dephosphorylation, and thus reduction of activation, by the Acuto lab. They found that in the absence of LAT, both Zap70 and CD3ζ showed an increase in phosphorylation, meanwhile, their downstream direct and indirect targets (PLCγ1 and Erk) actually showed decreased phosphorylation and activation [14]. This points to the LAT signalisome as a strict regulator of TCR signaling, in which it allows CD3ζ and Zap70 to stay phosphorylated and active, only as long as required for the signal to be passed on to the next set of effector proteins. Then, presumably, HPK1, a negative regulator of TCR signaling, and phosphatase associated with the LAT signalisome, dephosphorylates both Zap70 and CD3ζ [12]. This would effectively clamp TCR signaling and tightly regulate the LAT signalisome without the need for complex disintegration or protein degradation.

A final way Zap70 positively perpetuates TCR signaling is through inhibition of negative regulators. The Bierer lab was able to show that active Zap70 has the ability to inhibit CD2-dependent formation of the Dok1-Crk-L complex [29]. As this complex is known to down regulate kinase activity in the cell [30], blocking its formation allows signaling to propagate at the correct strength. However, it was revealed in 2007, that there is quite a bit of pushback from both Dok1 and Dok2 towards inhibiting TCR signaling at the level of Zap70 activation, as shown in Figure 1.2. The Yamanashi lab showed that knockdown of both Dok1 and 2 led to enhanced phosphorylation of Zap70,
LAT, and Erk upon CD3 stimulation. Meanwhile, the reciprocal was also true: over expression of Dok1 and 2 led to inhibition of Zap70 through competitive binding of the phosphorylated ITAMs on the CD3ζ chain; thus Zap70 could not bind to the TCR and remained inactivated [31].

1.2.3. The Regulation and Role of LAT and Slp-76

All further signal transduction (albeit amplified by CD28 help) is mediated by the formation of the LAT signalisome. Under normal signaling conditions, Zap70 will bind the phosphorylated TCRζ chain and phosphorylate LAT (as shown in Figure 1.3); the seeding of this relatively small protein with nine phosphorylated residues allows the binding of many additional proteins that themselves nucleate even larger multi-protein complexes which ultimately leads to the activation of second messengers including calcium and Ras. LAT forms the backbone for the appropriately named LAT signalisome (docking such SH2 motif containing proteins as Grb2, PI3K, PLCγ1, and Itk) [32] and phosphorylated SLP-76 (which is bound to LAT via Grb2) becomes the docking site for Vav1 and Itk. This dual docking of Itk is necessary to lock it in the signalisome and bring PLCγ1 into direct contact with Itk for phosphorylation [33], and is shown in Figure 1.3.

Another well characterized function of Slp-76 is its ability to enact LFA-1 and integrin signaling through its association with ADAP, demonstrated on the right side of Figure 1.3. In studies using Slp-76 deficient T cells, the cells lacked the ability to become motile, and showed severe inhibition of IL-2 production, as demonstrated by the Rudd lab [34]. The LAT signalisome, although crucial for bringing kinases into
proximity with their substrates, and essential for propagating TCR signaling, is not selective for only positive regulators, and the Michel lab generated a thorough report on the negative regulatory proteins that assemble into the complex immediately upon TCR stimulation [35]. Predominately, these are Src homology 2 domain-containing inositol polyphosphate 5'-phosphatase (SHIP-1) and downstream of kinase (Dok) 1 (both

Figure 1.3. The Regulation and Role of LAT and Slp76 in T Cell Signaling. Beginning on the left side of the diagram we show both LAT and Slp-76 are phosphorylated by Zap70, next they will bind several proteins to form the LAT signalosome. Key areas are the double binding of Grb2 and Itk, which aid in complex stability and allow for activation of PLCγ, respectively. Additionally, Slp-76 is shown as binding to ADAP, which stabilizes the complex within the Immune Synapse, and links TCR and integrin signaling.
inhibitory proteins are shown in the LAT signalosome in Figure 1.3) and Dok2. They first demonstrated that Dok1 and Dok2 were phosphorylated immediately following TCR binding to MHC. Interestingly, they found the inhibitors SHIP1 and Dok2 to be weakly associated in unstimulated T cells, however, with the administration of CD3, this association increased, and also led to the binding of SHIP1 to Dok1, as well. They next showed this association to be imperative for their recruitment into the LAT signalosome, and the inhibitory complex is recruited through SHIP1 binding to Grb2 [35]. Although the LAT signalosome will eventually become heavily laden with negative regulators, and the complex will disintegrate, this is long after the signal has disseminated to the next level: activation of PLCγ1.

1.2.4. The Regulation and Role of PLCγ1

PLCγ1 is one of the most important and pivotal molecules in the signaling cascade leading to T cell activation. It is known to be regulated at the gene expression level by the transcription factor Bcl11b/Ctip2, which also regulates expression of CD8 [36]. With respect to activation of PLCγ1, the Berg lab was one of the first labs to link Itk function to activation of PLCγ1 in 1998 [37]. While other labs had documented the decrease in calcium signaling associated with loss or down regulation of Itk, the Berg lab elucidated the mechanism. By showing that phosphorylation of PLCγ1 (and the subsequent cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and IP₃) was greatly reduced post TCR stimulation in Itk deficient T cells. Furthermore, they were able to show that this was a direct effect on PLCγ1, as neither the TCRζ chain nor Zap70 showed a reduction in tyrosine phosphorylation/activation in the absence of Itk.
The Yablonski lab took it a step further in 2007, when they showed that Slp76 was the main inducer and maintainer of Itk activity [38]. This lab delineated the mechanism by which a small pool of Itk was constitutively associated with Slp76, and that this fraction was responsible for 90% of the catalytic activity of Itk. They went on to demonstrate that separating the two proteins, even post activation of Itk, rendered Itk catalytically non functional; thus indicating that Slp76 not only played a role in the activation of Itk, but that it’s constant association was required to maintain that activation. Finally, they were able to show that Itk directly phosphorylated PLCγ1 at tyrosine residues 775 and 783, two sites critical for the activation of PLCγ1. Stabilization of Itk by Slp76 and phosphorylation of PLCγ1, by Itk, is shown in Figure 1.4A.

In 2006, the Samelson lab took a biochemical approach and completely mapped and assigned functional importance to each of the domains of PLCγ1 [33]. They were able to show that the SH2 domain at the N-terminal region of PLCγ1 was necessary, but not sufficient for recruitment of PLCγ1 to the LAT signalisome. They further evinced that the C-terminal SH2 and SH3 domains were required to maintain stability of the complex, and that all three SH domains were vital for the phosphorylation of tyrosine residue 783.

While dogma dictates that several proteins in the TCR signaling cascade (predominately LCK, Zap70, and Itk) are ultimately important for activation of PLCγ1, it was the Burkhardt lab in 2012 [39] that reinforced the importance of microcluster and IS formation in this process. More acutely, they were able to tease apart differences in actinomyosin dynamics, and show that it was F-actin retrograde flow (as opposed to
myosin IIA stability) that was required for phosphorylation / activation of PLCγ1. Most likely this effect is enacted through F-actin’s ability to govern Slp76 microcluster centralization, thus providing the correct docking substrate for PLCγ1, although it does not affect the phosphorylation status of Zap70 or Slp76. Additionally, this work seems to be the missing piece of the puzzle, as in 2010, the Boussiotis lab proved that RIAM (Rap1-GTP-interacting adaptor molecule) was necessary for correct localization of PLCγ1 [40], and RIAM, a MRL (Mig-10/RIAM/Lamellipodin) family member, plays a critical role in actin reorganization during IS formation following TCR ligation of an MHC I molecule loaded with the correct peptide. Therefore, RIAM is the perpetrator of PLCγ1 localization in the LAT signalosome by affecting PLCγ1’s interaction with the actin cytoskeleton; this is demonstrated in Figure 1.4B.

Vav1 is also a key molecular player in the regulation of PLCγ1. It was first shown by the Tybulewicz lab that Vav1 was required for full activation of PLCγ1 and the ensuing calcium influx [41], because Vav1 is the GEF responsible for the activation of Rac1, which is the GTPase that activates PI3K (which was reduced in Vav1-/- cells). From there, PI3K transforms PIP3 into PIP2, which brings Itk to the plasma membrane through association with Itk’s pleckstrin homology domain [42] (activated Itk was also deficient in Vav1-/- cells). From this discrepancy of Itk localization, it is a linear path to the additional downstream defects in activation of PLCγ1 and calcium signaling. Additionally, they realized that Vav1-/- cells not only had defects in activation of PLCγ1, but that PLCγ1 did not home to the LAT signalosome in the absence of Vav1. While originally this homing defect was thought to be an additional (PI3K independent) mechanism of regulation, this was later disproven by the Burkhardt lab. In 2009, they
brought the story full circle, when the demonstrated that HS1 (hemopoetic lineage cell-specific protein 1) co-immunoprecipitated with Itk in stimulated T cells [43]. This complex is illustrated in Figure 1.4B. This led to the new working model, wherein HS1 is an effector of Itk; meanwhile Itk is responsible for homing HS1 to the IS, where it orchestrates the spatial organization of the LAT signalisome. This explained how Vav1-/- could cause issues with the localization and activation of PLCγ1 entirely through a PI3K-dependent pathway, still involving Itk.

Extending the chain of defects as far up as PI3K, not only demonstrates how tightly controlled and intercalated these proteins and signaling events are, but additionally, it opens up the field to more distal negative regulators of PLCγ1. To this extent, it was of little surprise when the Wange lab reported that deletion, inactivation, or expression of a dominant negative PTEN (the main negative regulator of the PI3K pathway) led to constitutively active Itk and hyper responsive T cells [44], illustrated in Figure 1.4A.

The significance of Vav1 in activation of PLCγ1 was further trumpeted by the Braiman lab. They chose to focus on Vav1’s interaction with PLCγ1 during TCR activation, and found that truncation of the N-terminus of Vav1 greatly decreased phosphorylation of PLCγ1 through instability of the LAT signalisome [45]. This important finding was a bit counter intuitive, as Vav1 was already known to attenuate calcium signaling (far downstream of PLCγ1) through its interaction with Calmodulin [46]. Moreover, this specific mutant of Vav1 is known to be oncogenic and robustly activates many other pathways and transcription factors in T cells, all while abrogating calcium signaling.
Figure 1.4. The Regulation and Role of PLCγ in T Cell Signaling. In panel A, we show that PLCγ’s stability is regulated by LKB1 through direct (binding) and indirect (phosphorylation of Lck – pre-LAT signalosome formation. Additionally, we show how Vav interacts with the PLCγ pathway, by facilitating substrate materialization (PIP2), via activation of PI3K; and cleavage of PIP2 into the second messengers: DAG and IP3. In panel B, we show an additional binding partner RIAM, which is responsible for the correct localization of PLCγ in the IS. Also pictured is the path to PLCγ degradation enacted by Cbl-b.

An additional protein that regulates the association of PLCγ1 with the LAT signalosome is LKB1 [47], as shown in Figure 1.4A. This critical tumor suppressor gene, also known as STK11, is predominately recognized for the activation of AMPK family members in response to DNA damage and cellular stress. Moreover, in colon and lung cancer it is heavily mutated or silenced. In T cells, however, it seems to aid in TCR signaling, as it is immediately tyrosine phosphorylated by Lck at Y36, Y261, and Y365,
upon TCR-pMHC binding. The downstream effects of this interaction were elucidated using a CD4 lineage specific LKB1-/- mouse model, that displayed subpar phosphorylation/activation of PLCγ1, with the corresponding loss of calcium influx. Furthermore, colocalization experiments revealed that PLCγ1 was only associated with the LAT signalosome in the presence of LKB1, despite route of stimulation [47].

A final, negative, regulator of PLCγ1 in T cells is E3 Ubiquitin ligase Cbl-b, shown in Figure 1.4B. The Penninger lab was able to elucidate this role, by showing that Cbl-b-/- T cells were able to be fully activated, and had no difference in calcium signaling, despite high concentrations of ionomycin pretreatment, which would render WT T cells anergic [48]. They further went on to show that in the absence of Cbl-b, PLCγ1 not only showed an increase in phosphorylation / activation after restimulation, but total protein levels were also increased; thus Cbl-b plays a pivotal role in the ubiquitination and subsequent degradation of PLCγ1.

When TCR signaling proceeds through Zap70 and the LAT signalosome, PLCγ1 will be phosphorylated and become conformationaly active. From there, PLCγ1 cleaves phosphatidylinositol 4,5-bisphosphate into DAG (which will feed into the activation of NF-κB and AP-1) and IP3 (necessary for the generation of calcium influx [49].)

1.3. CD28 Signaling in T cell Activation

There is no doubt that costimulation of T cells is imperative for optimal T cell activation, and of the many co-stimulatory molecules, none are more storied and studied than CD28. CD28 is ligated by either CD80 or CD86 on APCs, and enhances TCR signaling at every level. The relatively small molecule (44kD) has an illustrious
cytoplasmic tail with several protein binding domains. Directly after ligation of CD28 by its cognate ligand CD80/86, CD28 is phosphorylated at Y191 by both Lck and Fyn [50]. CD28 has then been shown to bind to the p85 subunit of PI3K through phosphorylation of its YMNM motif, and, downstream, its proline rich domain binds the SH3 domains of Itk, Lck, and Grb2 [51, 52]. Interestingly, all molecules are known constituents of the LAT signalosome attached to the TCR; delineating just how difficult it can be to tease these two pathways apart, as shown in the left side of Figure 1.5.

Much focus is placed on the binding of CD28 to PI3K, as this produces PIP2 and PIP3, and localizes them to an area where molecules with pleckstrin homology domains can bind. One such molecule is phosphoinositide-dependent protein kinase 1 (PDK1) who conveys the activating phosphorylation onto PKCθ (allowing it to activate CARMA1 and canonical NF-κB signaling – discussed later) [53], although the Lee lab has since shown that PDK1 may also directly interact with CARMA1 [54]. Additionally, the Ghosh lab was able to clearly delineate that in T cells lacking PDK1, the activation of the NF-κB pathway is abrogated; meanwhile activation of AP-1 and NF-AT remains unhampered. They further went on to show that CD28 signals were integrated into TCR signaling via the molecule PDK1, whose localization and phosphorylation is dependent on CD28 [55]. They also demonstrated that PDK1 has the ability to localize both PKCθ and CARMA1 to the CD28 microcluster, and confirmed that, mechanistically, PDK1 has the ability to phosphorylate PKCθ at Thr538 leading to stabilization of PKCθ and laying the groundwork for activation of this protein [56], as shown in the top portion of Figure 1.5.

It was, in fact, the association of CD28 and PKCθ that was one of the first ways CD28 signaling was shown to positively impact TCR signaling, by aiding in the
localization of PKCθ to the c-SMAC of the IS. The Dustin lab showed, conclusively, that in the absence of CD28 signaling, PKCθ remained in the p-SMAC [57]. Recently, the Altman lab was able to mechanistically explain this phenomenon as the C-terminal sequence YQPYAPP of CD28 binds FLNa (a large actin–binding protein capable of binding over 70 proteins and mainly expressed in the hemopoietic lineage) which then binds PKCθ [58], as shown in Figure 1.5.

While the PI3K pathway is clearly an important part of CD28-mediated NF-κB activation, roles have also been shown for other binding proteins, including Grb2. While Grb2 has no intrinsic catalytic function, it is the adaptor that brings Vav1 (discussed extensively in the preceding section) into the CD28 microcluster, and leads to the direct activation of NF-AT and the indirect activation of NF-AT (through PLCγ1 regulation) and NF-κB (through a MEKK1 mediated pathway [59]).

Another role for CD28 signaling in T cells is to activate Tec (the founding member of the Tec family of kinases.) While Itk (another family member) has been established as the kinase responsible for activation of PLCγ1 [37], the Olive lab demonstrated that knock down of Tec abolished production of IL-2, while knock down of Itk only ablated production of IL-2 [60]. While this research delineates Tec as the more prominent Tec family kinase for TCR signaling, one caveat is that these studies were performed using PMA and ionomycin, a stimulation which bypasses the need for canonical TCR signaling and activation of PLCγ1. Because the family members Tec and Itk share structural characteristics, it is of little surprise that they bind the same proline rich motif of CD28; interestingly, they demonstrated that, quantitatively, the amount of Tec and Itk that bind to the CD28 receptor are equivalent. Paradoxically, the
main substrate for Tec appears to be Dok2, a known negative regulator of TCR signaling, that must be phosphorylated to be an effective adaptor [61]; this is demonstrated at the top of Figure 1.5. Meanwhile, Itk appears to perpetuate positive TCR signaling through phosphorylation of PLCγ1.

**Figure 1.5. The Regulation and Role of CD28 in T Cell Signaling.** Beginning on the left side of the diagram we show that CD28 is first phosphorylated by Lck and Fyn. Following the top panel (with TCR signaling) PKCθ and FLNa will bind CD28, bringing PKCθ into range of PDK1 for phosphorylation. From there, the CD28 complex will join the LAT signalosome to make one super macromolecular structure. The bottom panel, meanwhile, demonstrates how CD28 signaling has the power to activate NF-κB, even in the absence of TCR signaling.

Although activation of PKCθ and Itk are thought to be imperative for propagation of TCR signaling, and one of the key ways that CD28 signaling amplifies activation of T
cells, the Gajewski lab, in 2011, was able to link the pleiotropic effects of CD28 signaling to one molecule: Ras (shown in Figure 1.5). They first realized that cross linking of CD28 with a monoclonal antibody, or use of professional APC stimulation via B7H1 led to an increase in the activity of Ras (as measured by downstream phosphorylation of ERK, AKT and JNK) and that Ras was consistently associated with CD28 containing microclusters post stimulation [62]. The most convincing piece of evidence, however, came with the ability to replace CD28 signaling with activated Ras, as the use of the constitutively active molecule led to proliferation and IL-2 production with no additional costimulation. Moreover, while this effect relied on TCR signaling, it could accommodate a much lower antigen threshold. This demonstrated not only that activated Ras could be a surrogate for CD28 signaling, but reinforced the role of costimulation in amplifying weak TCR signals brought about by quantitatively or qualitatively subpar antigen [62].

More interestingly, work of late has shown that strong CD28 signaling (in the absence of TCR signaling) has the ability to activate NF-κB. Surprisingly, although the activation of NF-κB still requires full phosphorylation and activation of upstream constituents (including Vav1 and slp-76), there is no activation of other downstream transcription factors, NF-AT or AP-1. This illustrates that CD28 signaling is ultimately biased towards promoting the activation of NF-κB [59]. Evidence to explain this phenomenon comes from CD28’s ability to home and activate IKKα, independent of the IKK complex, and points to the role of non-canonical NF-κB signaling in T cell homeostasis and survival [63]. This pathway is depicted at the bottom of Figure 1.5.
1.4. Activation of AP-1, NF-κB, and NF-AT

![Diagram of signaling pathways involving AP-1, NF-AT, and NF-κB](image)

**Figure 1.6. Activation of AP-1, NF-AT, and NF-κB.** Beginning on the top left hand side (CD80/86) and top middle (TCR) this diagram illustrates all the key players in the signaling cascades leading to nuclear translocation (bottom right) of AP-1, NF-AT, and NF-κB.

1.4.1. Activation of AP-1

It became clear, between 1988 and 1991 that AP-1 was actually comprised of multiple protein subunits of the c-Jun and c-Fos families. Post nucleation of the LAT signalosome, Ras (p21) is activated and feeds forward to activate MAPK pathway proteins ERK and JNK which will ultimately phosphorylate and activate c-Jun [64].
However, from there the pathway becomes murkier, as c-Fos must actually be transcribed and translated before it can dimerize with c-Jun to activate transcription. This is accomplished when ERK-2, post phosphorylation by Elk-1, nuclear imports and binds to the promoter region of c-Fos [65]; illustrated in Figure 1.7.

**Figure 1.7. The Regulation and Role of AP-1 in T Cell Signaling.** Beginning at the top and moving down and to the left, all major proteins, post activation of Vav1 in the LAT signalosome, necessary for AP-1 signaling are illustrated, with special focus on the required transcription of c-Fos, facilitated by ERK2, before c-Fos and c-Jun can dimerize and translocate to the nucleus.
Outside of Ras activation in the LAT signalosome, AP-1 activation is also enhanced by the PKC pathway, through phosphorylation of the Fos kinase [66], and through the CD28 co-stimulatory pathway, through the activation of PI3K, and the resulting acidic sphingomyelinase [67]. Additionally, the Schmitz lab delineated a function for PKCθ in the Vav1 mediated activation of AP-1. They demonstrated synergistic activation of AP-1 when a constitutively active form of PKCθ and Vav1 were overexpressed in Jurkat T cells; and achieved the opposite phenomena when these molecules were knocked down, suggesting that the pathways leading to activation are independent, but not exclusive [68]. Moreover, they showed that Vav1 and PKCθ were constitutively associated in resting Jurkat T cells (depicted in Figure 1.7), and validated that it was Vav1’s ability to bind and orchestrate actin polymerization that homed both proteins to the correct macromolecular complex [69].

Another molecule bridging the gap between TCR ligation and activation of Ras leading to AP-1 nuclear translocation and transcriptional regulation is Src kinase-associated phosphoprotein 55kDa (SKAP55), shown in Figure 1.7. Experiments in the Mustelin lab demonstrated that if SKAP55 protein expression was deregulated, through overexpression or ablation, TCR signaling became uncoupled from the Ras activation as measured by decreases in Erk phosphorylation and AP-1 activity. Furthermore, this effect could be reversed by use of constitutively active Ras or Raf1, additional molecular experiments defined a mechanism whereby SKAP55 binds RasGRP1 and enhances the activation of Ras [70].

The Delovitch lab did a wonderful job, in 1999, of delineating how the activation of AP-1 is brought about through ligation of the TCR receptor. Through multiple
methodology, they were able to show that CD28 enhances the Zap-70 dependent phosphorylation of Vav1 [71]. This more activated form of Vav1 further charges the entire downstream pathway, by acting as a GDP releasing factor (GRF) for Rac-1 [72]. This allows Rac-1 to more easily adopt the (GTP bound) conformation of activation. From there, Rac-1 feeds forward to the activation of PAK1 and p38 MAPK [73], leading to the activation of c-Jun as outlined above. Moreover, the Crabtree lab showed a role for calcium in the activation of AP-1, as addition of cyclosporin A caused reduced activation of AP-1 [74] (interplay between signaling cascades will be discussed in further sections.)

An additional way that CD28 signaling enhances AP-1 function is through the activation of mitogen-activated protein kinase / extracellular-signal-related kinase kinase-1 (MEKK1), which will feed forward to phosphorylate JNK. The August lab showed that overexpression of MEKK1 had the ability to activate AP-1 and the CD28 response element; meanwhile knockdown of this protein, or use of a kinase dead mutant, ablated CD28 signaling to activate AP-1 [75]. They further demonstrated that this molecule likely integrated both TCR and CD28 signaling, and that AP-1 nuclear translocation and DNA binding involved MEKK1 activation of both NF-κB and JNK, as inhibitors against proteins in either pathway blocked the enhanced signaling witnessed in MEKK1 overexpressing cells. Ironically, pathways utilizing MAP kinase/ERK kinase1 (MEK1), NF-κB inducing kinase (NIK), Calcineurin, or p38 had no effect on this phenomena. Finally, they also delineated an opposing function for lymphocyte-oriented kinase (LOK), as overexpression of this protein also reversed the effect of MEKK1 activation [75]. This point is illustrated in figure 1.7.
Another negative regulator of AP-1 activation by TCR ligation is Cbl-b. The Bonvini lab evinced ablation of AP-1 activation by overexpression of Cbl-b, regardless of cell stimulation route (anti-CD3 or PMA). They further elucidated the mechanism responsible for these results, by showing that Cbl-b could competitively bind Grb2, blocking SOS ability to bind, and shunting Ras activation via SOS. Under physiological signaling conditions, Cbl-b is constitutively (weakly) associated with Grb2, until Cbl-b is modified, through phosphorylation and ubiquitination, and displaced post TCR ligation and initiation of TCR signaling [76], as shown at the top of Figure 1.7.

