

ABSTRACT

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REGULATION OF CALCIUM SIGNALING
AND CELLULAR LOCALIZATION OF NFAT
IN CD8⁺ ANERGIC T CELLS.

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Anergy is an important mechanism of maintaining peripheral immune tolerance. T cells rendered anergic, are refractory to further stimulation and are characterized by defective proliferation and IL-2 production. I have used a model of *in vivo* anergy induction in murine CD8⁺ T cells to analyze the initial signaling events in anergic T cells. Tolerant T cells displayed reduced PLC γ activation and calcium mobilization, indicating a defect in calcium signaling. This defect could be overcome by facilitating complete release of calcium from the ER. Reduced calcium signaling in CD8⁺ anergic T cells is therefore predominantly regulated by modulations in signaling events upstream of ER store release of calcium. Impaired calcium mobilization correlated with a block in nuclear localization of NFAT1 in anergic cells, upon stimulation. However, I found that stimulation of anergic, but not naïve T cells induced nuclear translocation of NFAT2. This suggested

that NFAT2 is activated preferentially by reduced calcium signaling, and I confirmed this hypothesis by stimulating naïve T cells under conditions of calcium limitation (by addition of the calcium chelator EGTA), or partial calcineurin inhibition. This phenomenon was independent of the phosphorylation activity of the NFAT1 and 2 kinases p38 and JNK, and resultant nuclear exit, as cellular translocation assays using specific kinase and nuclear export inhibitors caused no change in pattern of NFAT1 and NFAT2 nuclear localization. Proliferative and transcriptional changes were also observed under conditions of calcium limitation or sub optimal calcineurin activity. Thus, my work provides new insight into how T cell stimulation conditions might dictate activation of a specific member of the NFAT family and aid in differential transcriptional activation. My results have also led to the hypothesis that NFAT1 and NFAT2 transcriptional activation profiles in anergic T cells are different from responsive T cells. The preferential nuclear accumulation of NFAT2 in anergic T cells might be important for the regulation of genes related to anergy maintenance.

REGULATION OF CALCIUM SIGNALING AND CELLULAR LOCALIZATION OF
NFAT IN CD8⁺ ANERGIC T CELLS.

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

I have been blessed with parents who have let me be independent and make all my decisions, yet stood close by, to catch me if I fell, to hold me when I needed support, and to guide me when I lost sight of my goal. I dedicate this dissertation to my parents, Shanthi and K.V. Srinivasan. All I am is because of them; all I achieve is for them.

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Abbreviations

Ag- Antigen
APC – Antigen processing cell
ARRE-2 – Antigen receptor response element-2
BAPTA- 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CBP – CREB-binding protein
CD- Cluster of differentiation
CK-1 – Casein kinase-1
CRAC- Calcium release activated calcium
CREB – cAMP response element-binding protein
CSA – cyclosporin A
CTLA-4 – Cytotoxic T lymphocyte antigen -4
DAG – Diacyl glycerol
DC – Dendritic cells
DGK α – DAG kinase α
EGTA- Ethylene glycol tetraacetic acid
ELISA – Enzyme linked immunosorbent assay
ER- Endoplasmic reticulum
ERK –Extracellular signal responsive kinase
GEF – Guanine nucleotide exchange factor
GRAIL – Gene related to anergy in lymphocytes
GSK-3 – Glycogen synthase kinase-3
IFN γ – Interferon γ
IL-2 – Interleukin-2
IP₃ – Inositol 1, 4, 5 trisphosphate
ITAM – Immunoreceptor tyrosine based activated motif
ITIM – Immunoreceptor tyrosine based inhibitory motif
LAT- Linker for activation of T cells
MAP kinase – Mitogen activated protein kinase
MHC – Major histocompatibility complex
MKK – MAP kinase kinase
mTOR – mammalian Target of Rapamycin
NES- nuclear export sequence
NFAT- Nuclear factor of activated T cells.
NF- κ B- Nuclear factor- κ B
NHR – NFAT homology region
NLS- Nuclear localization signal
OVA – ovalbumin
PD-1 – Programmed death receptor-1
PI-3K – Phospho inositide – 3 kinase
PIP₂ – Phosphatidyl inositol bisphosphate
PKA – Protein kinase A
PKC – Protein kinase C

PLC γ - PhospholipaseC γ
RAG-2 – Recombination activation gene-2
RSD – rel similarity domain
SEB – Staphylococcus enterotoxinB
SH- Src homology
SMAC- Supra molecular activation complex
SOS- Son of sevenless
TAD – Transactivation domain
TCR – T cell receptor
TNF α – Tumor necrosis factor α
WASP- Wiscott Aldrich syndrome protein

1. Introduction

T lymphocytes are key components of the adaptive immune response. This constitutes the organism's antigen specific responses against both intracellular and extracellular infective agents, following the first wall of defense-innate immunity. T cells recognize a diverse array of antigens targeted to them by specialized antigen presenting cells (APCs). These antigens are peptide fragments processed by the APCs and bound to polymorphic molecules called the major histocompatibility complex molecules (MHC). Recognition and subsequent activation of the antigen specific T cell requires both peptide and MHC binding to the T cell receptor complex and other surface molecules.