1.4.2. NF-κB

1.4.2.1. The NF-κB Family of Proteins

The NF-κB family is comprised of 13 members. Five are transcriptionally active and consist of RelA (p65), RelB, cRel, p105/p50 (NF-κB1), and p100/52 (NF-κB2). While RelA and p50 are ubiquitously expressed, the expression of p52, cRel, and RelB is mainly limited to cells of the hemopoietic lineage [77]. Although many studies have aimed to divide this subgroup along canonical (p50, RelA, cRel [78, 79]) and non-canonical (RelB, p52) party lines, their high degree of homology makes redundancy in function an almost certainty, especially in lymphocytes where all five family members are expressed [77, 80]. Work by several groups have attempted to determine the role of each NF-κB family member through the use of knockout mice and specific inhibitors [81]. To date, we know that whole mouse deletion of IKKβ, IKKγ, and RelA are embryonic lethal due to liver degeneration [82, 83], thus most studies utilize tissue and
differentiation stage specific conditional knockout models, which will be discussed in further sections.

To further complicate matters, many of the transcription factors within the NF-κB family have been shown to regulate the expression of additional family members [84]. This makes it extremely difficult to diagnose whether deficiencies evinced in a single family member knock out study are the result of that missing transcription factor or the result of reduced expression of another family member whose transcription is regulated (and thus diminished) by the missing family member.

The next subgroup of proteins is the inhibitors of κB, whose main job is to retain transcriptional subunits in the cytoplasm until the correct stimuli allows for the degradation of these inhibitors and the release of the transcriptional subunits. The proteins are IκBα, IκBβ, IκBε, IκBζ, and Bcl-3. Once again, expression is tissue specific, with IκBα and IκBβ being the predominate repressors in lymphocytes [85]. Moreover, Cheng et al recently used a knock in system to express IκBβ under the IκBα promoter (thus concurrently knocking out IκBα) and showed that the two proteins had almost entirely redundant functions [86]. Additionally, it is believed that IκBε favors the repression of cRel and RelA containing homo and heterodimers [87], and that IκBζ has the ability to exert repression in the nucleus and cytoplasm [88].

The third class of proteins within the NF-κB family is the inhibitors of kappa kinase (IKK). This group of three members is divided into two activation members with kinase domains (IKKa and IKKβ) and one regulatory subunit (IKKγ aka NEMO). The purpose of this subgroup is to mark the IκBs for degradation, thus effecting the release of the transcriptional proteins of the family [85, 89]. This subgroup must form a complex
to exert its inhibitory effects and exact degradation of the IκBs; this can be a trimer composed of all three subunits, or a homodimer of two IKKβ subunits with one IKKγ subunit. Much work has been done to flush out the role of each activating kinase (IKKα and IKKβ) and current dogma holds that while IKKβ is the key member for transmitting signals pertaining to inflammatory stimuli and lymphocyte specific (TCR/BCR) signals [90], IKKα is more prevalent in signaling pertaining to growth receptors and the lymphotoxin pathway [91].

While it is clear that each kinase has its preferred targets and signaling pathways, conditional knockout studies have demonstrated that there is a great deal of redundancy between these two family members. The Verma lab showed that deletion of IKKβ reduced, but did not ablate, canonical activation of NF-κB, as long as IKKα was present, whereas elimination of both family members fully abrogated canonical signaling [92]. Finally, using conditional knockouts, the Pasparakis lab has been able to show that IKKγ is essential for T cell canonical activation of NF-κB, and that while IKKα can perform most of IKKβ’s duties, T-regulatory and NK-T cells are severely underrepresented in mice missing IKKβ [89].

### 1.4.2.2. The Role of NF-κB in T cells

The Molinero lab has recently proven what many people in the NF-κB community have long suspected: that basal NF-κB activation is required for survival of naive T cells, as shown in Figure 1.8 “Naïve”. They did this by showing that transcription of the IL-7 receptor (a required mediator for naïve T cell homeostasis) is dependent on NF-κB activation [93]. They were further able to elucidate that this mechanism is evolutionarily
conserved across human and mice, but is not related to tonic TCR signaling, as CD5 was not down regulated in the IκBα DN (super repressed NF-κB model.)

As NF-κB is very important for survival of antigen inexperienced T cells, it is also an important driver in survival of effector and memory T cell subsets. Work by the Croft lab has recently shown that OX40 plays a crucial role in this process [94]. OX40 is a costimulatory molecule of the Tumor Necrosis Factor Receptor (TNFR) super family, and it is normally up regulated 18 – 48 hours post activation of the T cell. Unlike other TNFR family members that normally activate NF-κB through MYD88, OX40 utilizes TCR based signaling pathways. Signaling through OX40 has been shown to nucleate a complex of TRAF2, RIP, the CBM complex (discussed in a later section), PKCθ, and the IKK complex, leading to canonical activation of NF-κB via degradation of IκBα [94]. This canonical activation is much stronger than non-canonical signaling and lasts longer than NF-κB activation generated through the standard TRAFF pathway. This is presumably based on trading of the CBM complex (discussed in depth in additional sections) for that of TRADD (the accepted ubiquitin ligase for TNF signaling to NF-κB.) This point is illustrated in Figure 1.8 “Memory.”

Finally, while many of the NF-κB family members have redundant functions [95], some have the ability to inhibit other family members, as has recently been shown for RelA and RelB. Dogma has dictated that these two family members had little interaction and were activated through distinct pathways and by different T cell surface receptors. RelA was known to be an activating family member controlled by IκBα, stimulated through TCR signaling, and the cornerstone of the canonical NF-κB pathway [96]. RelB, conversely, is the main player in the non-canonical NF-κB pathway, usually
activated through lymphotoxin signaling and believed to mainly repress transcription [97] [98]. The Neumann lab, however, evinced an additional way that RelB can exert its repressive properties: through binding to RelA. They nicely delineated that these heterodimers were incapable of binding κB sites in the DNA that would normally play

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**Figure 1.8. The Role of NF-κB in Naïve and Memory T Cells.** The left panel shows the importance of non-TCR activation of NF-κB in the transcription of the IL7R. the right panel demonstrates how memory cells utilize the signals from the OX40 receptor to chimerize canonical and non-canonical NF-κB signaling, to activate this important transcription factor more quickly and robustly than following only the non-canonical path.
host to RelA:p50 heterodimers. Moreover, they were able to show that this RelA:RelB heterodimer was found in the nucleus and the cytoplasm and did not appear to fall under control of IκBα [99]. It remains to be elucidated if NF-κB heterodimers of RelA:p50 or cRel:p50 must compete with RelA:RelB heterodimers, already in the nucleus, for κB binding sites once canonical NF-κB signaling is activated post TCR signaling.

Another family member with inhibitory properties is NF-κB2 (p100). Through TLR, or other non-canonical stimulation routes, p100 is cleaved into the active p52. When signaling through the TCR occurs, however, p100 is upregulated and maintains its full length form. The accumulation of this protein allows it to inhibit other family members through binding and cytoplasmic sequestration, thus functioning as a negative feedback loop to begin quenching TCR signals and return to cellular homeostasis post T cell activation [100].

Finally, while it is known that RelA and p50 are ubiquitously expressed and important for a plethora of canonical signaling pathways, cRel appears to be particularly important for T-lymphocytes. The expression of both the IL-2 cytokine and its receptor (IL-2R) are directly regulated by cRel [79]; moreover, expression of cRel is highly upregulated in T-regulatory cells and it has been shown to govern transcription of FoxP3 [101].

1.4.2.3. Using p50 -/-cRel-/- Mice to Explore the Role of Canonical NF-κB in T Cells

A great body of work has been completed by the Beg and Ohashi labs in determining the effect of p50 and cRel activation on gene transcription and activation of
T cells. The Ohashi lab was able to show that only cRel was necessary to push cells towards activation, and away from anergy; while the loss of p50 had little to no effect on any readouts of functional activation. Moreover, they were able to link PKCθ to cRel by demonstrating that many of the defects in PKCθ-/- T cells were due to decreased nuclear localization of cRel [102]. Additionally, they were able to track the deficiencies in cRel-/- T cells to a lack of the anti-apoptotic protein, Bcl-xL, and show that this reduction had a more substantial effect on CD8 T cells versus CD4 T cells [103].

The Beg lab, concomitantly, showed cRel to be the more important of the two family members, and attributed additional phenotypic defects, such as dearth of IL-2 production and reduced proliferation, to the loss of both p50 and cRel. Utilizing p50-/cRel-/- CD4 T cells, they evinced a necessary role for NF-κB in TCR-stimulated production of IL-2 and Bcl-2 (both required for T cell survival.) They also showed that post initial transcription of IL-2, NF-κB plays a minimal role in mediating IL-2 or AKT survival pathways [104]. Interestingly, while cRel deficient mice have been shown to have greatly reduced numbers of T-regulatory cells, the absence of this transcription factor does not appear to affect T-reg function, as there is no difference in suppressive ability between WT mice and their cRel-/- counterparts [105].

1.4.3. Activation of NF-κB

1.4.3.1. The Regulation and Role of Protein Kinase C (PKC) θ

One unique function of PKCθ, that is just beginning to emerge, is the role it plays in transcription, as the Rao lab recently shown a role for nuclear PKCθ. In several
elegant studies they showed PKCθ in a complex comprising RNA polymerase II, the adaptor 14-3-3z, and the histone kinase MSK-1. This activated nuclear complex was found on the promoter site of inducible immune response genes of the adaptive cell lineage and was associated with microRNA specific to cytokine regulation [106]. This phenomenon is illustrated in Figure 1.9D.

1.4.3.1.1. PKCθ in the Immune Synapse

PKCθ is the only PKC family member that is recruited to the IS upon TCR recognition of an APC presented target peptide [107]. Truncated mutants have shown that it is the N-terminal regulatory domain of PKCθ that it responsible for its lipid raft localization; the protein kinase Lck is also essential for this process [108]. Moreover, while it has long been known that PKCθ was required for the localization of CD28 in the IS [109], only recently did the Schwartzenberg lab reveal the protein responsible for aiding in the recruitment of PKCθ to the c-SMAC. They implicated two proteins SLAM (signaling lymphocytic activation molecule) and SAP (signaling lymphocytic activation molecule-associated protein) as being important for the homing of PKCθ to the c-SMAC, as shown in Figure 1.9B. SAP was already known for its role as chaperone of Fyn into the SLAM complex, but they elucidated a mechanism by which SAP was, additionally, constitutively associated with PKCθ in T lymphocytes [110]. Interestingly, they targeted R78 as the site of PKCθ binding to SAP, as this site was already known to be the site of Fyn docking, but that interaction requires a neighboring tyrosine residue be phosphorylated [111], whereas PKCθ binding does not. Furthermore, it appears that
Fyn binding has no effect on the SAP-PKCθ dimer being enfolded into the SLAM complex.

1.4.3.1.2. PKCθ in Signal Transduction

As early as 2000, scientists were aware that IKKβ was necessary for activation of NF-κB, and it was the work of the Nel lab [109] that showed physical association of PKCθ and the IKK complex in the IS of T cells during stimulation. Moreover, they were able to show a role for PKCθ in the activation of the CD28 Response Element; this has led to an explosion of research on this novel PKC protein.

Aside from its role in promoting the CBM complex (illustrated in Figure 1.9C) many additional functions of PKCθ, have been shown in T cells. One such function is the down regulation of TCR molecules to recycle and amplify the signaling motif. Outside of physical degradation of proteins, and down regulation of receptors, phosphatases are the number one agent in signal down regulation and termination. In 2006, the Mustelin lab showed Hematopoietic Protein Tyrosine Phosphatase (HePTP), was one such protein. Phosphorylated by PKCθ in the IS, this protein is then targeted to lipid rafts where it ablates TCR signaling by removing activating phosphate groups [112] The Geisler lab further illustrated a role for PKCθ in down regulating TCR proteins complexed to MHC, and showed a concurrent role for PKCα in the down regulation of TCR molecules unbound to MHC [113]. They delineated a mechanism whereby phosphorylation on Ser126 of the CD3γ chain (either by PKCθ or PKCα) makes the di-leucine motif available or binding by AP-2. It is this binding that will allow for endocytosis of CD3 [114]. The role of PKCθ in the degradation of the TCR is depicted in
Interestingly, further associations have been drawn to the overlapping functions of PKCθ and PKCα. Although PKCθ has long been touted as the novel PKC required for TCR activation of NF-κB, recent studies by the Baier lab have shown that PKCθ-/- PKCα-/- T cells caused much less GVHD in an allogenic transplant model (a measure of activation) than do single knockouts, whose phenotype is similar to WT T cells. Moreover, they were able to show that activation of NF-AT was seriously abrogated in the double knock out mouse model when CD3 stimulation was applied [115]. They mechanistically linked depression of NF-AT activation to hyperactive GSK3β due to reduced phosphorylation of ser9. As this modification was not evident in either single knockout, it appears both the novel PKCθ and the classical PKCα have the ability, and correct sub-spatial location, to enact the phosphorylation of this site on GSK3β and impact NF-AT activation [115]. The role of PKCθ in this process is illustrated in Figure 1.9A.

An additional function of PKCθ that positively regulates TCR signaling, is its ability to mediate the ubiquitination and subsequent degradation of Cbl-b, a known negative regulator of TCR downstream targets PI3K, PLCγ1, and, ironically, PKCθ [116]. Although the Baier lab was able to show that Cbl-b and PKCθ associated shortly after stimulation of the TCR, and they could prove that this association was only possible with an activated PKCθ protein, no clear mechanism linking PKCθ to degradation of Cbl-b was forthcoming. Although in the absence of PKCθ, Cbl-b levels remain high. Even more incriminating, the autoimmune enhanced deregulated phenotype of Cbl-b-/- mice can be rescued by concomitant knock out of PKCθ [48].
Figure 1.9. The Regulation and Role of PKCθ in T Cell Signaling. (A) Demonstrates the two ways that PKCθ can cause the degradation of TCR bound to pMHC, and how PKCα has the ability to down regulate un-bound TCR, an additional role for both PKCs in shown in the negative regulation of GS3Kβ. (B) Demonstrates that PKCθ’s negative regulator (PICOT) is constitutively bound to PKCθ, and shows a role for binding of SAP and SLAM in PKCθ’s subcellular localization along the actin cytoskeleton. (C) Demonstrates the role of PKCθ in the formation of the CBM complex. (D) demonstrates PKCθ’s ability to affect gene transcription through binding to the RNA Polymerase II complex.

One more negative regulator of PKCθ was found to be PICOT (PKC-interacting cousin of thioredoxin). Interestingly, PKCθ and PICOT were shown to be constitutively
associated in Jurkat T cells, and while stimulation altered the location of both proteins within the plasma membrane space, it did not break the association. However, over expression of PICOT was able to abrogate both AP-1 and NF-κB pointing to tight regulation of this pairing as crucial for controlled TCR signaling [117]; this is depicted in Figure 1.9A.

PDK-1, PLCγ1, and Vav 1 have all been established as upstream of PKCθ [118] and numerous binding partners have been identified for PKCθ. Perhaps PKCθ is most well known for its role in the CARMA1, Malt1, Bcl-10 (CBM) complex. This is perceived as the main upstream complex linking TCR engagement with activation of NFκB [54]. Current etiology holds that PDK1 phosphorylates PKCθ while simultaneously recruiting it to the lipid raft; although, counter evidence points to PDK1 phosphorylation of PKCθ as a constitutive event that occurs directly after protein synthesis [119]. PKCθ then phosphorylates CARMA 1, allowing for its conformational change and recruitment of Bcl10 [120]. These two phenomena are illustrated in Figure 1.9C.

1.4.3.2. The Regulation and Role of the CARMA1/Bcl-10/MALT1 (CBM) Complex

CARMA1 is a CARD domain containing member of the MAGUK family, which favor binding to other scaffolding proteins through CARD:CARD domain interactions and being tethered to the cytoplasmic membrane, respectively. Through initial studies in Jurkat T cells, it became clear that CARMA1 was essential for the activation of NF-κB, but had no bearing on AP-1 or NF-AT activation; furthermore, this effect had to be downstream of PKCθ, as use of PMA and ionomycin could not rescue the defect in NF-κB activation.
The Lenardo lab was the first to illustrate modifications of CARMA1 and its role in the activation of NF-κB by the protein casein kinase 1α (ck1α) [121], which is depicted in Figure 1.10. Interestingly, it seems to both positively and negatively regulate the process of complex assembly, as it positively associates with the CBM directly after TCR signaling and enhances cytokine production, but then also phosphorylates CARMA1, aiding in its subsequent phosphorylation and inactivation. Two other proteins known to positively regulate CARMA1 activation through phosphorylation are PKCθ and HPK1 [122, 123]. PKCθ has been identified as the kinase responsible for phosphorylating serines 564, 649, and 657 in the linker region of the CARMA1 molecule, while HPK1 is the newly identified culprit applying phosphate groups to serines 549, 551, and 552, in the same area. Both kinases have been proven necessary for full activation of CARMA1 and translocation of NF-κB heterodimers to the nucleus, as knocking out either PKCθ or HPK1 abrogates NF-κB signaling. Moreover, in each case, phosphorylation of only 2 (of 3) serine sites seems to be sufficient. With respect to PKCθ, mutation of either 564 or 657 abrogates CARMA1 activation, meanwhile mutation of S649A actually enhanced activation of IKKβ; demonstrating that this site may both positively and negatively regulate activation of CARMA1[124]. With respect to HPK1, serines 549 and 551, when mutated to alanine, abrogated activation of NF-κB in vivo (as gauged by IL-2 production); meanwhile, mutation of 552, only had an effect on CARMA1 activation in vitro [123]. These phosphorylation events are illustrated in Figure 1.10.

Importantly, CARMA1 has been revealed as the protein necessary for binding the Bcl-10/Malt1 protein complex (through the CARD domain on Bcl10) and homing it to the
plasma membrane [125]. It was later shown that Bcl-10 and Malt1 were constitutively associated in T cells, and that this association was indispensable for TCR signaling to NF-κB [126]; this occurrence is shown in Figure 1.10. This sets up a model whereby

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**Figure 1.10. The Regulation and Role of the CBM Complex in T Cell Signaling.** Beginning on the top, left side with signals from the TCR, the arrows depict all the phosphorylation and dephosphorylation events that must occur with respect to CARMA1 and Bcl-10 before they can bind using their CARD:CARD domains. Additional detail is given to proteins known to stabilize or detract from complex formation. The bottom of the diagram represents the fully assembled CBM, and shows its role in activating the IKK complex.
CARMA1 is responsible for the proper location of the CBM complex, and Bcl-10 and MALT perform the phosphorylation (of IKKβ) and the ubiquitination of (IKKγ), respectively, that are necessary for NF-κB activation [127]. However, it was additionally shown that dephosphorylation of Bcl10 by Calcineurin [128] is also crucial for the complex formation, as shown in Figure 1.10.

The Penninger lab was able to elucidate the next series of functions of CARMA1, by first showing that it was not required for proliferation or formation of the IS, as both were at WT levels in a CARMA-/- mouse model. It was, however, still crucial for activation of NF-κB and cytokine production. Furthermore, they delineated that while IKKβ appeared to migrate towards the focal contact point between the T cell and APC of its own volition, CARMA1 was necessary for the correct localization of IKKβ in the c-SMAC compartment. Moreover, they showed that IKKβ was not active in the absence of CARMA1 [129].

It was later in 2004, when the Vito lab would fully elucidate the physical and functional interactions between CARMA1 and the IKK complex: through association with IKKγ (NEMO.) They were able to pull NEMO down with CARMA1, in a stimulation dependant manner. They were also able to isolate Bcl-10 in NEMO immunoprecipitated fractions showing that the entire CBM complex was present, and further implicate MALT1 as the E3 Ubiquitin Ligase responsible for ubiquitination of IKKγ [130]. In 2007, the Kurosaki lab would implicate IKKβ in the additional phosphorylation of CARMA1 [131], furthering the complex and intricate interaction between these two protein complexes, which are demonstrated in Figure 1.10.
As CARMA1 has been shown to be the initiator of CBM signaling to NF-κB activation, a role for Bcl-10 is emerging as the protein responsible for down regulating the activation signal. The Krappman lab was able to show that approximately 3H after stimulation, there was a severe reduction in Bcl10 cytoplasmic levels. Through the use of multiple antibodies and interrogation of the detergent insoluble fraction, they illustrated that this reduction in protein level was due to degradation of the protein, not post translational modification or cellular translocation [132]. As PMA, but not LPS, induced this degradation, combined with evidence that Bcl-10 was additionally phosphorylated directly before degradation, scientists honed in on PKCθ as the culprit mediating Bcl10 degradation. Moreover, through mutational analysis, they were able to reveal that the CARD domain in Bcl10 was also necessary for degradation. Interestingly, they also showed through confocal microscopy that Bcl10 degradation did not track through the standard proteosomal degradation pathway, but instead homed to lysosomes for degradation [132]. Correspondingly they showed that ubiquitination was necessary for this trafficking to lysosomes and that this was mediated through the catalytic E3 ligases itch and NEDD-4, but not the RING finger E3 Ligase Cbl-b [132], which is known to promote the degradation of one of BCL-10’s negative regulators, PLCγ1. This model of degradation is illustrated in Figure 1.10.

However, as Newton has taught us, for every action, there is an equal and opposite reaction, and in this case it is an unlikely protein that staves off Bcl10 degradation: the COP9 Signalisome (CSN). This pleiotropic regulator of the ubiquitin/26S proteosome was known to be imperative for canonical NF-κB activation, as it controls degradation of IκBα. Using mutational analysis, the Krappman lab was
able to show that the CSN5 (one member of the CSN holocomplex) bound to ubiquitinated MALT1 post T cell activation, and only after the CBM complex had been formed [133]. Linear thought dictates that association of these two complexes would be necessary to bring IkBα to the CSN for proper degradation; however, proof of concept experiments utilizing knock down of CSN5 revealed that the protein serves additional functions. Namely, it enhances the activation of IKKβ, and stabilizes Bcl10 by staving off degradation, as shown in Figure 1.10.

Dogma explained AKTs involvement in the NF-κB pathway as negligible. Although there was always a correlation between the activation of the two pathways, it was believed that the only interplay was a rheostat function on the part of AKT. Essentially AKT was responsible for monitoring the “temperature of the cell”, and making sure the back ground landscape was in proper order to facilitate full activation of NF-κB [134]. Interestingly, the Kane lab has recently shown additional roles for Akt1 and Akt 2 in the formation of the CBM complex. Initially, they observed a reduction in transcription of NF-κB target genes when inhibitors against AKT1 and 2 were used. Working up the signaling chain, they determined that, in fact, phosphorylation of IKKβ and degradation of IkBα were reduced in the presence of inhibitors of Akt1 and Akt2, as well [135]. Finally they were able to show a disruption in the formation of the CBM complex, but no effect on upstream mediators of complex formation. Thus it was established that AKT1 and 2 play a positive regulatory role in the binding of CARMA1 to Bcl10, and resulting activation of NF-κB, although a direct mechanism remains to be elucidated, the event is depicted in Figure 1.10.
Two other proteins recently shown to be important for CBM formation are: ADAP (adhesion and degranulation-promoting adaptor protein) and PALS1 (Protein Associated with Lin Seven 1) [136, 137]. ADAP, which is recognized primarily for its ability to modulate integrin signaling via control of LFA-1 (through the interaction of ADAP / LFA1 / SKAP55) and maintenance of the T cell:APC interface [138], also seems to both positively and negatively impact the CBM complex. It is critical for honing TAK1 and CARMA1 to the PKCθ containing CD28 signalosome; yet several groups have shown that through a CARMA-mediated pathway, it also has the ability to disband the CBM [139]. PALS1, meanwhile, is known for participating in large multi-protein complexes that regulate cell polarity. The Bidere lab, however, illustrated that it is also important for activation of NF-κB, as CARMA1 was not found to be associated with Bcl10 in the absence of PALS1, in Jurkat T lymphocytes [140]. The role of these two proteins is illustrated in Figure 1.10.