1.1 The T cell repertoire

1.1.1 CD4⁺ T cell subset

Approximately 65% of the mature T cells present in the body are CD4⁺ helper T cells. These cells are considered the master regulators of the immune response, as they control B cell and CD8⁺ T cells responses to their respective antigens (adaptive immunity), as well as the macrophage mediated phagocytosis (innate response) (Figure 1.1). CD4⁺ T cells are activated by TCR engagement with MHC class II-peptide complexes. Activated T cells aid in the anamnestic or secondary immune response, by promoting B cell class switching and affinity maturation and by enhancing cytotoxic responses. A number of studies have confirmed the role of CD4⁺ T cells in promoting clonal expansion, and

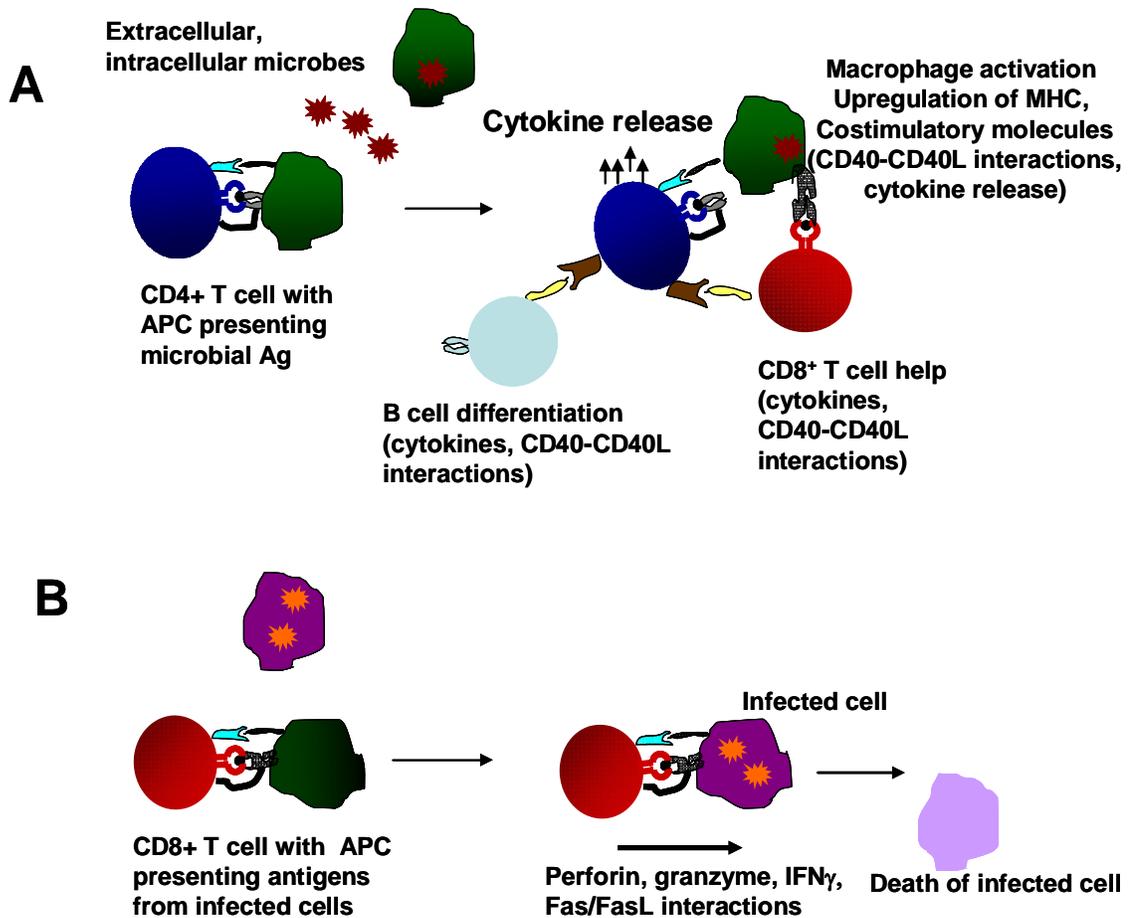


Figure 1.1: The CD4⁺ and CD8⁺ T cell subsets. The T cell repertoire consists of the two subsets of cells, CD4⁺ and CD8⁺ T cells. **A.** Antigens from both intracellular and extracellular microbes are presented to CD4⁺ T cells by antigen presenting cells (APCs) on MHC class II molecules. Activated CD4⁺ T cells perform a number of functions that mediate pathogen clearance. CD4⁺ T cells aid in B cell differentiation and class switching by CD40L/CD40 interactions and also by release of cytokines such as IL-4, IL-5 and IL-13. These cells also mediate maturation of macrophages and enhance their phagocytic capability, by release of cytokines such as IFN γ . CD4⁺ T cells also help in CTL

differentiation and resultant cytotoxicity by means of release of IL2, IFN γ and direct CD40L/CD40 interactions. Upregulation of MHC and costimulatory molecules on activated macrophages also enhances CD8⁺ responses. **B.** CD8⁺ T cells are activated by APCs presenting antigens from infected cells (viral and microbial infection, tumors) on MHC class I molecules. Activated CD8⁺ T cells or cytotoxic T lymphocytes (CTLs) mediate direct killing of infected cells by release of molecules such as granzyme B and perforin that induce apoptosis of infected cells and by release of IFN γ . Apoptosis of infected cells is also mediated by Fas-Fas ligand interactions.

effector functions during a primary CD8⁺ response [1, 2] and an efficient secondary memory recall response [3, 4]. Cytokine production and cell-cell interactions are often used ways by which CD4⁺ T cells communicate with the other immune cells involved in the response. The various CD4⁺ T cell produced cytokines that are employed in immune cell activation include IFN γ , IL-2, IL-4, IL-5 and IL-12. CD40/CD40 ligand interactions between T cells and B cells or T cells [5, 6] are another important activating mechanism.

1.1.2 CD8⁺ T cell subset