Once the CBM complex is correctly formed, the main function appears to be bi-functional in the activation of NF-κB: phosphorylation of IKKβ by the kinase TAK1, which always travels with the adaptor TAB1 [141], and ubiquitination of K63 on IKKγ, by the E3 Ligase Traf6 [142]. The importance of the CBM complex in T cell signaling cannot be overstated, as over expression of any of the constituents of the complex leads to spontaneous oligomerization and formation of the complex, which can lead to activation of NF-κB in the absence of upstream signal activation [143]. Moreover, the complex keeps expanding, and now it is known that both TRAF2 and TRAF6, along with UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1A) are required for the full activation of the IKK complex (phosphorylation of IKKβ and
ubiquitination of IKKγ) leading to destruction of IκBα [144], as shown at the bottom of Figure 1.10. This reveals the CBM as a point of intersection between the adaptive and innate immune responses, as it controls all canonical NF-κB activation, despite route of stimulation.

1.4.3.3. The Regulation and Role of the IKK Complex

1.4.3.3.1. The Role of IKKγ in T cell Activation

Dr. Karin has identified the IKK complex as containing two catalytic proteins (IKKα and IKKβ) and one regulatory subunit (IKKγ, aka NEMO) [85]. His work, along with that from Dr. Schmidt-Supprian placed the complex at the center of NF-κB activation via its ability to induce degradation of IκBα in 2000 [89]. Additional work by Dr. Li dictated that IKKγ had regulatory, not activating functions within the complex [145]. Three years later, it was Dr. Ting who first established the necessity for IKKγ in IL-2 production. Using a Jurkat T cell line with knock down of IKKγ, he showed specific defects in IL-2 production compared to WT cells. Moreover, he was able to trace this defect specifically to lack of phosphorylation of IKKβ, and loss of degradation of IκBα, rendering canonical NF-κB subunits unable to undergo nuclear translocation. Meanwhile, the AP-1 and NF-AT pathways remained unaffected [146].

1.4.3.3.2. The Role of IKKα in T cell Activation

Unfortunately, very little is known about the role of IKKα in T cell activation, and the general consensus of the field is that IKKβ is the required family member to illicit T
cellular activation from TCR ligation, while IKKα is a dispensable passenger [89]. In primary fibroblasts and COS cells, however, IKKα has been shown to have two unique functions that enhance NF-κB signaling, and could potentially be applicable to NF-κB activation in T cells via TCR signaling. In 2000, the Gaynor lab first showed that in fibroblasts IKKα could phosphorylate and induce destruction of IκBα. This is also true in T cells, but IKKβ has greater kinase activity and affinity for IκBα. The novel finding, however, was that IKKα seemed to be responsible for IKKβ’s increase in kinase activity through direct phosphorylation of IKKβ by IKKα [147]. Three years later, they would demonstrate a nuclear role for IKKα, in large complexes with RelA and CREB-binding protein. These complexes were formed upon cytokine treatment and honed to NF-κB responsive promoters where they would phosphorylate residues in histone H3, inducing subsequent acetylation [148]. Although these two roles will need to be verified in T cells, this dual functionality is completely plausible.

1.4.2.3.3. The role of IKKβ in T cell Activation

As early as 1999, the Nel lab conclusively proved that IKKβ was the premiere kinase between IKKα and IKKβ in TCR signaling. They also showed that primary CD4 T cells actually form two independent IKK complexes upon TCR stimulation, one heteromeric containing IKKα/ IKKβ / and IKKγ, and one homodimeric with two subunits of IKKβ and one subunit of IKKγ. Additionally, while overexpression of IKKβ leads to hyperactivated T cells, overexpression of IKKα has no effect. Moreover, inhibitors specific for IKKβ or knockdown of IKKβ ablated T cell activation, while interfering with expression or function of IKKα, did not inhibit T cell activation [149].
As with many discoveries in Science, the finding by the Janssen-Heininger lab, that IKKβ was a direct target for Nitric Oxide (NO) was accidental, as they first believed that NO’s suppressive effects were based solely on the ability of NO to disrupt NF-κB binding to DN [150]. Through a series of elegant experiments, they proved that it was the cysteine residue at aa179 that became S-Nitrosylated and attenuated the kinase activity of IKKβ, but only after IKKβ had been activated, as the phosphorylation of surrounding sites (Ser177 & Ser181) provides the imperative negative charge to the region. This was an important milestone in 2004 as it was one of the first papers to independently look at IKKβ (outside of the NF-κB super family in general [150].) This phenomenon is illustrated in Figure 1.11A. However, it would take until 2012 for dogma to view each member of the IKK complex (α,β,γ) as independent members of TCR induced NF-κB activation, when the Li lab [145] was able to show that IKKγ and IKKβ actually migrated to the IS (using detergent fractionation of lysates) at different kinetics and had disparate lengths of stay, showing that the complex was only formed right before degradation of IκBα.

Most of the field views the phosphatase PP2A as a negative regulator of NF-κB signaling, with the Krammer lab recently showing that IκBα shows a higher degree of phosphorylation (and there is an increase in T cell proliferation) when PP2A is silenced [151]. Contradictorily, however, the Kahn lab has shown a positive regulatory role for PP2A with respect to IKKβ, by showing that PP2A had the ability to reduce the amount of negative phosphorylation that IKKβ will endure during the activation process, and that PP2A exerts this effect through complexing with IKKγ [152]. The conflict between these two studies may be explained by the Krammer’s lab use of specific siRNA against only
one subunit of the PP2A complex, B56γ, while the Kahn lab utilized the popular PP2A inhibitor okadaic acid to block the entire complex. However, the conflict also draws light to the necessity of exact regulation of TCR signaling, and the fact that many proteins have the ability to be both positive and negative regulators, depending on where in the signaling chain they are located both spatially and temporally.

While it has long been accepted that the role of IKKβ in NF-κB signaling was to phosphorylate IkBα [153] and RelA [154] (to promote its degradation, and activation, respectively) in 2010, the Li lab showed an additional role for IKKβ outside of the activation of NF-κB, in the CD4 T cells of aged mice [155]. Here it was shown that IKKβ levels increase in CD4 T cells as the mouse ages, and that this increase correlates to, and appears to be the cause of, inhibited JNK activation, as shown in Figure 1.11C. This inhibition of JNK allows the cells to increase affinity for Bad to 14-3-3 (pro survival) while decreasing the interaction of Bad to Bcl-XL (pro apoptotic), thus rendering the cells insensitive to activation induced cell death (AICD). This age-associated increase in IKKβ also causes a corresponding increase in NF-κB activation, and aged T cells produce a much greater volume of proinflammatory cytokines than do their juvenile counterparts [155].

Moreover, the Krappmann lab demonstrated a role for IKKβ upstream of CBM complex formation. They were actually investigating the role of PKCθ in assembly of the CBM complex utilizing the PKCθ specific inhibitor Rotterlin, when they realized that their negative control (use of BAY11-7085, a specific inhibitor of IKKβ) also blocked Bcl-10 phosphorylation and CBM formation [156]. Further experimentation revealed that IKKβ was constitutively bound to Bcl-10 and Malt1 in unstimulated cells, placing this
important kinase even farther up the signaling chain, and proving that IKKβ was responsible for the assembly of the CBM, as phosphorylation of Bcl-10 (at multiple serine residues in the C-terminus, by IKKβ) is the event that allows CARMA1 to dock with Bcl-10 and Malt1. Additionally, through the use of several lineage specific knockout mice and shRNA, they were also able to show that IKKγ (the regulatory subunit of the IKK complex) or IKKα (a sister kinase in the complex – generally thought to be more impactful in TLR activation of NF-κB [157]) are not required for formation of the CBM; thus identifying this mechanism as an independent role for IKKβ, not a function of the IKK complex; these events are depicted in Figure 1.11B. They went on to further demonstrate that this phosphorylation event served to not only enhance CBM complex formation and activation, but also served as a negative regulator of this pathway, as the phosphorylation of Bcl-10 disrupted Malt1–Bcl10 binding and ultimately down regulated ubiquitination of IKKγ [156].

Interestingly, the Zhong lab has also demonstrated a negative regulatory role for IKKβ [158]. They used a constitutively activated form of IKKβ, only expressed under the CD4+ lineage, to evince that those T cells had extremely high levels of Fas, PD1, and Blimp1 expression, as shown in Figure 1.11E. The expression of these molecules is not only characteristic of an exhausted T cell, but also led to an increase in apoptosis and the corresponding decrease in survival. Interestingly, in culture, the caIKKβ CD4 cells demonstrated a moderate basal level of activation (measured by cell surface increase of CD69L and CD25) but were unable to induce strong up regulation of these molecules when treated with anti-CD3/CD28, while there WT counterparts could. They further tracked the activation defects in the cell toward more proximal signaling components.
and realized that phosphorylation of Zap70 and PLCγ1 were impaired, and that these defects led downstream to defects in calcium signaling and ERK phosphorylation [159].

Another potential way that IKKβ can negatively regulate T cell activation is through the phosphorylation / activation of Dok1. The Sylla lab demonstrated that IKKβ had the ability to phosphorylate Dok1 (shown in Figure 1.11D) in response to TNFα, IL-1, and γ radiation; through in vitro experiments, they were able to elucidate the sites of phosphorylation as S439, S443, S446, and S450 [160]. While this mechanism has not been tested in response to TCR signaling, it seems logical that this would be a viable method to quench TCR signaling as Dok1 is known to bind and enhance multiple other negative regulators of TCR signaling including SHIP1, Nck, and Csk [30] and attenuate tyrosine kinase signaling [161]. This down regulation of TCR signaling is ultimately required to begin the overwhelming task of migration to the infection or tumor site, which is known to be facilitated by Dok1 [162].

Meanwhile, a rebuttal to IKKβ as a negative regulator came in a 2013, Journal of Immunology paper from the Piccinini lab [163]. Where, to no one's surprise, they showed that IKKβ was imperative for activation of NF-κB. The novel aspect of this paper came in describing a new and necessary role for IKKβ in the activation of AP-1. They were able to show that IKKβ is responsible for the chromatin remodeling around the promoter sites of c-Fos and c-Jun which ultimately allow for transcription factor binding and AP-1 activation, as shown in Figure 1.11F. Moreover, by inhibiting NF-κB activation, but not IKKβ, they were able to preserve AP-1 translocation to the nucleus; thus indicating this was a specific role for IKKβ, independent of its role in NF-κB activation. Furthermore, they were able to show that this lack of AP-1 signaling in IKKβ
impaired T cells corresponded to the anergic T cell phenotype one would expect in the presence of NF-AT and absence of AP-1 activation [163]. Thus IKKβ performs multiple, crucial roles in the process of TCR signaling to T cell activation in both IKK complex-dependent and independent manners.

Figure 1.11. The Regulation and Role of IKKβ in T Cell Signaling. (A) Demonstrates that IKKβ can be inhibited directly by NO. (B) Demonstrates the positive and negative effects of IKKβ on Bcl-10 and formation of the CBM. (C) Shows that IKKβ, through inhibition of JNK, has the ability to promote cell survival, over activation induced cell death. (D) Shows that through phosphorylation/activation of Dok1, IKKβ can down regulate TCR signaling. (E) Shows that over expression of activated IKKβ amplifies cell surface expression of PD1, Fas, and Blimp1. (F) Shows a role for IKKβ in the chromatin remodeling of the promoter regions of c-Fos and c-Jun.
1.4.4. Activation of NF-AT

1.4.4.1. Calcium-Dependent NF-AT Activation

Long before a T cell will become activated, the landscape must be set to create the correct polarization of the membrane and cellular compartments to allow for robust and timely signal transduction. With respect to calcium signaling, this requires three main feats be accomplished. The first is keeping the cell in the right ionic state and that is accomplished via sodium (TRPM4) and potassium (Kv1.3 & K_Ca3.1) channels [164], and aided by cytoplasmic membrane bound calcium channels [165]. These channels (shown at the top of Figure 1.23) and pumps are activated and expressed at various levels and by a multitude of stimuli, but all function in concert to maintain the homeostasis of intracellular calcium at a low level and maintain the ionic potential across all cell membranes. The second is ensuring that proteins necessary for transducing the calcium signal are kept at appropriate levels and are compartmentalized correctly for easy access during the stimulation chain. Two major players in this process, whose levels are carefully monitored, are STIM1 and Orai1. In fibroblasts, these two genes have been shown to be regulated at the transcription level by NF-κB [166]. At the protein level, their fate lies in the hands of the ubiquitin ligase Nedd4-2, whose activation by AMPK depends on AMPK being kept in an unphosphorylated state, as phosphorylation by SGK1 leads to AMPK down regulation and a lack of Nedd4-2 activation [167]. This phenomenon is illustrated in Figure 1.12.

While other proteins levels are not monitored as scrupulously; there is a great need to make sure that they are correctly localized for the signaling chain to be
effective. One such protein, whose cellular localization has been extensively studied, is PLCγ1. In work by the Pareek lab, it has been shown that Coronin1 is responsible for pulling PLCγ1 into the TCR complex after phosphorylation by Cdk5 [168], as shown in Figure 1.12. Furthermore, the Domdkowski lab demonstrated that it was the protein RIAM that connected PLCγ1 to the cytoskeleton allowing it to relocate with the formation of the IS [40], as shown in Figures 1.4 & 1.12. Moreover, Julien Laconte demonstrated that I-COS was also necessary for this interaction and relocation of PLCγ1 along the actin cytoskeleton [169], Figure 1.12.

Finally, because T cell calcium influx is known to be regulated by Store Activated Calcium Entry (SOCE), the store must be stocked with calcium. This is accomplished by Sarco-Endoplasmic Reticulum (SERCA) pumps and STIM2 [170]. STIM2 especially is known to regulate the basal levels of calcium within the S-ER, by sensing levels within and then utilizing the pumps to keep levels steady, as shown in Figure 1.12.

Upon TCR ligation by an MHC with a cognate peptide, many events transpire in rapid succession, and after only ten seconds of contact, the first wave of calcium is being released. This is a direct result of the following cascade of phosphorylation: Lck phosphorylates ITAMs on the TCR zeta chain creating a docking site for Zap70 [171]; Zap70, once bound recruits and phosphorylates LAT and Slp76, which in turn activate the Ras pathway and PLCγ1 via phosphorylation by Itk [37]. From there, PLCγ1 cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG (which will feed into the activation of NF-κB) and IP3 [49]. IP3 will then bind to the IP3 receptor on the S-ER, causing a small leak of the stored calcium. This small amount of calcium has the ability to bind and change the conformation of STIM1. This change in protein shape redirects
the entire S-ER to the cell membrane where it is now in the correct configuration to bind to ORAI1 [172]. This opens the ORAI channel and calcium begins to influx from the extracellular space, as illustrated at the bottom of figure 1.12.

In addition to the ORAI channels that open to allow calcium to enter the cell, there is another family of channel proteins intimately linked to calcium signaling, the Transient Receptor Potential Cation (TRPC) channels [164]. Although these channels are non-selective for calcium, by allowing the flow of multiple ions across the membrane, they maintain osmotic pressure and drive the influx of calcium. TRPC1 is also known to be constitutively associated with ORAI1 [173]. Another, non-selective, family of channel proteins required for calcium signaling, is the purinergic family. These receptors respond to mechanical stress (created through the tethering of T cells to APCs) and allow flux of multiple ions across their path while contributing to ATP paracrine signaling and amplifying the calcium signal based on a limited number of TCRs [174]. Many labs have delineated roles for P2X1 and 4, and more recently P2X7 has risen to the forefront as indispensable for Calcium signaling [175]. This multitude of additional, non-selective, ion channels is depicted in Figure 1.12.

Once the extracellular influx of calcium has begun, the second avalanche of signaling begins. One of the first proteins modified by calcium interaction is Calmodulin. Once Calmodulin binds calcium, it phosphorylates Calmodulin Kinase II (CamKII), which is responsible for phosphorylating Calcineurin. This phosphorylation conveys an activating conformation, and Calcineurin can then dephosphorylate NF-AT allowing it to translocate to the nucleus [176], as shown in Figure 1.12. The NF-AT pathway represents the most straightforward path from influx of calcium to transcription factor
activation and cellular activation and response; however calcium can also impact the NF-κB pathway (as discussed in further sections).

![Diagram: The Regulation and Role of NF-AT in T Cell Signaling](image)

**Figure 1.12. The Regulation and Role of NF-AT in T Cell Signaling.** Beginning from the top (TCR) and moving clockwise with the arrows, this illustration demonstrates all the key proteins to enact calcium, influx, and in the left hemisphere dictates how the calcium signaling feeds into activation of NF-AT. Additional roles are shown for the regulation of Orai1 and STIM1 (bottom left) with regards to gene transcription and protein degradation.

An unlikely negative regulator of calcium signaling was shown to be AKT2. While both AKT1 and AKT2 are substrates of PI3K, and both are phosphorylated/activated,
almost immediately, following TCR stimulation. It appears that only AKT2 has the ability
to down modulate and decrease the duration of calcium signals. This inhibitory effect
seems to be defined by phosphorylated AKT2’s ability to competitively bind the IP$_3$R of
the S-ER, and block its interaction with Bcl-2 [177]. This event is illustrated on the right
side of figure 1.12.

A forthcoming, positive regulator of calcium signaling, shown by the Cao lab, is
Vav1. For many years, scientists have known that Vav1 homed to the LAT signalisome
during T cell stimulation, and roles have been established for this storied protein in the
activation of AP-1 and NF-κB [41, 59], but it was not until recently that it was proven to
also be instrumental in the calcium and NF-AT pathway. Through binding assays based
on Vav1 mutants, the Cao lab evinced that Vav1 would bind Calmodulin through its
calponin homology (CH) domain, but only during TCR stimulation, and only when
Calmodulin was bound to calcium, as shown in Figure 1.12. They were further able to
delineate that the Vav1-/ T cells and Vav1 with a mutated CH domain had similar
phenotypic profiles and detrimental calcium signaling during stimulation. Interestingly,
this does not seem to affect the phosphorylation / activation of PLCγ1, or the release of
calcium from intercellular stores [46]. These results point to a mechanism whereby
Vav1 – Calmodulin binding stabilizes and amplifies calcium signaling post calcium influx
from the extracellular space.

1.4.4.2. Calcium Independent NF-AT Activation

Interestingly, the Ras pathway has also been implicated as imperative for NF-AT
signaling in a calcium independent manner, as the use of a dominant negative Rac-1 or
MAPKK-1 rendered T cells unable to mount an NF-AT response, yet constitutively active MAPKKK-1 was not able to rescue the effects of absence of activated Ras [178]. This indicates that these two pathways must cross-talk at several levels upon the activation landscape. Moreover, they showed that Raf-1 was likely the MAPKKK that begins the phosphorylation cascade leading to the ultimate phosphorylation / activation of ERK-1 and ERK-2; this demonstrates the duality of Ras signaling, which appears to branch at Rac-1 / Raf1 [178].

Although it is widely accepted that NF-AT must be dephosphorylated for nuclear import, in 2004, the Yuan lab added another dimension of regulation with the discovery that NFAT1 is also sumoylated, as shown in Figure 1.12. Through elegant in vitro and in vivo experiments, they flushed out Lys684 and 897 as the sites for sumoylation. Furthermore, they evinced a mechanism by which Lys684 was required for nuclear import and for additional sumoylation at Lys897; while Lys897 regulates nuclear anchorage [179].

An additional, well known, negative regulator of NF-AT is Glycogen Synthase Kinase-3 (GSK3) which is known to re-phosphorylate NF-AT at sites of Calcineurin dephosphorylation [180]. This event is depicted in Figure 1.12. Interestingly, GSK3 has both cellular and nuclear roles. In the cytoplasm, there is dynamic interplay between GSK3 and Calcineurin (a constant phosphorylation/dephosphorylation of NF-AT) in resting T cells which keeps NF-AT inactivated until there is a strong calcium signal favoring Calcineurin based dephosphorylation. In the nucleus, GSK3 is responsible for the phosphorylation that will cause nuclear export of NF-AT [181].
1.4.5. Pathway Interplay

1.4.5.1. Dimers of AP-1 and NF-AT

One of the most accepted phenomenon in T cell biology is the effect of cooperative binding of AP-1 and NF-AT on DNA [182]. While many labs have shown that the proteins can dimerize, and it is dogma that many transcription factors will bind to the same gene, the interplay between AP-1 and NF-AT is special in that it allows for different results in gene transcription depending on whether NF-AT binds DNA alone, or if it binds as a dimer of NF-AT and AP-1. The Rao lab did a fantastic job of elucidating these two scenarios in 2000, when they illustrated that a tolerance phenotype was adopted by the cell in NF-AT only binding [183]. Concordantly, TNF and IL-13 were produced in abundance and the T cell was unable to undergo activation induced cell death (AICD). Conversely, they demonstrated that activation dependant gene transcription required the AP-1 / NF-AT dimer, as IL-2,IL-3,IL-4,GM-CSF, MIP1a, and Fas ligand mRNA could not be produced in the absence of AP-1 [183].

1.4.5.2. Calcium Impacts NF-κB Signaling

As early as 1994, the O’Neill lab demonstrated crosstalk between the calcium and NF-κB pathways. As they were able to show that activated Calcineurin had the ability to enhance nuclear translocation of the p50/p65 heterodimer, and that this was accomplished via enhanced degradation of the main repressor of NF-κB signaling, IκBα [184]. In 1995, they expanded their work to also include a role for the calcium activated serine protease, Calpain, in the physical degradation of IκBα [185]. It wouldn’t be until
2011, however, that the work of the Marienfield lab would hone in on the exact mechanism of Calcineurin’s positive impact on NF-κB signaling. They elicited a mechanism by which Calcineurin was not only responsible for NF-AT dephosphorylation, but that it also targeted dephosphorylated Bcl-10, and that this dephosphorylation was key to the formation of the CBM complex [128]. Because the CBM complex is upstream of IκBα degradation, this finally linked activated Calcineurin to activation of NF-κB, and explained the phenomenon demonstrated by the O’Neil lab seventeen years earlier. These two phenomena are depicted in Figure 1.13.

Once Calmodulin activates Calmodulin Kinase II (CamKII) another layer of NF-κB interplay is achieved, as multiple labs have shown intersection of CamKII with various components of the NF-κB pathway. One of the first interactions came from the Gundstrom lab in 2001, where they demonstrated that inhibition of CamKII resulted in a complete loss of IκBα phosphorylation when T cells were stimulated. Moreover, the reverse was also true, as over expression of CamKII led to activation of NF-κB in the absence of exogenous stimulation [186]. In 2010, they further delineated exactly how calcium was actually modifying the CBM complex. Ironically, they were able to show two opposing fates: Calmodulin (interacting with Bcl-10) was a negative regulator, as this disrupted CARMA1 and Bcl-10 binding because Calmodulin competitively bound Bcl-10’s CARD domain (CARMA1’s docking site [187].) Meanwhile, CamKII played a positive role in the pathway, as phosphorylation of CARMA1, by CamKII, leads to enhanced complex formation [188], as shown in Figure 1.13.

Calcium even helps to dampen the NF-κB response at the tail end of signaling, and help to “re-set” the cellular landscape, as post activation, there has been a role
shown for calcium bound Calmodulin in the nuclear export of cRel [189]. Furthermore, in a dazzling display of the incredible amount of overlap and interconnectedness of T cell pathways, the Billadeau lab, in 2007, showed that NF-AT1 is actually the protein responsible for transcriptional regulation of the NF-κB family member cRel [190], as illustrated in Figure 1.13.

**Figure 1.13. The Role of Calcium in NF-κB Signaling.** This diagram highlights key areas in which the calcium signaling pathway impacts the NF-κB pathway both positively and negatively.
1.5. Summary of T cell Activation

T cell activation is an extremely complex process that must be tightly regulated at every level to protect against unwanted activation and promote robust and timely activation in the correct setting. Conventional knowledge has dictated that most signals move linearly from the cell surface to the nucleus; this model is easily worked, and one can see each regulatory step in an orderly fashion. Recent research, however, is setting a landscape where pathways dynamically interact and signaling moves multidirectionally. Thus making the tight regulation of T cell activation, against a backdrop of seeming chaos, that much more impressive.

The first attempt at ordering the cellular chaos into distinct activation pathways is through the formation of the Immune Synapse. This sets the board and creates distinct subcellular pockets that will harbor or exclude various proteins based on size, charge, and the ability to bind the actin cytoskeleton. While most TCR signaling will be initiated in the periphery (p-SMAC), successfully bonded pMHC:TCR microclusters will soon join CD28 and PKCθ as they are shuttled towards the center (c-SMAC) of the IS for enhanced signaling potential (weak agonistic peptides) or degradation (strong agonistic peptides). Concurrently, larger molecules, such as CD45, will use retrograde flow, and move to the distal (d-SMAC) end of the IS. This makes the c-SMAC rich with positive regulators of T cell signaling, and repositioning of the mitochondria and S-ER, through cytoskeletal reorganization, stabilize calcium levels.

There are two main classes of proteins that coordinate the formation of the IS. Those that actually regulate the actin dynamics, such as WASP, WAVE2, and Talin, and those that are responsible for physically connecting specific TCR signaling proteins
to the actin cytoskeleton, such as TSG101 (responsible for TCR), RIAM (PLCγ1), FLNa (CD28), SAP & SLAM (PKCθ). Additionally, several proteins regulate the functions of these two classes of proteins (i.e. Vav activates Cdc42 and Rac1 which are required for WASp and WAVE mandated actin reorganization, respectfully; and Zap70 regulates F-actin via Talin.)

The second level of regulation is the transduced signal, itself. This is an intricate mix of protein interactions based predominately on the activities of kinases and phosphatases. Once the TCR ligates a MHC loaded with a cognate peptide, it will pull CD8 and Lck into the complex, and help the phosphatase CD45 to free Lck from Csk’s phosphorylating repression. Next, upwards of six sites will be phosphorylated, by Lck, on the CD3ζ/TCR associated ITAM. This creates a binding site for Zap70, already phosphorylation heavy from unidentified kinases. Zap70 will bind the TCR, auto-phosphorylate, and phosphorylate LAT and Slp76, thus nucleating the LAT signalisome, and, through Slp76, merging TCR and CD28 signaling.

PLCγ1, PI3K, and Itk all bind directly to phosphorylated LAT, which then expands its empire by bridging to Slp76 through their combined interaction with Grb2. This macromolecular structure now contains PDK1, PKCθ, Vav1 and ADAP via Slp76. Other proteins, such as LBK and HS1 provide anchorage for the signalisome, and Itk is additionally bound by Slp76 to solidify the stability of the complex. In addition to forming a stable signalisome, this also correctly localizes all the proteins necessary for the next wave of signaling. Through Vav1, Rac-1 will activate PI3K to transform PIP3 to PIP2, which will be cleaved into DAG and IP₃, by an Itk phosphorylated PLCγ1. The cleavage
of PIP2 into DAG and IP3 represents a critical branching of signal that will activate the NF-κB/AP-1, and NF-AT pathways, respectfully.

DAG will activate PKCθ, and both feed into an already Vav1/Rac-1, Ras activated pathway, which, through cascading kinases, will arrive at phosphorylated c-Jun. This pathway will also allow for phosphorylated ERK2 to undergo nuclear translocation and initiate the transcription and translocation of c-Fos. Once c-Fos is posttranslationally modified, via phosphorylation by the cleverly named Fos kinase, it will dimerize with c-Jun, and translocate to the nucleus as the fully activated AP-1 transcription factor.

Concurrently, DAG and PDK1 will phosphorylate PKCθ, who, along with PDK1, ck1a, CAMKII, IKKβ, and HPK1 will all convey activating phosphate groups onto CARMA1. Meanwhile, Calcineurin will begin stripping phosphate groups from Bcl-10 (already bound to Malt1) and IKKβ will add a strategically placed phosphate group to Bcl-10 that will allow for the binding of CARMA1 to Bcl-10 via their CARD domains, thus an activated CBM complex is formed, and stabilized by CSN5, AKT2, PALS1 and ADAP. The complex will keep growing in constituents by adding TRAF2/6, TAK1:TAB1, and two ubiquitin E2 ligases (UEV1A and UBC13) until it has all functionally active protein motifs to endorse the next stage of NF-κB activation.

Once the CBM+ complex has been formed, it will set to the task of fully activating IKKβ (through phosphorylation by TAK1) and ubiquitinating IKKγ (through MALT1). This will cause the degradation of IKKγ, and release IKKβ to phosphorylate IκBα. Phosphorylation of IκBα marks it for ubiquitination (by UEV1A and UBC13) and proteosomal degradation (by the calcium activated protease, calpain); thus releasing
the bound heterodimers of RelA:p50 or cRel:p50 to translocate to the nucleus and activate transcription.

Meanwhile, IP₃ generation will release calcium from intracellular stores and open the CRAC channels causing a surge in extracellular calcium to influx across the cytoplasmic membrane. Once Calmodulin binds calcium (and Vav1 binds Calmodulin), it will activate CamKII, who will then phosphorylate/activate Calcineurin. Calcineurin is the phosphatase responsible for removing the phosphate groups applied to NF-AT, by GSK3. With additional sumoylation, applied by an as yet undiscovered protein, NF-AT will nuclear translocate, where most molecules will dimerize with AP-1 to ensure proper transcription of immune activation related genes.

There are several negative regulators of TCR signaling. PTEN, HPK1 and SHIP1 are all phosphatases that have the ability to undermine the LAT signalosome. Moreover Dok1 and Dok2 competitively bind the phosphorylated TCRζ ITAMS and block Zap70 binding and activation. Finally, Cbl-b, itch, NEDD4, and Nedd4-2 are all ubiquitin ligases that encourage proteosomal degradation of signaling components at every level. Interestingly, these negative regulators are always present in active complexes, so there is always dynamic interplay between activating and deactivating signals, and TCR activation never proceeds at full tilt.

As explained above, negative regulators are always present in signaling complexes, but they generally do not gain the upper hand until the signal has propagated to the next level. While there is a great deal of overlap, in certain cases, there is a difference between proteins that are negative regulators (i.e. Dok) and proteins that are responsible for signal down regulation and termination (i.e., HeTPT).
TCR signaling is down regulated in multiple ways, several of which are modulated by PKCθ. PKCθ has the ability to phosphorylate Lck (and inactivate it); it can also directly phosphorylate the TCR or activate the phosphatase HePTP (both of which cause degradation of the TCR). Degradation of Zap70 (via the activated calcium-dependent protease, calpain) is also a standard route of signal down regulation. The CBM complex is additionally targeted for degradation through additional phosphorylation of CARMA1 (by ck1a), and phosphorylation and/or ubiquitination of Bcl-10 (by CamKII and itch/NEDD-4, respectively.)

1.6 Goal of Dissertational Research

The goal of this independent research has been to elucidate the variety of ways that T cells missing the NF-κB subunits p50 and cRel are defective with respect to TCR based activation, thus enhancing the knowledge base as to the roles and responsibilities of these two transcription factors in T cells. Through multiple assays, the interrogation of these cells, in a murine - CD8 setting, evinced that additional NF-κB family members (IKKβ and IKKγ) were also defunct in T cells lacking p50 and cRel. Moreover, it appears that many of the proximal defects (reduction in phosphorylation of TCR associated proteins and inhibited calcium influx) discovered in these cells are attributable to the reduction in expression and mis-localization of IKKβ. While later stage defects, such as dearth of IL-2 production and corresponding reduction in proliferation, appear to be the result of poor initial signaling from the TCR exacerbated by loss of key transcriptional units (p50 and cRel). Figure 1.14 illustrates the lackluster
signaling in p50/-/cRel/- T cells (top), juxtaposed against the bottom frame indicating

**Figure 1.14. T Cell Signaling in the Absence of p50/cRel.** The top diagram illustrates the key defects and absence of signaling cascades in cells that are devoid of p50/cRel, and is juxtaposed against the bottom diagram showing full activation of NF-κB.
how we believe IKKβ has the ability to enhance WT proximal TCR signaling through phosphorylation of Zap70.

1.7. Objectives

The purpose of this work is to flush out phenotypic and mechanistic deficiencies in T cell Activation in the absence of PKCθ and p50/cRel, as such; the following objectives will be undertaken:

1. Categorize deficits in PKC-/- and p50-/-cRel-/- CD8 T cell activation by strong foreign antigen in vitro and in vivo
2. Determine defects in gene regulation during activation of PKC-/- and p50-/-cRel-/- CD8 T cell by strong foreign antigen in vitro
3. Discover which transcription factors undergo ablation of nuclear translocation in PKC-/- and p50-/-cRel-/- CD8 T cell by strong foreign antigen or anti-CD3/anti-CD28 in vitro
4. Outline differences in the ability to influx calcium between WT, PKC-/-, and p50-/-cRel-/- CD8 T cells stimulated with strong foreign antigen or anti-CD3/anti-CD28 in vitro
5. Interrogate various calcium channel proteins at the mRNA and protein levels in unstimulated WT, PKC-/-, and p50-/-cRel-/- CD8 T cells
6. Use multiple inhibitors of calcium channel proteins and signaling to ascertain the role of various calcium channel proteins in WT, PKC-/-, and p50-/-cRel-/- CD8 T cells stimulated with strong foreign antigen or anti-CD3/anti-CD28 in vitro
7. Delineate differences in phosphorylation of TCR proximal signaling proteins in WT, PKC-/-, and p50/-/-cRel/-/- CD8 T cells stimulated with or anti-CD3/anti-CD28 in vitro

8. Assess the amount of IKKβ and IKKγ mRNA present in unstimulated WT and p50/-/-cRel/-/- CD8 T cells.

9. Assess the amount of IKKβ and IKKγ protein present in unstimulated WT and p50/-/-cRel/-/- CD8 T cells through western blotting and confocal microscopy.

10. Decipher differences in surface expression of CD3, TCR, and CD45 in WT and p50/-/-cRel/-/- CD8 T cells

11. Evaluate caIKKβ expressed in E6-1 Jurkat T cells for its ability to enhance:

   a. phosphorylation of TCR proximal proteins
   b. calcium influx
   c. activate NF-κB

12. Evaluate loss of IKKγ in 3T8 Jurkat T cells for its ability to ablate:

   a. phosphorylation of TCR proximal proteins
   b. calcium influx

13. Assess the ability of IKKβ to phosphorylate Zap70 in Hek 293 Cells
CHAPTER 2
A NOVEL ROLE FOR NF-κB IN PROXIMAL T CELL SIGNALING, REGULATING CALCIUM INFLUX & ACTIVATION OF NF-AT

2.1. Abstract

In the conventional paradigm of T cell activation, parallel signal transduction pathways activate the transcription factors NF-κB, NF-AT and AP-1 to drive clonal expansion of T cells in response to antigen. Genome-wide transcriptional profiling following antigen-induced CD8 T cell activation revealed that genes regulated by NF-AT were also reduced in the absence of NF-κB p50 and cRel subunits. Importantly, p50−/− cRel−/− CD8 T cells had significantly diminished NF-AT and AP-1 activation compared to WT or PKCθ−/− CD8 T cells. Attenuated NF-AT activation after TCR engagement was associated with reduced calcium influx, and subpar activation of PLCγ1. Interestingly, pharmacological bypass of PLCγ1 regulated pathways largely rescued p50−/−cRel−/− T cell proliferative defects. These results indicate a crucial and unexpected requirement for NF-κB p50 and cRel subunits in proximal TCR signaling and calcium responses. They further suggest that key defects in T cells in the absence of NF-κB pathway components

1 Portions of this chapter have been previously published in the European Journal of Immunology, and have been reproduced here with permission from Wiley Science. Additionally, all work presented herein was performed by Crystina Bronk, with the exception of the loading of the Microarray Chip and the Microarray Analysis which was performed by Sean Yoder.
may not be solely due to defunct gene transcription, but may also be due to impaired proximal T cell signaling defects.

2.2. Introduction

T cell activation is initiated by TCR binding to an MHC molecule loaded with cognate peptide and presented by professional Antigen Presenting Cells (APC) such as Dendritic Cells (DC). This interaction initiates a signaling cascade requiring several key kinases, phosphatases, and scaffolding proteins that together drive T cell activation [191]. Two key events in activation are phosphorylation of Zap70, which nucleates the formation of the LAT signalisome [192] and phosphorylation of PLCγ1, which then cleaves PIP2 into IP$_3$ and DAG [193]. IP$_3$ is crucial for calcium influx which in turn is necessary to activate the NF-AT transcription factor [193]. On the other hand, DAG activates PKCs which are thought to be important for activation of the transcription factors AP-1 and NF-κB [108, 194]. These seemingly parallel cascades of transcription factors lead to induction of key genes necessary for T cell proliferation, survival and cytokine production.

Canonical NF-κB signaling is initiated when CARMA1 forms a complex with Bcl10 and Malt1 [195, 196], which allows it to bind IKK complex proteins. Of the three IKKs, α/β/γ, IKKβ is primarily responsible for phosphorylating IκBα, an inhibitory protein responsible for cytoplasmic retention of NF-κB [197]. The phosphorylation of IκBα marks it for degradation leading to release of bound transcriptionally active heterodimers consisting of either p50+RelA or p50+cRel [197]. Studies of T cells have shown redundancy in function of these NF-κB subunits. While combined absence of
p50+RelA subverts mature T cell differentiation, the absence of p50+cRel does not impact thymic development, but instead impairs T cell function [198, 199]. PKC activity, in particular PKCθ, was believed to be crucial for NF-κB activation. However, recent studies, including our own, suggest that NF-KB can be activated in the absence PKCθ if T cells are activated by professional APC such as DCs [200]. Importantly, we also found that PKCθ−/− or p50−/−cRel−/− T cells are both incapable of initiating GVHD in allogenic recipient mice [200]. It is however not clear whether this impairment in alloreactivity is due to defects in similar or distinct signaling pathways. In particular, PKCθ deficiency negatively impacts NF-AT activation which likely contributes to the observed functional defects in PKCθ T cells [201].

In the accepted paradigm of T cell activation, proximal T cell signaling mediators such as Zap70 and PLCγ1 drive induction of downstream transcription factors. While investigating signaling defects in PKCθ−/− and p50−/−cRel−/− CD8 T cells in response to foreign antigen in a syngenic model of infection, we surprisingly found that activation of transcription factors NF-AT and AP-1 were severely impaired in p50−/−cRel−/− T cells. This defect was traced to impairment in calcium influx and sub-optimal PLCγ1 activation. Importantly, proliferative defects in p50−/−cRel−/− T cells were largely rescued through pharmacological bypass of PLCγ1. These important findings reveal an unexpected role for NF-κB in regulating proximal T cell signaling and further indicate that functional impairment created by the absence of NF-κB in T cells is also due to defects in activation of upstream signaling mediators.
2.3. Methods and Materials

2.3.1. Mice and Primary T cells

PKCθ-/-, OT-1 transgenic, and p50-/-cRel-/- mice have all been described previously [200]. All mice were maintained under specific pathogen free conditions, and all experiments using mice were carried out in accordance with institutional guidelines. T cells were isolated from spleens of control and experimental mice by CD4 and CD8 MACS beads (Miltenyi Biotec), and cultured in RPMI supplemented with 10% FBS, L-glutamine (2mM), Penicillin/Streptomycin (100 U/ml), β2ME (50mM), and Non-essential AA, Sodium Pyruvate, and HEPES (all 1mM). OT1 T cells were stimulated with BMDCs pulsed with 1μM SIINFEKL peptide. Briefly, Bone Marrow cells were flushed from the Femurs and Tibias of WT.B6 (syngenic) mice and after red blood cell lysis, the remaining cells were cultured at 1e^6/mL/well of 24 plate in the presence of supernatant (3% vol/vol) from J558L cells transduced with murine GM-CSF in RPMI supplemented with 5% FBS, L-glutamine (2mM), Penicillin/Streptomycin (100 U/ml), β2ME (50mM), and Non-essential AA, Sodium Pyruvate, and HEPES (all 1mM) for one week, with media change every other day. BMDCs were harvested through repeat pipetting, placed on low attachment plates at 1e^6/mL / well, and reharvested 24 hours later. Cells were then harvested, again, placed in 15mL eppindorf tubes and incubated at 37° with the peptide for at least 3 hours. Occasionally OT-1 T cells and all polyclonal cells were stimulated with either 1 or 10μg anti CD3/CD28 (ebioscince) and/or 50ng Phorbol 12-Myristate 13-Acetate (PMA) and 500ng ionomycin (both Fisher) depending on assay.
2.3.2. T cell Proliferation Assay

T cells were incubated at 1e5/ well in 96 well plates with culture medium and stimulants where indicated for 48 – 72 hours at 37ºC. 1μCi of Thymidine was added for the last 12 hours of culture. Plates were flushed onto fiberglass plates and Microscint (both Perkin Elmer) was applied and read on the Top Count NXT (Packard) scintillation counter.

2.3.3. In Vivo T cell Expansion and FACS Analysis

OT-1+/CD45.2+/CD8+ T cells from WT, PKCθ-/-, and p50-/-cRel/- mice were isolated from the spleen, and 1e6 were transplanted into CD45.1 immune competent hosts. 24hours later, the transplant and control mice were injected, IP, with 100μg OVA, 100μg anti-CD40, and 50μg PolyIC. Peripheral blood was acquired through submandibular bleed every 7 days thereafter. Peripheral blood was RBC lysed (ACK Buffer – Fisher) and FC blocked (eBioscience). WBCs were then stained with CD45.1, CD45.2, and CD8 (eBioscience) and KB-OVA tetramer (MLB International) in PBS (Fisher) supplemented with 0.5% Bovine Serum Albumin, BSA (Sigma) for 30m on ice. Finally, cells were washed twice and data was collected on an LSRII (Beckman Dickinson) and analyzed with FlowJO 9.6 (TreeStar). Gating strategy isolated only single cells (through Forward and Side Scatter) and DAPI- (live) cells.
2.3.4. Microarray Studies

2.3.4.1. Sample Processing for Microarray Analysis

One hundred nanograms of total RNA served as the mRNA source for microarray analysis. The poly(A) RNA was converted to cDNA and then amplified and labeled with biotin using the Ambion Message Amp Premier RNA Amplification Kit (Life Technologies, Grand Island, NY) following the manufacturer’s protocol initially described by Van Gelder et al[202]. Hybridization with the biotin-labeled RNA, staining, and scanning of the chips followed the prescribed procedure outlined in the Affymetrix technical manual as described previously [203].

2.3.4.2. Probe Arrays

The oligonucleotide probe arrays used were the Mouse Genome 430 2.0 Arrays. This array contains over 45,000 probe sets designed from GenBank, dbEST, and RefSeq sequences that were clustered based on build 107 of the UniGene database. The clusters were further refined by comparison to the publicly available draft assembly of the mouse genome. An estimated 39,000 distinct transcripts are detected including over 34,000 well substantiated mouse genes. Each gene is represented by a series of oligonucleotides that are identical to the sequence in the gene as well as oligonucleotides that contain a homomeric (base transversion) mismatch at the central base position of the oligomer, which is used to measure cross-hybridization.
2.3.4.3. Data Analysis

Scanned output files were visually inspected for hybridization artifacts and then analyzed using Affymetrix GeneChip Operating Software (GCOS) using the MAS 5.0 algorithm. Signal intensity was scaled to an average intensity of 500 prior to performing a comparison analysis between paired samples. Using the default settings, the GCOS software identifies the increased and decreased genes between any two samples with a statistical algorithm that assesses the behavior of 11 different oligonucleotide probes designed to detect the same gene [204].

Several comparisons were performed in order to identify the genes that changed in response to stimulation with the OVA peptide. First, genes that were affected by OVA treatment were identified in the wild-type mice, and then this list was further enriched for the genes were differentially expressed at an even greater degree in the knock-out mice after OVA treatment. Probe sets that yielded a change \( p \)-value less than 0.05 between each pair of conditions, and with a fold change of greater than 1.4 (increased or decreased) were identified as changed. A gene was identified as consistently changed if it was identified as changed in all replicate experiments. Lastly, genes that showed expression at or below background levels across all conditions (signal intensity below 100) were filtered from the final list. Data from the resulting lists of differentially expressed genes were visualized in the form of heat maps using the Cluster 3.0 and Java TreeView software applications [205, 206].
2.3.5. Calcium Assay

T cells were suspended at 1e6/mL in Calcium Buffer (No-Phenol Red RPMI (Fisher) with 2%FBS and 10% Probenecid (Thermo-Scientific)) and stained with 4μM Fluo-4 (Invitrogen) for 45m at 37˚C. They were washed three times in Calcium Buffer (above) and replated at 2e5/well in a 96 well flat-bottom plate. Cells were then rested for 20m at RT, and incubated at 37˚C for 10m immediately preceding the assay. Where indicated, cells received the following: 10μg/mL anti-CD3/CD28 (mouse & human-ebioscience), 500ng ionomycin (Fisher), and 3mM EGTA (Sigma). Samples were read on a Wallac EnVision 2102 Multilabel Reader from Perkin Elmer.

2.3.6. Electrophoretic Mobility Shift Assay (EMSA)

2 X 10^7 cells were washed in PBS then suspended in 10mM HEPES (pH7.9), 10mM KCL, 0.1mM EDTA, 0.3% NP-40, and 1x Protease inhibitors for 5min. at 4°C, then centrifuged for 5 min at 1600g. The supernatant (cellular extract) was removed and the remaining pellet was washed twice in the above buffer (minus the NP-40.) Finally the pellet was suspended in 20mM HEPES (pH7.9), 0.4M NaCl, 1mM EDTA, 25% Glycerol, and vortexed intermittently as it incubated for 10min. at 4°C. After centrifugation (16,000g for 5min.,) the supernatant (nuclear extract) was collected. To assay NF-κB activity, the following hairpin oligonucleotide probe was used: GAGAGGGGATTCCCCGATTACCTTTCGGGGAATCCCCTCT. NF-AT: F-ACGCCCAAGAGGAAAATTTTGTTC; R-TGTATGAAACAAATTTTCCTCTTTGGG. Briefly, the oligonucleotide probe was annealed and end-labeled with Gamma-ATP using T4 polynucleotide kinase according to the manufacturer’s instructions (New
England Biolabs). Labeled probes (100,000cpm/rxn) were incubated with 5μg of nuclear extracts, Binding Buffer (50mM Tris (pH7.6), 250mM NaCl, 2.5mM EDTA, 5mM DTT, 1mg/mL BSA, 25% Glycerol) and 1μg poly-dIdC for 20min at room temperature and separated on a 10% non-denaturing polyacrylamide gel with 0.25X TBE running buffer. After electrophoresis, DNA-protein complexes were dried for two hours onto 3MM Chromatography paper (Whatman.) The paper was then exposed to film for 4 - 18 hours.