The CD8⁺ subset is the immune system's defense against intracellular microbes such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and viruses which invade the cell (Figure 1.1). Presentation of antigens from these organisms or other intracellular viruses is mediated by the proteosomal machinery of the cell. All translation products inside the cell undergo a defined amount of proteosomal degradation and the degraded peptides are presented by the MHC class I molecules to the T cell population circulating in the peripheral lymph nodes. When the cell produces virally encoded proteins, which differ from the host cell, or when the antigens from the microbes residing in organelle compartments of the cell are presented to the CD8⁺ T cells, a productive response ensues. CD8⁺ T cells are also essential components of the immune system's tumor surveillance mechanisms. Tumor cells are transformed cells which might express modified versions of host proteins. Since the TCR specificity for antigens is defined by a very narrow range of residues, any mutations in the amino acid compositions of these self proteins could trigger a T cell response. Graft rejection is the CD8⁺ response to non autologous tissue or organ transplants. Since the MHC molecules themselves are highly polymorphic, MHC-I

molecules present on the surface of the transplanted tissue could act as foreign antigens and cause rejection of the graft.

1.2 Signal transduction during T cell activation

Antigen-MHC recognition by the T cell leads to a cascade of signal transduction events, culminating in the activation of transcription factors, which regulate the expression of different proliferative and effector cytokines and proteins [7]. Signal transduction after T cell engagement with MHC-peptide presenting APCs or with antibodies can be broadly divided into the proximal events that take place immediately after TCR engagement, and the distal signaling processes that are activated as a result of these proximal events.

Signal transduction downstream of T cell receptor stimulation consists of arrays of signaling cascades that intersect with each other at various points to generate a complex network of events that determine the various functional outcomes of T cell stimulation (Figure 1.2).

1.2.1 Proximal signaling events

When the T cell receptor complex is engaged by the MHC antigen complex, the accessory molecules such as the CD4⁺ or CD8⁺ molecules, the costimulatory CD28 molecule, and the T cell surface integrins aggregate around the TCR complex. This forms the supra-molecular activation complex (SMAC) or immunological synapse, which has been proposed to increase the affinity of the interaction and initiates the biochemical events which start the signal transduction cascade. These are defined by a number of trans-phosphorylation events. The first step in the pathway is the phosphorylation of the.