2.3.7. Relative Quantitative RT-PCR (RT-qPCR)

Table 2.1 Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>TCAAGAACGAAAGTCGGAGG</td>
<td>GGACATCTAAGGGCATCACA</td>
</tr>
<tr>
<td>P2PX1</td>
<td>CGTCATCGGTTGGGTCTCATA</td>
<td>AGGGCGCGGATGTCGCTCA</td>
</tr>
<tr>
<td>P2PX4</td>
<td>CGGGACCACAGCAACGGGAGTCT</td>
<td>TGTATGAGCGCCGGCGAAGGAGTA</td>
</tr>
<tr>
<td>P2PX7</td>
<td>CCGGCCACAACTACACCACAG</td>
<td>GGCCAGACCAGATAGGAGAG</td>
</tr>
<tr>
<td>ORAI</td>
<td>CATAAGGGCATGGATTACCAG</td>
<td>CGGGTACTGGTACTGCGTCT</td>
</tr>
<tr>
<td>STIM1</td>
<td>CTCTAACACGCCCACCTCST</td>
<td>CTTCTGTGCTCTTTCTCACGC</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>CAGGTGACCCAACCTTCAGT</td>
<td>CAGTCCTTGAGGAGGAG</td>
</tr>
<tr>
<td>IL-17</td>
<td>ATCCCTCAAGGCCTAGCCTGTC</td>
<td>GGGCTTTTGACGGGAGGAG</td>
</tr>
</tbody>
</table>

Total RNA was extracted with the RNeasy Plant Kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from total RNA (0.2 μg) using the TaqMan® ReverseTranscriptase kit (Applied Biosystems). All RT-qPCR reactions were
performed in an ABI Prism 7300 Sequence Detection system (Applied Biosystems,) with Power SYBR® according to SYBR protocol (Applied Biosystems). All experiments were conducted in triplicate and SDs are indicated in the figures. The primers used were IL-2, IL-21, and IFNγ (Realtime Primers) and the following sequences from IDT DNA:

### 2.3.8. Western Blotting

Whole Cell Extracts were prepared in 25mM Tris-HCl pH7.6, 2mMEDTA, 150mM NaCl, 0.5% NP-40, (Lysis Buffer) with late addition of 1mM PI, DFP, NaF, and NaVO₃. A Bradford Assay (Applied Biosystems) was performed to standardize proteins on the DU 350 (Beckman Coulter) and samples were mixed with a 0.5% SDS loading buffer (Fisher) and run on a 10% acrylimide-bis gel and transferred to nitrocellulose paper. Membranes were blocked in Tris-buffered saline, 0.1% Tween 20 (TBS-T) with 3% BSA and 2% Milk (Carnation). Then blots were incubated overnight at 4˚C with primary antibodies at 1:1000 in 5%BSA.TBS-T. After three TBS-T washes, the membrane was incubated with the proper secondary at 1:3000 in 5%Milk.TBS-T for 1.5 hours at room temperature. The specific bands were revealed by chemiluminescence (Western Lightning ® Plus-ECL, Perkin Elmer) and visualized by autoradiography (Amersham Hyperfilm-ECL, GE). Before reprobing, blots were stripped by incubation in 0.1 M 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8) for 30 min at 65˚C and the process began again, with blocking. Primary western antibodies include: p65, NF-ATc1 (Santa Cruz), ORAI (ProSci Incorporated), PLCγ1 (BD), pPLCγ1 (Y783), STIM1, anti-c-Jun (9165) (Cell Signaling). HRP conjugated-β-actin, and all secondary antibodies (mouse, rabbit, and goat) were from Santa Cruz.
2.3.9. Statistical Analysis

All figures represent results from at least two (usually three) independent experiments. When appropriate, data is given as the mean ± standard deviation. P values are expressed as • ≤ 0.05  ** ≤ 0.01; *** ≤ 0.001.

2.4. Results

2.4.1. p50<sup>-/-</sup>cRel<sup>-/-</sup> CD8 T cells Exhibit Pronounced Clonal Expansion Defects In Vitro and In Vivo

In previous studies, we found that absence of either PKCθ or p50 and cRel in T cells greatly impairs GVHD in allogenic recipients [200]. To better understand the roles of these proteins in regulating T cell function in response to cognate antigen, we crossed PKCθ<sup>-/-</sup> and p50<sup>-/-</sup>cRel<sup>-/-</sup> mice to OVA<sub>257-264</sub> (SIINFEKL) specific OT1 TCR Tg mice. Stimulation of CD8 T cells from these mice with SIINFEKL (OVA) peptide-pulsed dendritic cells (DC) showed that while PKCθ<sup>-/-</sup> T cell proliferation was reduced compared to WT T cells, the most severe defect in proliferation was in p50<sup>-/-</sup>cRel<sup>-/-</sup> T cells (Fig. 2.1A). In addition, IL-2 mRNA expression (used as a marker for activation of T cells) was also most significantly reduced in p50<sup>-/-</sup>cRel<sup>-/-</sup> T cells (Fig. 2.1B). Therefore, p50<sup>-/-</sup>cRel<sup>-/-</sup> T cells have a more significantly defective in vitro proliferation response and greatly reduced IL2 expression compared to PKCθ<sup>-/-</sup> T cells.

To determine the in vivo effect of T cell-specific absence of p50+cRel and PKCθ, we adoptively transferred CD45.2<sup>+</sup>/OT1<sup>+</sup> WT, PKCθ<sup>-/-</sup> or p50<sup>-/-</sup>cRel<sup>-/-</sup> CD8 T cells into
congenic WT CD45.1+ hosts. 24h later, CD45.2 transplant recipients, along with naïve CD45.1 mice, received TriVax, a vaccine formulation with peptide, anti-CD40, and PolyIC [207]. Every 7 days, thereafter, peripheral blood samples were acquired and analyzed to assess the degree of clonal expansion. As expected, WT T cells expanded vigorously, and by day 7, 60% of peripheral WBCs were the transplanted CD45.2- OVA-specific T cells. Mirroring in vitro findings, PKCθ−/- T cells expansion was significantly reduced; however, the greatest impairment in expansion was in p50−/-cRel−/- T cells (Fig. 2.1C) which was evident at all time-points tested (Fig. 2.1D).

Detrimental consequences of activation of large numbers of T cells are well documented (e.g., in the context of super-antigen administration or adoptive cell therapy with anti-tumor T cells [208] ). To this effect, injection of BiVax (100μg OVA and 50μg PolyIC) vaccine 38 days after initial TriVax treatment of WT OT1 transplanted mice resulted in death of all four WT mice at day 3 (Fig 2.1E). The PKCθ−/- group survived somewhat longer, and had to be euthanized on day six due to IACUC governing of moribund state; in contrast, mice receiving p50−/-cRel−/- T cell were completely protected from lethal effect of T cell re-stimulation. Interestingly, even the severely repressed proliferation of the p50−/-cRel−/- - OT1 transplanted T cells was enough to squash the endogenous proliferation, as shown in Figure 2.1F, in 85% of mice. These results indicate that absence of p50+cRel results in substantially more pronounced impairment of T cell activation than PKCθ absence. Thus, overall signaling responses controlled by p50+cRel may be distinct from those regulated by PKCθ, even though they are colloquially placed in the same pathway.
Figure 2.1. Activation and proliferation are defective in the p50/-cRel/- T Cells. (A) In vitro proliferation of WT, PKCθ/-, and p50/-cRel/- CD8 T-cells cultured alone or in the presence of DC ± OVA; values represent H3 incorporation. (B) Relative amount of IL-2 mRNA present in untreated (WT) and WT, PKCθ/-, and p50/-cRel/- CD8 T-cells post 18 hours culture with DC and OVA, normalized to 18S Ribosomal RNA. (C) Clonal expansion of OVA-Tet+ cells. Peripheral blood was analyzed 7 days post Tri-Vax vaccination; values represent % of endogenous (45.1) and transplanted (45.2) OVA-Tet+ CD8 T Cells / PBMN. (D) Line graph representing percentage of transplanted (CD45.2) OVA-Tet+ CD8 T Cells / PBMN over 5 weeks. (E) Survival of mice post BiVax boost. (F) Percentage of endogenous OVA-Tet+ CD8 T Cells on Day 7 for two experiments. The data are representative of three independent experiments; for (C-F), three mice per genotype received Tri Vax. *<0.05, **<0.01, ***<0.001 days later.
2.4.2 Differential Impact of p50+cRel versus PKCθ Absence on Activation-Induced Gene Expression in T cells

To develop a broader understanding of the consequences of p50\(^{-/-}\)cRel\(^{-/-}\) and PKCθ\(^{-/-}\), we performed genome-wide analysis of gene expression. To this end, OT1 CD8 T cells from WT mice were co-cultured with un-pulsed DC and OT1 CD8 T cells from the three different genotypes were stimulated with SIINFEKL-pulsed DC, at 18h, we utilized magnetic column sorting to deplete DC from the culture, and mRNA from remaining T cells was used for microarray using Affymetrix Mouse Genome 430 2.0 Array platform. First, genes that were affected by OVA peptide DC treatment versus no peptide (unstimulatory) DC were identified in the WT mice. Probe sets that yielded a change (\(p\)-value less than 0.05) between each pair of conditions, and with a fold change of greater than 1.4 (increased or decreased) were identified as changed. In addition, a gene was identified as consistently changed if it was identified as changed in replicate experiments. Lastly, genes that showed expression at or below background levels across all conditions (signal intensity below 100) were filtered from the final list. Using the above criteria, we identified genes differentially regulated in OVA-DC stimulated PKCθ\(^{-/-}\) or p50\(^{-/-}\)cRel\(^{-/-}\) T cells compared to OVA-DC stimulated WT T cells.

Out of a total of 45,101 probe sets, 10,400 were changed after WT T cell activation, with 4144 up-regulated and 6256 down-regulated in 2 replicate experiments. Focusing on the up-regulated 4144 probe sets, expression of 73 (corresponding to 55 known genes; Table 2.2) was impaired in PKCθ\(^{-/-}\) while 146 probe sets (112 genes; Table 2.2) were impaired in p50\(^{-/-}\)cRel\(^{-/-}\) T cells. A modest level of overlap in PKCθ and
p50+cRel regulated genes was seen (34 probe sets; 22 genes), which included previously defined targets such as IL2 (Table 2.2).

**Table 2.2.** Genes deregulated in the absence of p50/cRel are shown in the left (light grey) box. Genes deregulated in the absence of PKCθ are shown in the right (grey) box. Genes regulated by both p50/cRel and PKCθ are where the two boxes overlap (dark grey.)
Notable amongst p50+cRel target genes were several key cytokines (IL17A, IL21, IL23A, IL3, and IFNG), signal transducers (STAT5A, STAT5B, E2F1 and PRKCA), and cell surface receptors (CTLA4, CD2, TNFRSF4 and IL12RB2) (Table 2.2).

Figure 2.2. Confirmation of Microarray Reported Deregulated Genes. (A) Relative amount of IFNγ, IL-17, CTLA-4, and IL-21 mRNA present in untreated (WT) and WT, PKCθ-/-, and p50-/-cRel/-/- CD8 T-cells post 18hours culture with DC and OVA, normalized to 18S Ribosomal RNA.

Impaired expression of several of the above genes, including, IFNG, IL17A, CTLA4, and IL-21 were confirmed by RT-PCR (Fig. 2.2A). Overall, these results suggest that despite severe defects in activation and clonal expansion, a relatively small percentage of transcripts are impacted in the absence of PKCθ (1.7%) or p50+cRel (3.5%). IL2 is known to require NF-κB, NF-AT and AP-1 transcription factors for optimal induction.
Interestingly, in addition to IL2, transcription of several genes known to be regulated by calcium/NF-AT signaling, including IL3, STAT5B, IFNγ and CTLA4 [209-211] were found to be reduced in p50/-cRel/- T cells. These results are suggestive of potentially broader defects in absence of p50+cRel and, in particular, warranted a closer examination of NF-AT/calcium signaling in p50/-cRel/- T cells.

2.4.3. p50-cRel T cells Have Defects in NF-AT and AP1 Activation and Calcium Influx

We next determined activation of NF-AT, AP-1 and NF-κB in CD8 T cells from all three genotypes after stimulation with OVA-pulsed DCs. WT T cells showed strong nuclear translocation of all three transcription factors (Fig. 2.3A) when stimulated with OVA-pulsed DC. Consistent with our previous results, NF-κB levels were not significantly impacted in PKCθ-/- T cells [200]; however, a more substantial reduction in NF-AT and AP-1 was seen (Fig. 2.3A). As expected, p50-cRel-/- T cells showed significant reduction in NF-κB activity. Remarkably, both NF-AT and AP-1 levels were also greatly reduced in p50-cRel-/- T cells (Fig. 2.3A). A similar trend was also seen in polyclonal CD8 T cells from WT, PKCθ-/-, and p50-cRel-/- mice that were stimulated with anti-CD3/CD28 (Fig. 2.3B). Interestingly, in the polyclonal setting, p50/-cRel/- T cells underperformed PKCα-/-θ-/- T cells; even though this somewhat redundant duo of classical and novel PKC isoforms is known to impact all three major transcription factors [212]. In addition, western blotting of nuclear extracts showed greatly reduced presence of NF-ATc1 in p50-cRel-/- T cells after anti-CD3/CD28 stimulation (Fig. 2.3C). The reduction in NF-AT induction was, in fact more pronounced than that of NF-κB RelA/p65
subunit (Fig. 2.3C). These results therefore suggest that the substantially reduced responsiveness of p50-cRel T cells compared to PKCθ T cells may be due to graver impairment in activation of multiple transcription factors in the former.

![Figure 2.3](image)

**Figure 2.3. Activation of NF-AT and Calcium Influx are Reduced in the p50-cRel T Cells.** (A) Untreated (WT) and WT, PKCθ, and p50-cRel OT1 CD8 T-cells post 18 hours culture with BMDC and SIINFEKL were used to prepare nuclear lysates and an EMSA was performed. (B) Process was the exact same as A, but polyclonal WT, PKCθ, and p50-cRel CD8 T Cells were used, and stimulated with 1μg/mL CD3/CD28; a PKCa/θ mouse was added as an additional control. (C) Western blot on nuclear fraction of unstimulated and 18 hours culture with 1μg/mL CD3/CD28 of WT and p50-cRel CD8 T Cells. (D) WT, PKCθ, and p50-cRel polyclonal CD8 T-cells were analyzed for the ability to influx Calcium: The first 60 sec represent a baseline after which the following components were added to the culture: (*) 10μg/mL CD3/CD28 and (#) 9mM EGTA. Points are Arbitrary Fluorescent Units (AFU) and lines represent one of three repeats within each assay. (E) Again polyclonal CD8 T cells were assayed for ability to influx calcium, this time they received 2μM of Thapsigargin, followed by 5mM CaCl2, where indicated.
As NF-AT activation requires calcium signals [213], we next determined calcium influx in T cells after stimulation. WT T cells showed rapid increase in calcium influx when stimulated with anti-CD3/CD28 (Fig. 2.3D). Importantly, calcium influx was reduced in PKCθ-/- but the reduction was most severe in p50-/-cRel-/- T cells. Additionally, this defect was preserved when cells were treated with thapsigargin (a SERCA pump inhibitor that forces activation of the CRAC pathway of calcium influx), Fig. 2.3E, demonstrates that even forced influx of calcium in PKCθ-/- and p50-/-cRel-/- CD8 T cells cannot achieve the capacity seen in WT CD8 T cells. We next determined calcium influx in T cells after stimulation with un-pulsed or OVA-pulsed DCs. WT T cells stimulated with OVA-pulsed DC showed substantially enhanced calcium influx compared to T cells with un-pulsed DCs (Fig. 2.4A). This difference was substantially reduced in PKCθ-/- T cells (Fig. 2.4B). Strikingly, no difference in calcium influx was seen in p50-/-cRel-/- T cells stimulated with un-pulsed versus OVA-pulsed DCs (Fig. 2.4C). In the above studies, we determined calcium influx after TCR engagement of naïve T cells. However, calcium influx is also important for continued T cell proliferation and cytokine expression [193, 214]. Importantly, calcium influx was also substantially reduced in p50-/-cRel-/- T cells post 48h stimulation with 1µg/mL anti-CD3/CD28 (Fig. 2.4D), although some recovery was evident. Moreover, it appears that, at least for calcium influx, p50 and cRel serve a redundant function as knock out of each NF-κB family member, independently, led to only a mild reduction in calcium influx, as shown in Figure 2.4E. Additionally, the mild depression of calcium influx in each of the single knock outs was mirrored by only a slight decrease in proliferation (Fig. 2.4F). Together, our results
indicate that impaired NF-AT activation in p50<sup>+/−</sup>-cRel<sup>+/−</sup> is likely due to defects in calcium influx.

Figure 2.4. Defects in Calcium Influx Persist Despite Route of Stimulation or Duration, but Require Absence of p50<sup>+</sup>-cRel. (A-C) Same procedure as Fig. 2.3. D, but OT-1 T Cells were used and graphs compare calcium influx based on T Cell:DC interaction ± OVA; therefore, there is no baseline, and no need for anti-CD3/CD28. (D) Same as Fig. 2.3. D, but polyclonal CD8 cells were cultured for 48 hours with 1μg/mL anti CD3/CD28, rested for 2 hours, and then calcium assay was performed. (E) Same as in Fig. 2.3. D, but single knockouts (p50<sup>−/−</sup> and cRel<sup>−/−</sup>) were evaluated. (F) In vitro proliferation of WT, p50<sup>−/−</sup>-cRel<sup>−/−</sup>, p50<sup>−/−</sup>, and cRel<sup>−/−</sup> CD8 T Cells untreated or stimulated with 1μg/mL anti CD3/CD28; values represent H<sub>3</sub> incorporation. The data are representative of three independent experiments.

2.4.4. Defects in Calcium Influx Are Due to Impaired PLCγ1 Activation, Not a Reduction in Calcium Channel Proteins in p50<sup>−/−</sup>-cRel<sup>−/−</sup> CD8 T cells

Given the severity of the calcium defects in p50<sup>−/−</sup>-cRel<sup>−/−</sup>, we considered potential impairment in additional calcium response regulators in T cells. In lymphocytes, entry of
extracellular calcium is primarily mediated by calcium release-activated calcium (CRAC) channels. Two key regulators of calcium influx are STIM1/2 (ER Ca\(^{2+}\) sensors) and ORAI (a subunit of the CRAC channel) [193, 214]. Previous studies have implicated NF-κB as regulating both STIM1 and ORAI expression in fibroblasts [215]. However, we did not see any reduction in STIM1 or ORAI mRNA (Fig. 2.5A) or protein (Fig. 2.5B) expression in unstimulated p50\(^{+/−}\)cRel\(^{+/−}\) T cells. Moreover, we could not ascertain any differences between mRNA of the puronergic calcium channels either (Fig. 2.5C) suggesting that calcium influx defects are more likely associated with impairment in proximal TCR signaling.

Upon TCR engagement, PLCγ1 cleaves PIP2 into IP\(_3\) (leading to calcium influx and NF-AT activation) and DAG (leading to PKC and AP-1/NF-κB activation). Since p50\(^{+/−}\)cRel\(^{+/−}\) T cells have defects in both NF-AT and AP-1 activation, we next determined whether this was due to impaired PLCγ1 activation. While total PLCγ1 levels were equivalent, a decrease in activation-induced phosphorylation (Y783) in pPLCγ1 was seen in p50\(^{+/−}\)cRel\(^{+/−}\) CD8 T cells (Fig. 2.5D). These results suggest that defects in proximal TCR signaling in p50\(^{+/−}\)cRel\(^{+/−}\) T cells may be responsible for defects in calcium/NF-AT and AP-1 activation. Since calcium/NF-AT and AP1 also contribute to T cell activation and proliferation, we hypothesized that observed defects in p50\(^{+/−}\)cRel\(^{+/−}\) T cells may also be due to impaired PLCγ1 signaling rather than p50+cRel absence per se. To test this possibility, we used two agents that can bypass the need for activated PLCγ1 mediated IP\(_3\) and DAG generation: ionomycin (IO; calcium ionophore) and PMA (PKC inducer). It is worth mentioning that while the actual second messengers DAG and IP3 are molecules that endure extreme turnover rates, and are marked easily for
Figure 2.5. The Role of Calcium Channel Proteins and PLCγ in Mediating Defects in p50-/-cRel-/- T Cells. (A) Relative amount of Orai1 and STIM1 mRNA present in untreated WT and p50-/-cRel-/- CD8 T Cells, normalized to 18S Ribosomal RNA. (B) WT and p50-/-cRel-/- CD8 T cells, untreated, were used to make whole cell extracts and western blotting was performed. (C) Relative amount of P2PX1, P2PX4, and P2PX7 mRNA present in untreated WT, PKCθ-/-, and p50-/-cRel-/- CD8 T Cells, normalized to 18S Ribosomal RNA. (D) WT and p50-/-cRel-/- untreated and 5,10, and 20m 10µg/mL anti-CD3/CD28 stimulation lysates were made and western blotting was performed. (E) In proliferation of WT and p50-/-cRel-/- CD8 T cells cultured alone or with 1µg/mL anti-CD3/CD28 and/or 50ng PMA/ 500ng ionomycin; values represent H3 incorporation and are given as mean ± SD. (F) Same as Fig. 2.3. D, but polyclonal CD8 cells were stimulated with either 1µg/mL anti-CD3/CD28 or 50ng PMA/ 500ng ionomycin. (G) WT and p50-/-cRel-/- CD8 T cells, untreated, or stimulated with 50ng PMA/ 500ng ionomycin were used to make nuclear extracts and western blotting was performed.
degradation, the pharmalogical mimetics PMA and Ionomycin do not suffer the same fate. Therefore, using this approach not only generates stronger initial activation of the PKC and calcium pathways, but continues to signal, long after the endogenous second messengers would have been destroyed and down regulated [216].

Intriguingly, IO+PMA largely rescued the proliferative defects in p50⁻/⁻cRel⁻/⁻ T cells (Fig. 2.5E). Moreover, the use of IO+PMA also had the ability to enhance calcium influx in the p50⁻/-cRel⁻/- CD8 T cells, almost to WT levels (Fig. 2.5F). Additionally, this increase in calcium led to the corresponding increase in NF-AT and AP-1 nuclear translocation, as shown in Figure 2.5G. Therefore, a significant component of defects in proliferation of p50⁻/⁻cRel⁻/⁻ T cells stem from impaired proximal T cell signaling leading to activation of PLCγ1. Importantly, these results further indicate that key defects in T cell responses in the absence of NF-κB pathway components can be due to defects in proximal T cell signaling.

2.5. Discussion

Our results show an unexpected function of NF-κB in calcium influx and NF-AT activation. We further show that this function is at least partly controlled through optimal activation of the crucial T cell signaling protein: PLCγ1. Pharmacological rescue of p50⁻/⁻cRel⁻/⁻ T cell calcium influx, transcription factor nuclear localization, and proliferation evinced that a substantial component of defects in these cells are due to impaired PLCγ1 activation. However, we do not believe that p50⁻/⁻cRel⁻/⁻ cells are universally incapable of activation-induced signaling: first, expression of only a small set of transcripts was impacted in p50⁻/⁻cRel⁻/⁻ T cells (3.5%) indicating that the vast majority of
activation-induced genes are not impacted. Second, rescue by IO+PMA indicates that p50+/cRel+/ T cells are fully capable of robust proliferative responses, if stimulated in a manner that bypasses specific functional defects. Our results also indicate the novel function for NF-κB described here appears to be redundantly controlled by p50 and cRel, since CD8 T cells lacking either p50 or cRel showed only a slight reduction in calcium influx and proliferation.

A key question is how PLCγ1 activation and calcium/NF-AT signaling is regulated by NF-κB. Given the fact that calcium influx was measured minutes after stimulation, it is unlikely that this is due to defective gene induction by activation-induced NF-κB. Instead, regulation of calcium influx likely represents a function for constitutive NF-κB activity. We believe that similar, albeit less severe, defects in PKCθ−/− T cells seen here may suggest some possible answers. Both NF-κB subunits and PKCθ associate with the IKK complex [194], which is comprised of IKKα/β/γ. Importantly, IKKβ/γ can localize to the immune synapse (IS) where the key proximal signaling events are initiated [217, 218]. It is possible that absence of either PKCθ or NF-κB subunits can somehow impact IKKβ/γ activation, localization, or stability. While IS presence of IKKβ is thought to be crucial for NF-κB activation, it is interesting to speculate that IKKβ regulates additional events in TCR signaling, which are adversely impacted by PKCθ or p50+cRel absence. Regardless of the precise mechanism, our results demonstrate a crucial role for NF-κB p50 and cRel subunits in calcium/NF-AT signaling that is mediated through optimal activation of the proximal TCR signaling mediator, PLCγ1. Previous studies have defined a crucial role of the NF-κB pathway in promoting T cell survival [197]. The
results shown here indicate that in addition to promoting survival, NF-κB is also crucial for early T cell activation responses.
CHAPTER 3
LOSS OF p50 AND cRel LEAD TO REDUCTION OF IKKβ AND DEFICIENT PHOSPHORYLATION OF PROXIMAL TCR SIGNALING PROTEINS

3.1. Abstract

In the accepted model of T cell activation, signals are transduced in a feed-forward direction, and although there have always been negative and positive feedback loops, it is generally thought that downstream molecules do not impact upstream precursors during the first original signaling cascade. In the previous chapter, I demonstrated that p50-/-cRel-/- CD8 T cells had significantly diminished NF-κB, NF-AT and AP-1 activation compared to WT or PKCθ-/- CD8 T cells, and was able to demonstrate that this was a direct result of reduced phosphorylation / activation of PLCγ1, leading to attenuated calcium influx. In our current work, we focused on the molecular basis of sub optimal PLCγ1 activation, and discovered that both LCK and Zap70, and subsequently LAT phosphorylation were also reduced in the p50-/-cRel-/- T cells. Importantly, we were also able to show that loss of p50 and cRel reduced IKKβ and IKKγ at both the mRNA and protein levels in murine, naïve CD8 T cells. By expanding our study to Jurkat T cells, we teased apart the impact of IKKβ and IKKγ on proximal TCR signaling. Where, with the KO of IKKγ (and reduction of the IKK complex

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as a whole), we correctly phenocopied the p50/-/-cRel/-/- T cell’s defects in activation. Moreover, through expression of a constitutively activated IKKβ (IKKβEE), we were able to generate the opposite phenotype, and increase proximal TCR protein phosphorylation and activation of Jurkat T cells. Finally, through transduction of Zap70 and IKKβEE in Hek293 cells, we were able to show that IKKβ can phosphorylate Zap70 in vitro. These results indicate a crucial and unexpected requirement for NF-κB subunits p50 and cRel in the stability and correct localization of the IKK complex; furthermore, they demonstrate an important role for IKKβ in the enhancement of proximal TCR signaling leading to greater calcium influx and fully activated NF-AT and NF-κB.

3.2. Introduction

T cell signaling through the TCR is an extremely flexible, yet highly regulated, process that requires not only the correct subcellular localization of a myriad of proteins, but that several of these proteins be phosphorylated or dephosphorylated to propagate the chain. Interestingly, the Weiss lab demonstrated that quite a bit of TCR signaling occurs in the basal state of naïve T cells, in the absence of TCR binding to an MHC loaded with a cognate peptide [19]. This assertion explains the rapid response rate of T cells when faced with antigen, as they have several components of the signaling chain already in an “on” position. This also creates a space in which proteins are in constant flux of being phosphorylated and dephosphorylated and shuttled in and out of varying subcellular locations as complexes are created and dissolved moment to moment.

Moreover, it is well documented that basal activation of NF-κB is required for T cell survival and homeostasis [94, 219]. Because several of the pathways activated by
NF-κB to promote survival involve proteins that also overlap with signaling chains eliciting activation of T cells, and because previous results had shown grave defects in the activation of p50-/-cRel-/- T cells, we hypothesized that NF-κB activity in the basal state of T cells could also positively impact T cell activation at the earliest of time points.

Utilizing the OT1 transgenic trope, we previously evinced that our p50-/-cRel-/-CD8 T cells fell prey to a grave reduction in proliferation and cytokine production in response to strong antigen (both in vitro and in vivo.) We further elucidated that these cells also had impaired activation of NF-AT, which appeared to be sequela to abrogated calcium influx and a diminution of PLCγ1 phosphorylation. In the current work, we continued to catalogue additional proximal signaling proteins that showed a reduction in phosphorylation/activation. To this end, we discovered that LCK, Zap70, and LAT phosphorylation were also impaired in the p50-/-cRel-/- T cells. Furthermore, we discovered that absence of p50 and cRel negatively impacted the IKK complex leading to a reduction in the mRNA and protein levels of IKKβ and IKKγ, while concurrently showing that less IKKβ localized to lipid rafts in p50-/-cRel-/- T cells, in a basal state. By exploiting the human, CD4 Jurkat T cell lines (3T8 & E61) we were able to demonstrate that the defects in phosphorylation of proximal signaling proteins displayed in the p50-/-cRel-/- T cells were likely due to the paucity of the IKK complex, as knockdown of IKKγ (and the resulting complex degradation) recapitulated the activation defects in the p50-/-cRel-/- T cells. Moreover, expression of a constitutively active IKKβ (IKKβEE) resulted in enhanced phosphorylation of proximal signaling proteins and activation of T cells. Additionally, the use of an NF-κB super repressor, IκBα, had no effect on proximal phosphorylation or other early activation events, validating paucity of
the IKK Complex as the perpetrator of decreased TCR signaling, not the lack of NF-κB activation. Finally, in a separate in vitro system, utilizing Hek 293 cells, we were able to show that IKKβ can directly phosphorylate Zap70.

3.3. Methods & Materials

3.3.1. Mice and Primary T cells

OT-1 transgenic, and p50/-/-cRel/-/- mice have all been described previously [200]. All mice were maintained under specific pathogen free conditions, and all experiments using mice were carried out in accordance with institutional guidelines. T cells were isolated from spleens of control and experimental mice by CD4 and CD8 MACS beads (Miltenyi Biotec), and cultured in RPMI supplemented with 10% FBS, L-glutamine (2mM), Penicillian /Streptomycin (100 U/ml), β2ME (50mM), and Non-essential AA, Sodium Pyruvate, and HEPES (all 1mM). OT-1 and polyclonal CD8 T cells were stimulated with either 1 or 10μg anti CD3/CD28 (ebioscince) and/or 50ng Phorbol 12-Myristate 13-Acetate (PMA) and 500ng Ionomycin (both Fisher) depending on assay.

3.3.2. Cultured Cells, Transfections, and Infections

The parental 3T8 cells and 3T8 cell lines transduced with IKKγ K.D. (3T8.8221) and GST (3T8.8221.11), and IKKγ put back (3T8.8221.16) were a kind gift from Dr. Adrianne Chang. Jurkat E6-1 Cells were purchased from the ATCC and both Jurkat subsets were cultured in RPMI supplemented with 10% FBS, penicillin (100 U/ml),
streptomycin (100 U/ml), and L-glutamine (2 mM). Hek293 T cells were purchased from the ATCC and cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 U/ml), and L-glutamine (2 mM). All transient (Hek293 – HA-Zap70, MiG, and IKKβEE) and stable (Jurkat - MiG, IκBα, and IKKβEE - described here) ([220]) transfections were performed using Fugene 6 (Roche Diagnostic Systems). Briefly, 25μL of Fugene was incubated with RPMI for 5m at RT. Mixture was then added to plasmid DNA and incubated for 15m at RT. Finally plasmid and Fugene were added to Cells and incubated overnight. Cells were sorted or used 48 hours post transfection. Stable transfection of the Jurkat T cell line was accomplished through initial transfection of the MiG, IκBα, or IKKβEE plasmids into Hek293 cells with 4μg of the pCL-Eco packaging vector and Fugene 6. Cells were cultured for 48h, and viral containing supernatant was collected. This Retrovirus was used to infect the E6-1 Jurkat cells using high speed centrifugation. Lines were confirmed and established through sorting and expanding of GFP positive Cells for the MiG, IκBα, and IKKβEE Jurkat lines.

3.3.3. Electrophoretic Mobility Shift Assay (EMSA)

2 X 10^7 Cells were washed in PBS then suspended in 10mM HEPES (pH7.9), 10mM KCL, 0.1mM EDTA, 0.3% NP-40, and 1x Protease inhibitors for 5min. at 4°C, then centrifuged for 5 min at 1600g. The supernatant (cellular extract) was removed and the remaining pellet was washed twice in the above buffer (minus the NP-40.) Finally the pellet was suspended in 20mM HEPES (pH7.9), 0.4M NaCl, 1mM EDTA, 25% Glycerol, and vortexed intermittently as it incubated for 10min. at 4°C. After centrifugation (16,000g for 5min.,) the supernatant (nuclear extract) was collected.
To assay NF-κB activity, the following hairpin oligonucleotide probe was used: GAGAGGGGATTCCCCGATTACCTTTCGGGGGAATCCCCTCT. Briefly, the oligonucleotide probe was annealed and end-labeled with Gamma-ATP using T4 polynucleotide kinase according to the manufacturer’s instructions (New England Biolabs). Labeled probes (100,000 cpm/rxn) were incubated with 5 μg of nuclear extracts, NFκB Binding Buffer (50 mM Tris (pH 7.6), 250 mM NaCl, 2.5 mM EDTA, 5 mM DTT, 1 mg/mL BSA, 25% Glycerol) and 1 μg poly-dIdC for 20 min at room temperature and separated on a 10% non-denaturing polyacrylamide gel with 0.25× TBE running buffer. DNA-protein complexes were then dried for two hours onto 3MM Chromatography paper (Whatman.) The paper was then exposed to film for 4 - 18 hours.

3.3.4. Relative Quantitative RT-PCR (RT-qPCR)

Table 3.1 Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>TCAAGAACGAAAGTCGGAGG</td>
<td>GGACATCTAAGGGCAGTCAACA</td>
</tr>
<tr>
<td>IKKα</td>
<td>CTGAGGTTGGTGTAGCGAGG</td>
<td>CAGAATCTGTGTAGCAGGCC</td>
</tr>
<tr>
<td>IKKβ</td>
<td>GTGTCAGCTGTATCCCTTC</td>
<td>GCTCCACAGCCTGCTCC</td>
</tr>
<tr>
<td>IKKγ</td>
<td>CTTTTGCGGAGATGCGG</td>
<td>GGTTAAATACACATCGGTCTG</td>
</tr>
</tbody>
</table>

Total RNA was extracted with the RNeasy Plant Kit (Qiagen), according to the manufacturer’s instructions. cDNA was synthesized from total RNA (0.2 μg) using the TaqMan® ReverseTranscriptase kit (Applied Biosystems). All RT-qPCR reactions were performed in an ABI Prism 7300 Sequence Detection system (Applied Biosystems,) with
Power SYBR® according to SYBR protocol (Applied Biosystems). All experiments were conducted in triplicate and SDs are indicated in the figures. The primers listed in the table were all from IDTDNA.

3.3.5. Flow Cytometry

Spleens were taken from WT, PKCθ-/-, and p50/-cRel/- mice, crushed, RBC lysed (ACK Buffer – Fisher), and FC blocked (eBioscience). WBCs were then stained with CD45, CD3, and CD8 (eBioscience) and KB-OVA tetramer (MLB International) in PBS (Fisher) supplemented with 0.5% Bovine Serum Albumin, BSA (Sigma) for 30m on ice. Cells were then washed twice and data was collected on an LSRII (Beckman Dickinson) and analyzed with FlowJO 9.6 (TreeStar). Gating strategy isolated only single Cells (through Forward and Side Scatter) and DAPI- (live) Cells.

3.3.6. Western Blotting

Whole Cell Extracts were prepared in 25mM Tris-HCl pH7.6, 2mMEDTA, 150mM NaCl, 0.5% NP-40, (Lysis Buffer) with late addition of 1mM PI, DFP, NaF, and NaVO₃. Occasionally, lysates were incubated for 30m at 37°C with 400U Lambda Phosphatase (New England Biolabs) / 30µg protein. A Bradford Assay (Applied Biosystems) was performed to standardize proteins on the DU 350 (Beckman Coulter) and samples were mixed with a 0.5% SDS loading buffer (Fisher) and run on a 10% acrylimide-bis gel and transferred to nitrocellulose paper. Membranes were blocked in Tris-buffered saline, 0.1% Tween 20 (TBS-T) with 3% BSA and 2% Milk (Carnation). Then blots were incubated overnight at 4°C with primary antibodies at 1:1000 in 5%BSA.TBS-T. After
three TBS-T washes, the membrane was incubated with the proper secondary at 1:3000 in 5%Milk.TBS-T for 1.5 hours at room temperature. The specific bands were revealed by chemiluminescence (Western Lightning ® Plus-ECL, Perkin Elmer) and visualized by autoradiography (Amersham Hyperfilm-ECL, GE). Before reprobing, blots were stripped by incubation in 0.1 M 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8) for 30 min at 65°C and the process began again, with blocking. Primary western antibodies include: pLck (Y394) and anti-NF-ATc1 (H-110) (Santa Cruz); anti-pLAT Y132 (4476) & anti-pLAT Y171 (73205) both from Abcam; PLCγ1 (BD), IKKα, IKKβ, IKKγ, pIKKα/β, Zap70, pZap70 (Y319), pPLCγ1 (Y783), anti-LAT (9166) (Cell Signaling). HRP conjugated-β-actin, and all secondary antibodies (mouse, rabbit, and goat) were from Santa Cruz.

3.3.7. Spectrophotometer Based Calcium Assay

T cells were suspended at 1e6/mL in Calcium Buffer (No-Phenol Red RPMI (Fisher) with 2%FBS and 10% Probenecid (Thermo-Scientific)) and stained with 4μM Fluo-4 (Invitrogen) for 45m at 37°C. They were washed three times in Calcium Buffer and replated at 2e5/well in a 96 well flat-bottom plate. Cells were then rested for 20m at RT, and incubated at 37°C for 10m immediately preceding the assay. Where indicated, Cells received the following: 10μg/mL anti-CD3/CD28 (mouse & human-ebioscience), 3mM EGTA (Sigma) and 1μM Thapsigargin (Thermo-Scientific), and 5mM CaCl₂ (Fisher). Samples were read on a Wallac EnVision 2102 Multilabel Reader from Perkin Elmer.
3.3.8. Confocal Microscopy

2e^6/2mL T cells were incubated for 10m at 4ºC with 2ng Cholera Toxin Subunit B Alexa Fluor 488 (Invitrogen)/mL PBS. Cells were then washed twice and plated on Poly-Lysine D coated coverslips (Fisher) after settling for 10m, 150μL of 37º BD Cytofix was added to the cell mixture and incubated for 30m at RT. Coverslips were washed gently 2X in PBS. 0.5% Triton-X (Sigma) in PBS was applied to the Cells for 5m at RT to permeabilize. Cells were then washed in PBS, 2X and blocked in 3%BSA – PBS for 30m. Next, T cells were stained with indicated primary antibody (IKKβ or IKKγ – Cell signaling) at 1:50 in 3%BSA – PBS overnight, in the dark, at 4ºC. Cells were then washed 3X and incubated with Goat-anti rabbit Alexa Fluor 647 (Invitrogen) at 1:500 in 3%BSA – PBS for 1.5h, in the dark, at RT. Finally Cells were washed 3X, and Vectashield with Dapi (Vector Labs) was used to affix them to slides. Images were captured on the Leica TCS SP5 (Leica Microsystems) and analyzed using Definiens Tissue studio 3.0.

3.3.9. Statistical Analysis

All figures represent results from at least two (usually three) independent experiments. When appropriate data is given as the mean ± standard deviation. P values are expressed as * ≤ 0.05  ** ≤ 0.01; *** ≤ 0.001.
3.4. Results

3.4.1. Proximal T cell Signaling is Impaired in the Absence of p50 and cRel

As previous work from the lab established that activation / proliferation deficiencies in the p50-/−cRel-/− CD8 T cells appeared to result from reduced phosphorylation / activation of PLCγ1, we next determined if other proximal signaling proteins also displayed a paucity of phosphorylation in the p50-/−cRel-/− CD8 T cells. As you can see in Figure 3.1A, when stimulated with 10μg CD3/CD28, the p50-/−cRel-/− T cells evince a reduction in phosphorylation of three proximal signaling proteins: PLCγ1 (Y783), Zap70 (Y319) and the activating site on LCK (Y394). In Figure 3.1B, we additionally show that reduction in phosphorylation of Zap70 does diminish its activity level, as evinced by a reduction in phosphorylation of LAT at tyrosine residues 132 and 171, which are direct targets of Zap70. The inhibitory phosphorylation site on LCK (ser 505) seemed to be equivalently occupied, but at different kinetics (Fig 3.1A). It has been suggested by the Alexander [221] and Weiss [19] labs that LCK activity is based on the phosphatase CD45, and that the inhibitory site is much more vulnerable to dephosphorylation than the activating site. When we assessed total levels of CD45 using a pan antibody against all 8 isoforms in T cells [222], we found no difference in expression between WT and p50-/−cRel-/− CD8 T cells (Fig. 3.1C). While we cannot exclude the possibilities that CD45 may be less active or excluded from the proper compartments in the p50-/−cRel-/− CD8 T cells, we do know that total expression of CD45 is unchanged.
Figure 3.1. Phosphorylation of Proximal Signaling Proteins is Impaired in the p50-/cRel/- T Cells. (A) WT and p50-/cRel/- untreated and 5, 10, & 20m of 10μg/mL CD3/CD28 stimulation lysates were made and western blotting was performed. (B) Same as A, but only the unstimulated and 5m timepoints were utilized. (C) Unstimulated WT and p50-/cRel/- T Cells were isolated from spleens and stained for expression of CD45, CD3, and OVA-Tet; gating reflects live (DAPI-), single (Forward / Side Scatter) splenocytes. The data are representative of three independent experiments.

Next, we began exploring T cell activation signaling from the top, down. As one of the first events after TCR – MHC.peptide ligation is the strength of signal dependant phosphorylation of 6 sites in the TCR associated CD3 zeta chain [223], we initially
assessed its cell surface expression. When using the strong antigen system, OT1, we were able to show no difference in expression of CD3ε or OVA.Tetramer+ TCR (Fig. 3.1C) in splenic CD8 T cells from WT or p50-/-cRel-/- OT1 mice. Therefore, it appears that the discrepancies between WT and p50-/-cRel-/- activation signaling are not based on differential protein expression, as evinced through western blotting and flow cytometry, but based on phosphorylation differences at the level of LCK and Zap70, which in turn affect phosphorylation of LAT and PLCγ1.

3.4.2. IKKβ/γ Protein Levels are Reduced in the Absence of p50 and cRel

As we began to catalogue which proteins were affected in the p50-/-cRel-/- CD8 T cells, we also began to investigate other NF-κB family members that would have the ability to affect proximal T cell signaling, and could possibly be impacted by loss of p50 and cRel. Several labs have shown that NF-κB family members participate in large molecular weight complexes, and mathematical modeling has implicated multiple transcription factors in the regulation of their own family members [224, 225]. Moreover, it is known that the IKKα/β/γ regulatory complex must be brought into the Immune Synapse for ultimate degradation of IκBα and translocation of NF-κB transcription factors to the nucleus [226]. We therefore hypothesized that (a) absence of p50 and cRel may transcriptionally or post-transcriptionally impact levels of IKKα/β/γ, and (b) IKKα/β/γ may have imperative functions, including altering the proximal TCR signaling cascade, beyond exclusive control of NF-κB activation: both hypotheses were investigated.
Figure 3.2. Expression of IKKβ & IKKγ Are Reduced in the p50/-cRel/- T Cells. (A) Relative amount of IKKα, IKKβ, and IKKγ mRNA present in untreated WT and p50/-cRel/- CD8 T-Cells, normalized to 18S Ribosomal RNA. (B) WT and p50/-cRel/- untreated lysates were made and western blotting was performed. (C) Amount of Cytoplasmic IKKβ, by confocal microscopy, in untreated WT and p50/-cRel/- CD8 T Cells. (D) Amount of Cytoplasmic IKKγ, by Confocal Microscopy, in untreated WT and p50/-cRel/- CD8 T Cells. (E) Representative Visual Image of IKKβ and IKKγ intensity from Confocal Microscopy. (F) Amount of IKKβ Colocalized with Lipid rafts in untreated WT and p50/-cRel/- CD* T Cells.
As mathematical modeling predicts that IKKβ has a cRel binding site near its promoter [224], we first assessed whether or not transcription of IKKα, β, or γ was diminished in the p50-/-cRel-/- CD8 T cells. As you can see in Figure 3.2A, levels of IKKβ and IKKγ (but not IKKα) were reduced in the polyclonal p50-/-cRel-/- when compared to WT T cells, and standardized by 18S ribosomal RNA. Next, as shown in Figure 3.2B (western) and Figure 3.2C, D, and E (immunofluorescence) there was also a reduction in protein levels of IKKβ and IKKγ, sequela to the diminution of mRNA levels. While further CHIP analysis would be needed to delineate that p50 and/or cRel are directly controlling expression of IKKβ and IKKγ, it is clear that the amount of these proteins is diminished when both p50 and cRel are absent. Moreover, as shown in Figure 3.2F, there is a larger fraction of IKKβ associated with lipid rafts in unstimulated WT T cells than in the p50-/-cRel-/--. Thus indicating that in WT T cells, a fraction of IKKβ is basally associated with lipid rafts to encourage rapid, robust TCR signaling. Meanwhile, the impairment in the subcellular localization of IKKβ in the p50-/-cRel CD8 T cells, is an additional defect to the diminution of total IKKβ levels, and could explain the much lower level of activating phosphorylation seen in these cells.

3.4.3. Using Jurkat T cells to Explore Molecular Mechanisms and the Role of IKKβ/γ

To assess whether the plethora of defects we witnessed in the p50-/-cRel-/- CD8 T cells could be recapitulated in a model where p50 and cRel were present and only the IKK complex was reduced, we began using the Jurkat 3T8 line, which has been transduced with a KD of IKKγ (3T3.8221). As shown in Figure 3.3A, the T cells missing
IKKγ perfectly mirror the reduction of phosphorylation seen in PLCγ1, LCK, and Zap70 that we witnessed in the p50-/-cRel-/- CD8 T cells. Moreover, this reduction in activation of key signaling proteins also led to a decrease in calcium influx, as shown in Figure 3.3B. Having demonstrated, in two models, that a reduction in IKKγ and IKKβ have a profound effect on the activation of Jurkat T cells, we next decided to evaluate overexpression of activated IKKβ in the E6-1 Jurkat line.