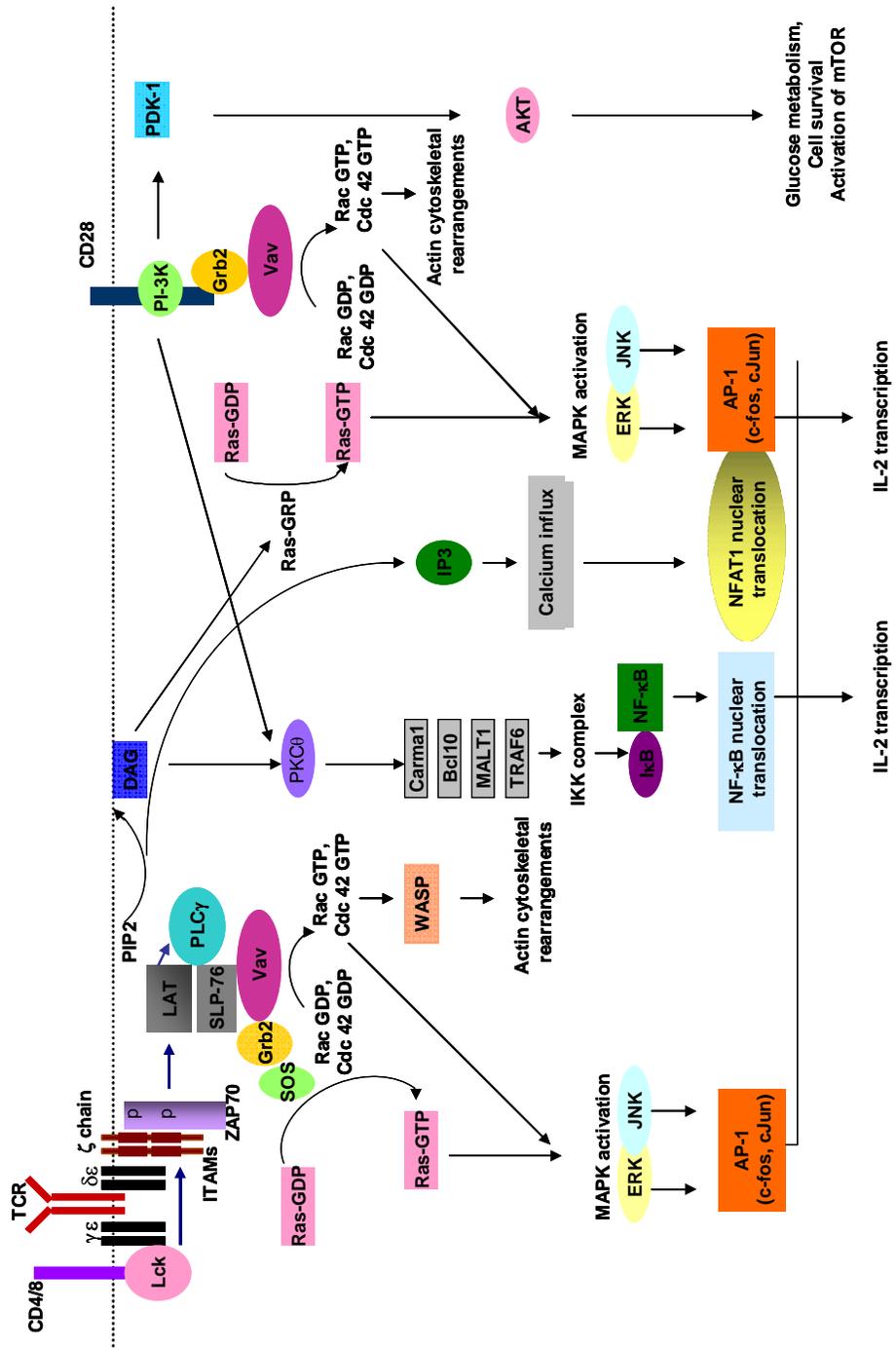


Figure 1.2: Signaling events mediated by T cell stimulation. TCR and CD28

engagement lead to a network of downstream events that lead to transcriptional activation of genes involved in productive immune responses. The MHC-peptide complex interacts with the TCR complex and CD4/8 leading to the phosphorylation of the cytoplasmic ITAMS of the ζ chains of the TCR by Lck, associated with CD4/8. ITAM phosphorylation recruits ZAP-70 that then undergoes phosphorylation. Phosphorylated ZAP-70 mediates the phosphorylation of LAT, which then binds SLP-76. These adaptor proteins, lead to recruitment of effector proteins such as Grb2, Vav and PLC γ . Vav mediates GTP binding of Rac1 and Cdc 42, which upon further interaction with WASP leads to actin cytoskeleton reorganization. Rac1-GTP and Cdc42-GTP also contribute to the activation of the MAP kinase pathway. Grb2, upon LAT binding recruits the GEF SOS, which activates Ras. Ras-GTP also contributes to the MAP kinase pathway. PLC γ is activated by phosphorylation and mediates the conversion of PIP₂ to IP₃ and diacylglycerol (DAG). IP₃ generation is the first step in the signaling cascade leading to the influx of extracellular calcium (see Figure 3). DAG leads to the activation of PKC θ , leading to the recruitment of the Carma1, Bcl-10, MALT1 proteins that further recruit the IKK complex. This complex mediates the degradation of I κ B, freeing the transcription factor NF- κ B and leading to nuclear translocation of NF- κ B. DAG also activates Ras-GRP another GEF that leads to the activation of Ras. CD28 engagement results in the recruitment and activation of the p85 subunit of PI-3K to the membrane, causing Akt activation. Akt has many downstream functions including regulation of glucose metabolism, regulation of cell survival and activation of mTOR. Activated PI-3K also aids in the activation of PKC θ contributing to the activation of NF- κ B. CD28 stimulation

also leads to Grb2 recruitment. The MAP kinase pathway leads to the formation of the transcription factor AP-1. Calcium signaling leads to the nuclear translocation and activation of the NFAT family of transcription factors. NFAT, AP-1 and NF- κ B are the three main transcription factors that are involved in regulation of gene expression following T cell stimulation.

tyrosine residues on the ITAMs of the CD3 and ζ chain polypeptides by the Src family kinase Lck. Lck is bound to the cytosolic tail of the CD4⁺ or CD8⁺ molecules.