We began the next series of experiments by retrovirally infecting the E6-1Jurkat line with three separate plasmids: MiG (the control vector) and IKKβEE (overexpression of IKKβ with glutamic acid replacing the serines 177 and 181 to lock the protein in an active conformation resulting in higher NF-κB activation, and for our purpose, a way of evaluating whether or not activated IKKβ could have a role outside of NF-κB activation) [220], and IκBa, a super repressor of NF-κB, known to be downstream of proximal signaling and the IKK complex. As shown in Figure 3.3C (western) the phosphorylation of PLCγ1, Zap70, and LCK are augmented with overexpression of activated IKKβ; there was no further reduction in phosphorylation of proximal proteins between the MiG and the IκBα (data not shown). Figure 3.3D shows the corresponding increase in calcium influx in the IKKβ transfected Jurkat T cells. Once again, there was no discernible difference between the MiG and IκBα with respect to calcium influx. This suggests that repression of the canonical NF-κB pathway is not able to recapitulate the defects seen in the p50-/-cRel-/- T cells; indicating that loss of NF-κB activation is not the main driver for the impaired activation we see in the absence of p50 and cRel, and that there must be a more proximal mediator, with current data suggesting that reduction in IKKβ is the actual culprit.
Figure 3.3. Using Jurkat T Cells to Explore Molecular Mechanisms and the Role of IKKβ & IKKγ in T Cell Signaling. (A) Parental 3T8 and 3T8 with IKKγ-/- Jurkat T Cells untreated and 5, 10, & 20m of 10μg/mL CD3/CD28 stimulation lysates were made and western blotting was performed. (B) Parental 3T8 and 3T8 with IKKγ-/- Jurkat T Cells were analyzed for the ability to influx Calcium: The first 60sec represent a baseline after which the following components were added to the culture: (^)10μg/mL CD3/CD28 and (#) 9mM EGTA. Points are Arbitrary Fluorescent Units (AFU) and lines represent one of three repeats within each assay. (C) E6-1 transfected with control vector: MiG, super NF-κB repressed: IκBα, or super NF-κB activated: IKKβEE Jurkat T Cells untreated and 5, 10, & 20m of 10μg/mL CD3/CD28 stimulation lysates were made and western blotting was performed. (D) Same as “B”, but E6-1 transfected with control vector: MiG, super NF-κB repressed: IκBα, or super NF-κB activated: IKKβEE Jurkat T Cells were used. (E) Relative amount of IL-2 mRNA present in untreated and two and six hour 50ng PMA/ 500ng Ionomycin treated MiG and IKKβEE Jurkat T Cells, normalized to 18S Ribosomal RNA.
In order to evaluate the level of repression of NF-κB in the IκBα line, and assure that the non-reduction in phosphorylation of proximal signaling proteins was truly representative of an NF-κB non-activated T cell, we performed qPCR, western blotting, and an EMSA on Jurkat T cells stimulated with PMA and Ionomycin, to bypass the proximal signaling cascade. As you can see, all three transfected lines responded appropriately to stimuli with the IKKβEE line showing a massive increase in IL-2 and IκBα showing a 5 fold reduction from the MiG (Fig. 3.3E). Moreover, as shown by EMSA in Figure 3.4A, the IKKβEE transfected cells not only increased NF-κB activity with PMA/Ionomycin treatment, but also had an elevated baseline (Lane 7). Incidentally, this also led to an increase in basal levels of nuclear NF-AT, as shown in Figure 3.4B, lane 7. Meanwhile, the IκBα line was unable to initiate nuclear translocation of NF-κB subunits at any time point or stimulant concentration (Lanes 5 & 6). Finally, through western blotting of nuclear extracts for NF-ATc1, we can see that IKKβEE has the ability to increase NF-AT nuclear translocation and activation (both basally and with the addition of PMA and ionomycin), but repression of NF-κB transcription (transfection of IκBα) has no bearing on activation of NF-AT (Fig. 3.4B). These results seem to indicate that impaired proximal signaling in p50/-/cRel/- T cells, leading to reduced calcium influx and activation of NF-AT, is likely due to the diminution, instability, or mis-location of IKKβ, not to the transcriptional requirements of p50 and cRel, per se; although these missing transfection subunits certainly exacerbate the defects.
Figure 3.4. Evaluation of IκBα Transduced Jurkat T Cells  (A) E6-1 transfected with control vector: MiG, super NF-κB repressed: IκBα, or super NF-κB activated: IKKβEE Jurkat T Cells untreated and two and six hour 50ng PMA/500ng Ionomycin treated were used to prepare nuclear lysates and an EMSA was performed. (B) Same as in “A”, but nuclear lysates were used for western blotting.

3.4.4. Using Hek 293 Cells to Explore the Relationship Between IKKβ and Zap70

In order to show a mechanism of action for IKKβ enhancing phosphorylation / activation of proximal TCR proteins, we chose to move into the Hek293 system. Here, through transfection of HA-Zap70 and IKKβ, we could properly assess phosphorylation
Figure 3.5. IKKβ Phosphorylates Zap70 In Vitro. (A) Hek 293 cells were transiently transfected with 1μg HA-Zap70 with and without 1μg IKKβ. 24 hours later, lysates were made in the presence or absence of phosphatase inhibitors. “Upper band” indicates a mobility shift in Zap70. (B) Select lysates (from “A”) were incubated for 30m at 37°C with phosphatase buffer in the presence or absence of Lambda Phosphatase. “Upper band” indicates a mobility shift in Zap70. (C) Whole cell lysates from a second experiment were made, and probed for pY319 on Zap70, indicating autophosphorylation/activation of the protein.

changes in Zap70, without confounding protein interaction that would be found in T cells. This way, any change would be directly attributable to IKKβ. As you can see in Figure 3.5A, cotransfection of HA-Zap70 and IKKβ did, in fact, lead to a mobility shift in
the migration of the HA-Zap70 protein. Moreover, as shown in Figure 3.5B, when the cotransfected lysates were treated with Lambda Phosphatase, the “upper band” disappeared, indicating that the way in which IKKβ is modifying Zap70 is through phosphorylation. Additionally, as you can see in Figure 3.5C, the addition of calIKKβ in the Hek293 system also has the ability to enhance the autophosphorylation (Y319) of Zap70. This demonstrates that the possible direct phosphorylation of Zap70 by IKKβ does increase Zap70’s total phosphorylation status and results in a more activated protein.

3.5. Discussion

Dogma states that phosphorylation of IKKβ (leading to degradation of IκBα and activation of NF-κB) lies many interactions downstream of activation of PLCγ1 [130, 227]. And the work of the Marienfield lab demonstrated that you needed not only PLCγ1 activation, but a hearty influx of calcium to activate Calcineurin and create the secondary signaling complex (CBM) which would activate IKKβ [128]. It is, therefore, quite easy to trace activation of PLCγ1 to phosphorylation of IKKβ and nuclear translocation of p50/cRel and p50/p65. Our results, however, indicate that IKKβ is actually affecting T cell activation much farther upstream in the proximal T cell signaling cascade.

In previous studies we elucidated that many of the phenotypic discrepancies in p50/-/-cRel/- activation were a result of greatly reduced calcium influx and activation of NF-AT, sequela to suboptimal phosphorylation/activation of PLCγ1. In this study, we expanded our research to find mechanistic insight as to why phosphorylation of PLCγ1
(a proximal signaling protein) would be impacted in a system deplete of two transcription factors. Here, we demonstrated that not only was PLCγ1 phosphorylation impaired, but LCK, Zap70, and LAT phosphorylation were also abrogated. This led us to look at other NF-κB family members as possible culprits, and to that end, we found a decrease in mRNA and protein levels (by both western blot and immunofluorescence of IKKβ and IKKγ in the p50/-/-cRel/-/- CD8 T cells.

Figure 3.6. IKKβ Enhances Phosphorylation of Proximal TCR Signaling Proteins. Working model showing signaling to the three main T Cell transcription factors: AP-1, NF-κB, and NF-AT; highlighting the novel impact of IKKβ on proximal T Cell signaling.
By branching out into the human CD4 Jurkat T cell line, we were able to recapitulate our system in a different species (murine vs. human) and a different T cell type (CD8 vs. CD4); moreover, we could easily manipulate genes and pathways of interest to tease apart complex mechanisms. In Figure 3.3 A&B, we showed that KD of IKKγ (and the resulting reduction of the total IKK complex) was able to mimic the defects we see in the p50-/-cRel-/- CD8 T cells, with respect to phosphorylation of proximal T cell signaling proteins and calcium influx. Moreover, when a constitutively active IKKβ was overexpressed, in a different model, we generated the exact opposite results compared to the p50-/-cRel-/- and IKKγ KD data. Yet, inhibiting the NF-κB pathway (through transfection of super repressive IκBα) did not achieve the same phenotype as KD of IKKγ, or absence of p50 and cRel in T cells. This highlights key differences between abolishment of NF-κB subunits and ablation of NF-κB activation, and implies that regulation or loss of certain NF-κB proteins can have dramatic effects on other family members. Moreover, through in vitro work in the Hek293 system, we were able to show that IKKβ has the ability to enhance phosphorylation of Zap70. This indicates a mechanism of action for IKKβ enhancing proximal TCR protein phosphorylation and activation through possibly direct phosphorylation of Zap70.

The striking finding of this study comes in two parts, the first being the unexpected decrease in IKKβ seen at both the mRNA and protein expression level in the p50-/-cRel-/- CD8 T cells. One possible explanation for this is simply that p50 (or more likely cRel) plays a part in the transcription of IKKβ [224]. Another possibility is that the entire IKK complex is reduced in the p50-/-cRel-/- mice; this is a more likely scenario, in which an overall less responsive cell would require many less units of such
an important signaling complex. As the IKK complex theoretically exists for the sole purpose of IκBα degradation, and IκBα is only produced in high quantities after stimulation. This theory is also supported by the similar decrease in protein levels of IKKγ. Although we did not witness an effect on IKKα, many groups have shown IKKβ to be the superior and more represented kinase of the pair with respect to canonical NF-κB signaling through the TCR [228]. Whether IKKβ itself is reduced in these p50-/cRel-/- T cells, leading to a reduction in complex formation, or the complex as a whole is less transcribed, the reduction in mRNA is a telling sign that this gene is being impacted at the transcriptional level and not only through post translational modifications or enhanced degradation of the IKK complex in p50-/cRel-/in T cells.

The other striking finding of this research is the impairment in phosphorylation of early T cell signaling proteins, and the fact that in vitro, IKKβ has the ability to enhance phosphorylation of Zap70. While the defects in proliferation, transcription and effector function can all be linearly attributed to the lack of the transcription factors p50 and cRel, the reduction in early phosphorylation events and calcium influx bears no direct action from these missing transcription factors. However, it does reveal that the p50-/cRel-/ T cells may have additional defects, and that these alterations or reductions in signaling ability are present in the basal state of the cell. The first proximal defect we documented was phosphorylation of Zap70, one of many proteins complexed around the TCR signaling cluster, and necessary for the phosphorylation of PLCγ1 [71], another protein we see with decreased phosphorylation in this system. Interestingly, it has been proposed by Yang et al [229] that the first step for Zap70 to become fully activated is phosphorylation at Ser-520. Furthermore, he went on to espouse, in his
thesis [230], that the sequence surrounding Ser-520 is S\textsuperscript{520}DVWS\textsuperscript{524}. He notes that this sequence is highly conserved evolutionarily, but for our purposes, it must also be noted that SDVWS is the exact sequence that IKKβ targets when phosphorylating IκBα. Although further experimentation is needed to evince that IKKβ is responsible for attaching the phosphate group to Ser520 on Zap70, it is circumstantially plausible, especially in light of the enhanced phosphorylation of Zap70 we see when IKKβEE is transfected into Jurkat T cells, or when Zap70 and IKKβ are cotransfected into Hek293 cells. This data has led us to working model (Figure 3.6) in CD8 T cells, in which IKKβ is not only indispensable for NF-κB activation, but also plays a role in enhancing proximal TCR signaling.

Regardless of the mechanism of direct action that IKKβ may utilize to affect the phosphorylation/activation of Zap70, we have conclusively shown that IKKβ levels are reduced in p50-/−cRel-/− T cells, and that this reduction correlates with a diminished phosphorylation of Zap70. With this event, many of the discrepancies and reductions in signaling seen farther down the chain are easily explainable. A less active Zap70 would leads to sub-par phosphorylation of LAT, which reduces the nucleation potential of the LAT signalosome; this leads to a decrease in phosphorylation of PLCγ1. PLCγ1 functioning at a less than optimal state creates a paucity of IP\textsubscript{3}, which lowers the amount of calcium influx, therefore reducing the amount of activated NF-AT. From here, any further readout (dearth of proliferation and IFNγ and IL-2 production) are easily accounted for by the lack of p50 and cRel and the reduction of NF-AT, three storied and fundamental transcription factors for CD8 T cell activation.
CHAPTER 4
CONSTITUTIVELY ACTIVE IKKβ DOES NOT INCREASE EXPRESSION OF CRAC OR PURINERGIC CALCIUM CHANNEL PROTEINS

4.1 Abstract

Previous work from our lab has shown that transducing Jurkat T cells with constitutively active IKKβ has the ability to enhance proximal TCR phosphorylation, and influx greater volumes of calcium, leading to superior activation of NF-AT and amplifying positive downstream effects, such as proliferation and IL-2 production. Moreover, NF-κB subunits p50/p65/p52 have recently been shown to regulate the CRAC proteins, STIM1 and ORAI1 in mast cells [215]. In this chapter, we attempt to elucidate the mechanism by which calKKβ amplifies calcium signaling, by delving deeper into the proteins responsible for calcium import: the CRAC and purinergic calcium channels. We chose to assess the ability of activated NF-κB to modulate these two families of calcium channel proteins, with respect to mRNA and protein levels, and protein function. Additionally, a holistic view of calcium channel protein modulation by NF-κB was created by also generating cell lines in which NF-κB was repressed through overexpression of IκBα.

The results generated herein confirm that IKKβEE cell lines have superior calcium signaling, and delineate a possible role for constitutively active IKKβ in
enhancing the function of the CRAC channel protein STIM1, as use of the specific
STIM1 inhibitor Suramin had a more profound effect on calcium influx in the calKKβ
expressing cells. They do not indicate a role for activated NF-κB in affecting the
function of ORAI1’s pore forming ability, however. Additionally, NF-κB does not appear
to modulate the function of purinergic calcium channel proteins: P2X1, P2X4, or P2X7.
Contradictory to published findings, we could not validate that NF-κB was the element
responsible for transcriptional regulation of ORAI1 and STIM1; this discrepancy,
however, is likely due to differences in cell type, NF-κB subunit, and stimulation route.
In accordance with NF-κB’s inability to modify the function of purinergic channel
proteins, NF-κB activity level also had no effect on the mRNA or protein levels of this
calcium channel family.

4.2. Introduction

Calcium is one of the most powerful and versatile second messengers in T cell
signaling, and the previous decade has represented a flood of knowledge as to the
complex protein interactions required to induce this cascade via influx of extracellular
calcium. Thirty years ago, The Store Operated Calcium Entry (SOCE) was one of the
first pathways identified as crucial for calcium signaling in a multitude of nucleated cells,
including T cells [231]. SOCE signaling is initiated by binding of IP₃ (generated via
cleavage of PIP2 by PLCγ1) to the IP₃R on the Sarcoendoplasmic reticulum (S-ER).
This allows for a small amount of calcium to seep out from the S-ER into the cytoplasm
where it binds to STIM1 causing a conformation change that will allow for docking with
ORAI1 (the pore forming unit) and the translocation of the entire protein complex (and S-ER) to the cytoplasmic membrane [232].

Although Transient Receptor Potential Cation (TRPC) channels are not selective for calcium, the Muallem lab has solidified the role of TRPC1 as intimately linked to the functional interaction of ORAI1 and STIM1, as knockdown of any of these three proteins rendered the SOCE pathway inactive, and thwarted further extracellular influx of calcium. Moreover, they demonstrated that TRPC1 is constitutively associated with ORAI1 [173]. Furthermore, the Lang lab, through elegant experimentation in HEK293 cells, was able to show that both ORAI 1 and STIM1 were transcriptionally regulated by NF-κB subunits p65/p50/and p52; as augmentation of these proteins led to increased transcript genesis and a corresponding increase in Calcium, while use of the NF-κB inhibitor, Wagonin, had the opposite effect [215].

Another indispensable family of calcium channel proteins, demonstrated by the Junger lab, is the purinergic receptors. They illustrated a crucial role for ATP-gated calcium channel proteins (P2X1 and P2X4) in sustaining the necessary calcium level for activation of NF-AT and various downstream targets including production of IL-2 [233]. Through time-lapse confocal microscopy, they were able to demonstrate that both P2X1 and P2X4 home to the Immune Synapse during T cell activation. Additionally, if these proteins were inhibited, calcium influx was abrogated, even in the presence of a fully functional SOCE pathway. Moreover, they went on to delineate a role for P2X7, which although it does not home to the Immune Synapse, still seems indispensable, as P2X7 gene transcripts are highly upregulated post TCR stimulation, and ablation of P2X7 fully inhibited calcium signaling in T cells [234].
In light of the known roles of these two important calcium channel protein families, we sought to discover the effect of activated or repressed NF-κB on calcium signaling and CRAC and purinergic protein levels and function. To modify NF-κB activity, Jurkat E6-1 T cells were transduced with either MiG (control vector), IκBα (super repressed NF-κB), or IKKβEE (super activated NF-κB) and interrogated using several techniques. To assess the ability of NF-κB to modulate CRAC and purinergic protein expression, we first employed qPCR to delineate any change in transcript level. Sequela to that line of interrogation, cell lines indicating transcript upregulation of CRAC and purinergic proteins were further subjected to western blotting to determine if this upregulation would translate into an embellishment of protein levels. To assess the ability of NF-κB to modify CRAC and purinergic protein function, several inhibitors were used to block various components of the calcium signaling pathway. The two CRAC channel protein inhibitors used were: 2-aminoethoxybiphenyl borate (2-APB), known to restrain CRAC based calcium influx by inhibiting TRPC function [235], and 1-(b- (3-(p-methoxyphenyl)-propyloxy-p-methoxyphenyl)-1H-imidazole hydrochloride (SK&F 96365), known to disrupt SOCE at the level of cytoplasmic membrane receptors [236]. The P2X inhibitors utilized were: Suramin, a non-selective antagonist of purinergic receptors [237]; 8,8’-(carbonylbis(imino-3,1-phenylene carbonylimino) bis(1,3,5-naphthalenetrisulfonic acid) (NF023), a Suramin analogue specific for P2X1, with little to no activity against P2X4 and P2X7 [238]; and 2’- & 3’-O-(4benzoylbenzoyl)-ATP (o-ATP), an antagonist specific for P2X7 [239]. By comparing the effect on calcium by each inhibitor (with regard also paid to the NF-κB activity level of the cell) we were able
to assess not only the contribution of individual channel proteins to intracellular calcium levels, but delineate the role of NF-κB in their function.

Through these studies, we were able to show that IKKβEE has the ability to moderately enhance the mRNA levels of STIM1, P2X1, and P2X7 in unstimulated cells. This increase in mRNA levels, however, does not translate to increased protein levels. Additionally, while the use of various CRAC and purinergic channel protein inhibitors did validate the requirement of these proteins for proper calcium influx, only the function of STIM1 appears to be moderately enhanced by super activating the NF-κB pathway through expression of constitutively active IKKβ, although many further studies will be required to validate this effect and evince a mechanism.

4.3. Methods and Materials

4.3.1. Cultured Cells, Transfections, and Infections

Jurkat E6-1 Cells were purchased from the ATCC and both cultured in RPMI supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 U/ml), and L-glutamine (2 mM). All stable (Jurkat - MiG, IκBα, and IKKβEE described here ([220]) transfections were performed using Fugene 6 (Roche Diagnostic Systems). Stable transfection of the Jurkat T cell line was accomplished through initial transfection of the MiG, IκBα, or IKKβEE plasmids into Hek293 cells with 4μg of the pCL-Eco packaging vector and Fugene 6. Cells were cultured for 48h, and viral containing supernatant was collected. This Retrovirus was used to infect the E6-1 Jurkat cells using high speed
centrifugation. Lines were confirmed and established through sorting and expanding of GFP positive Cells for the MiG, IκBα, and IKKβEE Jurkat lines.

4.3.2. Relative Quantitative RT-PCR (RT-qPCR)

Total RNA was extracted with the RNeasy Plant Kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from total RNA (0.2 μg) using the TaqMan® ReverseTranscriptase kit (Applied Biosystems). All RT-qPCR reactions were performed in an ABI Prism 7300 Sequence Detection system (Applied Biosystems,) with Power SYBR® according to SYBR protocol (Applied Biosystems). All experiments were conducted in triplicate and SDs are indicated in the figures. ORAI1 and STIM1 were purchased from Realtime Primers, all other primers were from IDTDNA with sequences listed below:

Table 4.1 Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>TCAAGAACGAAAGTCGGAGG</td>
<td>GGACATCTAAGGGCATCACA</td>
</tr>
<tr>
<td>P2PX1</td>
<td>CGTCATCGGGTGGGTGTTTCTCTA</td>
<td>AGGGCGCGGGATGTCGTCA</td>
</tr>
<tr>
<td>P2PX4</td>
<td>CGGGACCCACACGCAACGGAGTCT</td>
<td>TGTATCGAGGCGGAAGGAGTA</td>
</tr>
<tr>
<td>P2PX7</td>
<td>CCGGCCACAACTACACCACGA</td>
<td>GGCCAGACCGAAGTAGGAGAG</td>
</tr>
<tr>
<td>ORAI2</td>
<td>CATAAGGGCATGGATTACCG</td>
<td>CGGGTACTGGTACTGCGTCT</td>
</tr>
<tr>
<td>STIM2</td>
<td>CTCTAACACGCCACCTCAT</td>
<td>CTTCTGTGCCTTTTCAAGC</td>
</tr>
</tbody>
</table>
4.3.3. Western Blotting

Whole Cell Extracts were prepared in 25mM Tris-HCl pH7.6, 2mMEDTA, 150mM NaCl, 0.5% NP-40, (Lysis Buffer) with late addition of 1mM PI, DFP, NaF, and NaVO₃. A Bradford Assay (Applied Biosystems) was performed to standardize proteins on the DU 350 (Beckman Coulter) and samples were mixed with a 0.5% SDS loading buffer (Fisher) and run on a 10% acrylimide-bis gel and transferred to nitrocellulose paper. Membranes were blocked in Tris-buffered saline, 0.1% Tween 20 (TBS-T) with 3% BSA and 2% Milk (Carnation). Then blots were incubated overnight at 4°C with primary antibodies at 1:1000 in 5%BSA.TBS-T. After three TBS-T washes, the membrane was incubated with the proper secondary at 1:3000 in 5%Milk.TBS-T for 1.5 hours at room temperature. The specific bands were revealed by chemiluminescence (Western Lightning ® Plus-ECL, Perkin Elmer) and visualized by autoradiography (Amersham Hyperfilm-ECL, GE). Before reprobing, blots were stripped by incubation in 0.1 M 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8) for 30 min at 65°C and the process began again, with blocking. Primary western antibodies include: ORAI 1 (Pro Sci Inc - #ABIN1030562), STIM1 (Cell Signaling - #5668), and P2X7 (Cell Signaling - #13809). HRP conjugated-β-actin, and all secondary antibodies (mouse, rabbit, and goat) were from Santa Cruz.

4.3.4. Spectrophotometer Based Calcium Assay

T cells were suspended at 1e6/mL in Calcium Buffer (No-Phenol Red RPMI (Fisher) with 2%FBS and 10% Probenecid (Thermo-Scientific)) and stained with 4μM Fluo-4 (Invitrogen) for 45m at 37°C. They were washed three times in Calcium Buffer
and resuspended in Calcium Buffer with or without indicated calcium channel inhibitors for 2 hours. Cells were then washed twice and replated at 2e5/well in a 96 well flat-bottom plate. Cells were then rested for 20m at RT, and incubated at 37˚C for 10m immediately preceding the assay. Where indicated, Cells received the following: 10μg/mL anti-CD3/CD28 (ebioscience), 3mM EGTA (Sigma) and 1μM Thapsigargin (Thermo-Scientific), and 5mM CaCl₂ (Fisher). Samples were read on a Wallac EnVision 2102 Multilabel Reader from Perkin Elmer.

4.3.5. Statistical Analysis

All figures represent results from at least two (usually three) independent experiments. When appropriate data is given as the mean ± standard deviation. P values are expressed as * ≤ 0.05  ** ≤ 0.01; *** ≤ 0.001.

4.4. Results

4.4.1. The Effect of Enhanced and Repressed NF-κB on Calcium Release and Activation Channel (CRAC) Proteins

As previous work from the lab established that calcium influx could be enhanced in Jurkat T cells transduced with NF-κB super activating IKKβEE; several labs have reported inhibition of calcium influx when NF-κB was abrogated; and NF-κB has been shown to transcriptionally regulate CRAC channel proteins, we next determined if mRNA expression of ORAI and STIM proteins could be impacted by alterations in NF-
κB activity. As you can see in Figure 4.1A-D, only basal levels of STIM1 appear to be

![Graphs and images](image)

**Figure 4.1. The Effect of Enhanced and Repressed NF-κB on Store Operated Calcium Entry (SOCE) Receptors.** (A - D) Relative amount of ORAI1 (A), ORAI2 (B), STIM1 (C), and STIM2 (D) present in untreated and two and six hour 50ng PMA/500ng Ionomycin treated MiG, IκBα, and IKKβEE Jurkat T Cells, normalized to 18S Ribosomal RNA. (E) Untreated and two and six hour 50ng PMA/500ng Ionomycin treated MiG, IκBα, and IKKβEE Jurkat T Cells whole cell extracts were made and western blotting was performed.

slightly enhanced in the IKKβEE, as compared to MiG controls, and not to a significant degree. Even more consternating, by western blotting (as depicted in Figure 4.1 E) it appears that the NF-κB repressed line (IκBα) actually shows an increase in protein levels of STIM1 in the basal state, and at all time points post stimulation. However, the
highest protein expression in the series was ORAI1, in the IKKβEE line, after two hours of stimulation with PMA and Ionomycin.

After determining that NF-κB activity did not directly impact the level of mRNA or protein expression of CRAC channel proteins, we next sought to determine if protein function could be impacted by NF-κB status. To this end, each of the three transduced lines was assessed for their ability to influx calcium under forced (but physiologically relevant) conditions, and in the presence or absence of known CRAC channel inhibitors.

**Figure 4.2. The Effect of SOCE Inhibitors on NF-κB Transduced Jurkat T Cells (A)** MiG, IκBα, and IKKβEE Jurkat T Cells were analyzed for the ability to influx Calcium in the presence or absence of 100μM SK&F: The first 30sec represent a baseline after which the following components were added to the culture: (2) 3mM EGTA, (3) 2ng Thapsigargin, and (4) 5mM CaCl₂. Points are Arbitrary Fluorescent Units (AFU) and lines represent one of three repeats within each assay. **(B)** Graphical representation of percent calcium influx inhibition by 100μM SK&F. **(C)** MiG, IκBα, and IKKβEE Jurkat T Cells were analyzed for the ability to influx Calcium in the presence or absence of 100μM 2-APB: The first 30sec represent a baseline after which the following components were added to the culture: (2) 3mM EGTA, (3) 2ng Thapsigargin, and (4) 5mM CaCl₂. Points are Arbitrary Fluorescent Units (AFU) and lines represent one of three repeats within each assay. **(D)** Graphical representation of percent calcium influx inhibition by 100μM 2-APB.
the IκBα cell line, with respect to having a larger pool of sarcoplasmic calcium (as indicated by the larger spike in calcium release when thapsigargin degrades the SERCA pump (Fig 4.2A (3))) and the ability to influx extracellular calcium at a faster and more robust rate (Fig 4.2A (4)). However, when 100µM SK&F was added to the cells, the percent of inhibition was equivalent (as shown in Figure 4.2B) indicating that the increased calcium influx in the IKKβEE cell line is likely not due to enhanced cytoplasmic pore formation by ORAI.

Next, in an attempt to interrogate whether or not, the enhanced performance by the IKKβEE cell line, was indicative of increased function of STIM1 and/or STIM1/ORAI1/TRPC1 complex formation, we utilized the inhibitor 2-APB. Here the results are much more drastic, as use of 100µM 2-APB was able to completely abrogate calcium influx in all three cell lines, while the forced release of calcium by thapsigargin induced depletion of the SERCA pumps still revealed a greater starting pool in the IKKβEE cell line (Figure 4.2C). Plotted graphically (Figure 4.2D) it suggest that the IKKβEE cell line likely owes a portion of its enhanced calcium function to increased activity of STIM1, (compared to only 45% for MiG and IκBα) in calcium influx when these processes are inhibited by 2-APB.

4.4.2. The Effect of Enhanced and Repressed NF-κB on Purinergic Calcium Receptors.

Utilizing the same three cell lines as above, we next sought to determine the impact that NF-κB may have on the purinergic family of calcium channel proteins. As you can see in Figure 4.3.A-C, P2X7 does show the expected increase in transcript
level, post stimulation, and both P2X1 and P2X7 mRNA appear to be upregulated in the presence of IKKβEE at the basal state, although neither was shown to be statistically significant. Moreover, when we sought to find out if the increase in mRNA levels would translate to an increase in protein level, it did not, as shown in Figure 4.3 D.

Figure 4.3. The Effect of Enhanced and Repressed NF-κB on Purinigeninc Calcium Receptors.  (A - C) Relative amount of P2X1 (A), P2X4 (B), and P2X7 (C) present in untreated and two and six hour 50ng PMA/ 500ng Ionomycin treated MiG, IκBα, and IKKβEE Jurkat T Cells, normalized to 18S Ribosomal RNA. (D) Untreated and two and six hour 50ng PMA/ 500ng Ionomycin treated MiG, IκBα, and IKKβEE Jurkat T Cells whole cell extracts were made and western blotting was performed. (E) Graphical representation of percent calcium influx inhibition by 100μM Suramin.
Next, we sought to determine if the function of purinergic receptors could be enhanced by increasing the activity of NF-κB. To this end, we first utilized inhibitors specific for particular purinergic receptors P2X1 (NF023) and P2X4 (o-ATP).

![Diagram](image)

**Figure 4.4. The effect of Purinergic Inhibitors on NF-κB Transduced Jurkat T Cells**

(A) MIG, IκBα, and IKKβEE Jurkat T Cells were analyzed for the ability to influx Calcium in the presence or absence of 100μM Suramin: The first 30sec represent a baseline after which the following components were added to the culture: (1) 10μg/mL CD3/CD28, (2) 3mM EGTA, (3) 2ng Thapsigargin, and (4) 5mM CaCl₂. Points are Arbitrary Fluorescent Units (AFU) and lines represent one of three repeats within each assay. (B) MIG, IκBα, and IKKβEE Jurkat T Cells were analyzed for the ability to influx Calcium in the presence or absence of 10μM NF023 or o-ATP: The first 30sec represent a baseline after which the following components were added to the culture: (2) 3mM EGTA, (3) 2ng Thapsigargin, and (4) 5mM CaCl₂. Points are Arbitrary Fluorescent Units (AFU) and lines represent one of three repeats within each assay.
Interestingly, neither NF023 nor o-ATP had any effect on the ability of T cells to flux calcium at 10μM (Figure 4.4B); whereas a 100μM concentration of either inhibitor proved lethal (data not shown.) Interestingly, the pan purinergic inhibitor, Suramin, had grave effects on all three cell types. This resulted in highly erratic readings (as shown in Figure 4.4A), but overall, the percent calcium influx inhibition in the IKKβEE T cells was not statistically significantly different than that of the effect on MiG or IkBα cells (Figure 4.3 E). This data reveals that while the purinergic receptors are certainly important for T cell calcium influx, it does not appear that NF-κB status impacts the total protein level or function of this crucial family of calcium channel proteins.

4.5. Discussion

Unexpectantly, we were not able to show that enhanced activation of NF-κB (through constructively active IKKβ) was able to upregulate gene transcription of ORAI or STIM1. This data sits in opposition to recent findings that NF-κB was responsible for transcription of these two crucial CRAC channel proteins [215]. This likely points to key differences between the studies: the Lang lab utilized Hek 293 cells, while we used E6-1 Jurkat T cells; we augmented the pathway leading to NF-κB subunit nuclear translocation, whereas the Lang lab transfected in additional p50/p65/ and p52. The transfection of the p52 subunit, by the Lang lab, is especially controversial, as this non-canonical family member would not be induced in normal TCR signaling until 18-24 hours post stimulation, whereas calcium signaling occurs in the first few seconds; therefore, p52 was not a factor in our transfection model. Additionally, although we saw modest increases in P2X1 and P2X7 mRNA in the IKKβEE line, it did not translate to
increased protein levels, so based on our data; activated NF-κB can not increase protein levels of CRAC or purinergic calcium channel proteins.

In terms of modifying calcium channel protein function, our results indicate that only STIM1 activity is possibly enhanced in the IKKβEE cell line, as calcium influx showed a greater amount of inhibition compared to the MiG and IκBα cell lines when cells were incubated with 2-APB, a STIM1 specific inhibitor; whereas SK&F, an ORAI inhibitor, affected all three transduced cell lines equivalently. With respect to the purinergic receptors, although use of the inhibitors showed that this family of calcium channel proteins is indispensable (Suramin caused huge inhibition of calcium influx, and NF023 and o-ATP were lethal at equivalent concentrations), it does not appear that NF-κB activity can modulate their function. Low dose (10μM), specific inhibition of P2X1 (NF023) and P2X7 (o-ATP) had no effect on calcium influx in any cell line, and Suramin inhibited all three cell lines equally.

An interesting caveat to our results of the effect of caIKKβ on calcium must be considered based on work done in neurons exploring the relationship between IKKβ and TRPC channels. Here, the Tegeder lab demonstrated that IKKβ mediated negative effects on the TRPC channels, as TRPC channel sensitivity, calcium influx, and neuronal excitability were all increased when IKKβ was inhibited [240]. Interestingly, this would point to a contradictory situation in which activated IKKβ would decrease effectiveness of TRPC components of the SOC pathway, while concurrently leading to nuclear translocation of NF-κB which may induce upreglation of gene transcription of the ORAI and STIM, thus enhancing those components of the SOC pathway. Although
this would need to be recapitulated in T cells, and evaluated for the effect of activated IKKβ on TRPC1, specifically.

Additionally, our current studies may bear repeating with use of anti-CD3/CD28 in lieu of PMA/Ionomycin stimulation, as the Junger lab conclusively showed that TCR stimulation was required to elicit an upregulation in purinergic (P2X) channels (especially P2X7) [234], and PMA/Ionomycin bypass the TCR. However, we did see a greater increase in P2X7 mRNA in the IKKβEE cell line, post stimulation; although this did not generate increased protein levels. Moreover, the main focus of this study was on basal expression (both mRNA and protein levels) and how that could impact the immediate early calcium influx, thus rendering stimulation route irrelevant.
CHAPTER 5
CONCLUDING REMARKS AND ATTRIBUTIONS OF NF-κB IN T CELL SIGNALING

NF-κB represents one of the most critical protein families in, not only cells of the hemopoetic lineage, but all nucleated cells, and across multiple organisms. It regulates the transcription of genes important for almost every cellular process from growth and homeostasis, to effector functions, to both survival and apoptosis. In the studies presented in this work, we chose to focus on NF-κB’s role in T cell activation, predominately in naïve CD8 T cells; and, in vitro, specifically focusing on the first 20 minutes (earliest activation) and two to eighteen hours post stimulation (late-early activation) to gain a full spectrum readout on a T cell’s ability to: 1.) phosphorylate proximal TCR signaling proteins 2.) flux Calcium 3.) activate transcription factors, and 4.) regulate and transcribe genes. Utilizing primary murine CD8 T cells that were WT, PKCθ-/-, or p50-/-/cRel-/-, the Jurkat E6-1 and 3T8 CD4 cell lines that expressed caIKKβ or drIKBα and IKKγ-/-, respectively, and Hek293 cells, the studies presented here define a novel role for NF-κB in T cell signaling. Moreover, they reveal that primary, murine, naïve CD8 T cells that lack p50 and cRel have lower levels of IKKβ and IKKγ and display serious detriments in their ability to phosphorylate proximal TCR proteins, influx calcium, and activate NF-AT. Moreover, these defects amplify as they move from extracellular receptor ligation towards transcription regulation in the nucleus leaving these cells virtually incapable of displaying effector functions or proliferating.
Studies in Jurkat T cells allowed us to mechanistically unravel the above phenomena and indicate that the lack of the IKK complex is most likely the culprit for reduction in proximal TCR protein phosphorylation. These studies were additionally backed up by studies in Hek293 cells which indicated that IKKβ has the ability to enhance phosphorylation of Zap70. This indicates that a lack of IKKβ results in decreased phosphorylation / activation of Zap70, and that this “weak” start generates far less activated PLCγ1, which ultimately reduces the level of IP₃. PLCγ1, can generate, and diminishes calcium influx. Aside from this monumental effect on PLCγ1 (and the direct impact this has on decreasing calcium influx and further downstream effector functions) we have also shown that IKKβ may have the ability to modify the function of the CRAC channel protein STIM1, which is necessary for extracellular calcium influx.

5.1. A Novel Role for NF-κB in Proximal T Cell Signaling, Regulating Calcium Influx & Activation of NF-AT

In the second chapter, we examined the effect of absence of PKCθ and p50/cRel on primary CD8 T cells during activation. We documented that while absence of PKCθ had moderately depreciative effects on calcium influx, transcriptional regulation, and proliferation, loss of p50/cRel had gravely detrimental impacts on the above listed factors. Through microarray, we were able to tease apart the somewhat inclusive role played by PKCθ and p50/cRel in regulating gene transcription. Surprisingly, only a small amount of activation induced genes were disrupted in either knockout model (3.5%: p50-/-cRel-/- and 1.7%: PKCθ). In the p50-/-cRel-/- T cells, however, this small
percentage of deregulation had the ability to all but shut down activation elicited through anti CD3/CD28 treatment or culture with strong antigen pulsed APC.

Interestingly, many genes of the small subset which were impacted in the p50-/-cRel-/-, are classically defined and molecularly confirmed targets of the transcription factor NF-AT, such as IL3, IFNγ, and CTLA-4 [209-211]. This led us to explore the ability of p50-/-cRel-/- T cells to activate NF-AT upon stimulation. The resulting data categorized their ability as abysmal, with the p50-/-cRel-/- showing barely any presence of NF-AT in the nucleus through EMSA. Moreover, those cells also evinced the expected loss of nuclear NF-κB, and a somewhat more abrogated loss of nuclear AP-1. The PKCθ-/- T cells again displayed a more mild reduction in nuclear translocation of NF-AT NF-κB, and AP-1, as compared to p50-/-cRel-/-, although use of PKCα-/-PKCθ-/- T cells enhanced the diminution. Once again, this phenomenon was consistent, despite route of stimulation.

As calcium signaling is the main driver of NF-AT activation and nuclear translocation, we next looked to see if that would also be hampered in the p50-/-cRel-/-CD8 T cells, and, in fact, it was. As the p50-/-cRel-/- were barely able to bring in extracellular calcium, compared to the slight reduction in the PKCθ-/- T cell’s ability to flux calcium. The inequality in calcium influx ability points to a huge difference in the function of PKCθ and p50/cRel, two entities presumed to activate the same pathways. Arguably, there may be redundancy of function in other members of the PKC family that mask the true capabilities of PKCθ in the context of cellular activation, or the use of strong antigens may bypass the requirement for PKCθ in the assays we have selected to ascertain function.
In an attempt to delineate the cause of calcium influx blockade in p50-/-cRel-/- T cells, we first evaluated expression of two calcium channel proteins believed to be regulated by NF-κB: STIM1 and Orai1. Disappointingly, there was no perturbation of these calcium signaling agents at the mRNA or protein levels. Additionally, P2PX1, P2PX4, and P2PX7 (all purinergic calcium channel proteins) were also expressed at WT levels in the p50-/-cRel-/- CD8 T cells. Next, we looked to activation of PLCγ1 (via phosphorylation status of Y783) as a possible potentiator of poor calcium influx and signaling. Here, we discovered a key defect (reduction in phosphorylation of PLCγ1) in the p50-/-cRel-/- T cells that would explain the downstream deleterious effects of reduced calcium influx, transcription activation, and proliferation. Further substantiating that the signaling deficient phenotype of p50-/-cRel-/- T cells was due to defects in PLCγ1 activation, the use of PMA and ionomycin (pharmacological agents that activate T cells via a method that bypasses PLCγ1) was able to rescue the proliferation defects of the p50-/-cRel-/- T cells, and returned calcium influx and transcription factor nuclear localization to WT levels. This sheds light on two important points: 1.) Many of the activation defects of the p50-/-cRel-/- CD8 T cells are due to subpar activation of proximal TCR proteins and immediate-early signaling cascades, not simply lack of transcriptional regulation of genes by p50 and cRel and 2.) p50-/-cRel-/- T cells can activate and proliferate (robustly) once the initial hurdle of activation is overcome.

This work represents a thorough evaluation of T cell activation in the absence of PKCθ or p50/cRel, and concretely defining the functionality of these proteins in this arena is a top priority, as the ability to limit or exploit T cell activation (and both entities
are drugable targets) is crucial for prevention of GVHD and T cell based autoimmunity and control of infection and cancer, respectively.

5.2. Loss of p50 and cRel Lead to a Reduction of IKKβ and Deficient Phosphorylation of Proximal TCR Proteins

In Chapter Two, we cataloged deficiencies in p50-/-cRel CD8 T cells proliferation, transcription of immune activation related genes, and activation of transcription factors, calcium influx, and phosphorylation/activation of PLCγ1. In Chapter Three, we attempted to elucidate the underlying mechanism that would disrupt proximal TCR signaling in the absence of p50/cRel. To this end, we first assessed whether or not other proximal proteins also displayed a reduction in phosphorylation/activation, and discovered that Zap70, LAT, and to a lesser extent Lck, phosphorylation was also diminished in the p50-/-cRel-/- T cells. Next, we looked at expression of cell surface proteins required for TCR signaling, by Flow Cytometry. Here, we could evince no difference between WT and p50-/-cRel-/- T cells with respect to TCR, CD3ε, or CD45; thus pinpointing the subpar phosphorylation of Zap70 as the proximal defect in p50-/-cRel-/- T cells.

In light of the above findings, we expanded our research to look at other NF-κB family members that could be involved in this proximal level of T cell signaling: mainly, the IKK complex. This heterotrimer composed of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ) is known to home to the Immune Synapse during T cell activation; thus placing it in the correct subcellular compartment to affect proximal TCR signaling. Here, we discovered another key defect in the p50-/-cRel-/- T cells, as
they had reduced expression of IKKβ and IKKγ at both the mRNA and protein levels. Moreover, the localization of these two proteins also seemed to be altered in the p50-/-cRel-/- T cells, as through confocal microscopy, we demonstrated that unstimulated WT T cells had a greater volume of IKKβ colocalized with lipid rafts, than did p50-/-cRel-/- T cells.

Armed with the knowledge that a reduction in the levels of IKKβ and IKKγ could be causing the plethora of defects in the p50-/-cRel-/- T cells, we moved into the human, CD4 Jurkat T cell line and manipulated protein expression to help flush out a mechanism. We first looked at T cell activation responses in the 3T8 Jurkat line that had a knockout of IKKγ. Pleasantly, we found that loss of IKKγ perfectly recapitulated the phenotype of p50-/-cRel-/- T cells with respect to proximal TCR protein phosphorylation and calcium influx.

Next, in E6-1 Jurkat T cells, we retrovirally infected cell with either a constitutively activated IKKβ to (to enhance NF-κB signaling) or degradation resistant IκBα (to severely repress NF-κB signaling.) With respect to the caIKKβ plasmid, the cells performed beautifully, and were able to greatly enhance every level of activation assessed (proximal TCR protein phosphorylation, calcium influx, and transcription of IL-2) over cells transfected with a control (MiG) vector. The transfection of IκBα on the other hand, had no deleterious effect on activation based signaling in the Jurkat model, although they demonstrated complete repression of NF-κB, as judged by the inability of NF-κB to undergo nuclear translocation in response to stimuli. Moreover, these cells produced a dearth of IL-2, demonstrating that while repressed NF-κB signaling may not effect proximal activation of T cells, it has a tremendous negative effect on gene
transcription (distal activation). The lack of proximal activation impairment in the \( \text{IKB}\alpha \) cells sheds light on the fact that within our calIKK\( \beta \) cells, the positive signaling effects we were demonstrating could most likely be attributed to activated IKK\( \beta \), not the downstream enhancement of the NF-\( \kappa B \) pathway. Finally, for proof of principal, we adopted the Hek293 system, and here, through cotransfection of IKK\( \beta \) and Zap70, we were able to show that IKK\( \beta \) has the ability to enhance phosphorylation of Zap70 directly.

5.3. Constitutively Active IKK\( \beta \) Does Not Increase Expression of CRAC or Purinergic Calcium Channel Proteins

As Orai1 and STIM1 have both been described as targets of NF-\( \kappa B \), we originally hypothesized that these genes would be deregulated and under transcribed in the absence of p50/cRel. However, in chapter three, this was proven false, as no difference was shown between WT and p50-/-cRel-/- mRNA or protein levels of these two CRAC channel proteins. In the fourth chapter we attempted to sus out the mechanism behind this phenomenon utilizing human, CD4, Jurkat T cells. To this end, the cell line was retrovirally infected with either MiG (control vector), \( \text{IKB}\alpha \) (super repressed NF-\( \kappa B \)), or IKK\( \beta \)EE (super activated NF-\( \kappa B \)) and assayed for mRNA and protein levels of CRAC and purinergic calcium channel proteins unstimulated or after two or six hours of treatment with PMA and ionomycin. Although IKK\( \beta \)EE appeared to slightly enhance the mRNA transcripts of STIM1 (CRAC) and P2X1 and P2X7 (purinergic), this did not translate to increased protein levels.
To ascertain whether or not NF-κB was able to modulate the function of these proteins, the same three cell lines were assayed for their ability to influx calcium in the absence or presence of pre-incubation with several known independent inhibitors of CRAC and purinergic calcium channel proteins. In this arena, only the function of STIM1 appeared to possibly be enhanced by constitutively activated IKKβ, as the 2-APB inhibitor (known to inhibit STIM1 function) led to much greater inhibition in the IKKβEE cell line, compared to controls, indicating that this pathway could be important for enhanced calcium influx in the caIKKβ cell line, although many additional experiments are required for validation.

5.4. Concluding Remarks

Activation of T cells by interaction of the TCR with an MHC molecule loaded with a cognate peptide is one of the most intricate and regulated biological processes to be discovered. Decades of work have helped to outline the proteins involved in this process, and painstaking experiments have parsed each of these proteins into specific catalytic and regulatory domains, even highlighting the exact amino acids required for phosphorylation or additional post translational modifications. The scope of this dissertation has been to define the role of NF-κB subunits and overall activity with respect to T cell signaling. To that end, we have discovered that NF-κB subunits generally congregate in large supermacromolecular complexes, and ablation of one subunit (p50 or cRel) can affect stability of other subunits (IKKβ or IKKγ) within the family.
Additionally, this work demonstrates that phosphorylation of proximal TCR proteins, calcium influx, and activation of NF-AT are all inhibited in the absence of p50 and cRel, and that this is likely due to the corresponding decrease in IKKβ. Moreover, utilizing the human, CD4, Jurkat T cell line, we determined that knockdown of IKKγ and the accompanying decrease in all IKK complex members (α/β) recapitulated the p50-/-cRel-/- T cell activation defects; whereas constitutively active IKKβ could enhance phosphorylation of proximal TCR proteins, calcium influx, and activation of NF-AT. Utilizing Hek293 cells we were also able to delineate that IKKβ has the ability to directly enhance the phosphorylation of Zap70, and we believe that this is the mechanism of action for enhanced proximal signaling in WT compared to p50-/-cRel-/- T cells.
CHAPTER 6

CITATIONS


230. Yang, Y., Regulation of protein tyrosine kinase ZAP-70 by serine phosphorylation Division of Experimental Medicine, Department of Medicine McGill University, Montreal, Canada, 2003.


Appendix A. Institutional Animal Care and Use Committee Approval

DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM
TO:Amer Beg,
FROM:Institutional Animal Care & Use Committee
Division of Research Integrity & Compliance
DATE:4/19/2013
PROJECT TITLE:Elucidating the Function of PKC-theta in Alloreactivity and GVHD
FUNDING SOURCE:National Institutes of Health
IACUC PROTOCOL #:R IS00000138
PROTOCOL STATUS:APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 4/18/2013:

Mouse: kB-luciferase (5 weeks and older/both sexes.)90
Mouse: C57BL/6 CD45.1 congenic (5 weeks and older/both sexes.)60
Mouse: NF-kB p50 knock-out (5 weeks and older/both sexes.)90
Mouse: NF-kB cRel knock-out (5 weeks and older/both sexes)90
Mouse: C57BL/6 (5 weeks and older/both sexes)375
Mouse: OT-1 transgenic mice (5 weeks and older/both sexes.)90
Mouse: 129 (5 weeks and older/both sexes.)285
Mouse: 2C transgenic mice (5 weeks and older/both sexes)90
Mouse: BALB/c (5 weeks and older/both sexes)60
Mouse: PKC theta knock-out (5 weeks and older/both sexes.)90

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol. Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.

• All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.
• All costs invoiced to a grant account must be allocable to the purpose of the grant. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.
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