Phosphorylation of the ITAMs recruits ZAP70 to the synapse, that then binds to the tyrosines at its SH2 domains [8]. Activation of ZAP70 by phosphorylation results in subsequent phosphorylation of its substrates LAT and SLP76. LAT is a transmembrane protein important for docking of various signaling proteins to lipid rafts formed in the immunological synapse. The LAT and SLP-76 adapter proteins, by their concerted action recruit molecules such as Grb2/Gads, Vav, PI-3K and PLC γ to the plasma membrane [9]. From this signaling node, the various proteins interacting with LAT and SLP-76, initiate signaling events that contribute to the various structural and functional changes that the T cell undergoes when activated.

Phosphorylated SLP-76 promotes the phosphorylation of Vav, which leads to Nck recruitment. The Vav-Nck complex then enables GTP-bound Rac-1 and Cdc42 to bind WASP. WASP then promotes active actin nucleation and cytoskeletal rearrangement. This rearrangement aids in the stabilizing of the immunological synapse and strengthens adhesion. The SH3 domains of Grb/Gads bind to the Ras guanine nucleotide exchange factor (GEF), SOS, which activates the Ras signaling pathway by catalyzing GDP release and subsequent GTP binding. Ras-GTP recruits the serine/threonine kinase Raf that further leads to the activation of the MEK/ERK MAP kinase signaling pathway [10]. PLC γ is activated by phosphorylation, leading to the cleavage of Phosphatidyl inositol biphosphate (PIP₂) which is an integral plasma membrane lipid. PIP₂ is cleaved into the second messenger inositol trisphosphate (IP₃) and membrane component diacylglycerol

(DAG). Signaling events downstream of DAG generation also feed into the MAP kinase pathway.

1.2.2 The MAP kinase signaling pathway

DAG is the membrane integral subunit of PIP2 and is the upstream effector involved in the activation of NF- κ B and AP-1, two transcription factors, which are necessary for complete activation of the T cell [11]. DAG recruits RasGRP1, that functions as a GEF in catalyzing GTP binding and activation of Ras, which leads to a step wise kinase cascade. MEK1, 2 are the MAP kinase kinases involved in activation of the ERK MAP kinase. ERK activation leads to the synthesis of the Fos subunit of the AP-1 heterodimer by activation of the transcription factor Elk-1. p38 MAP kinase is also involved in Fos generation. This protein is activated by kinase activity of MKK3 and 6, a downstream target of the Rac1/Cdc42 pathway that is activated by LAT-Vav interactions. AP-1 is an important transcription factor that regulates essential gene expression after T cell stimulation. AP-1 is a heterodimer consisting of the c-Fos subunit and the c-Jun subunit. The c-Jun subunit of AP-1 is phosphorylated by the JNK MAP kinase. This is activated by MKK 4 and 7, targets of the Rac pathway [12].

DAG is also responsible for the plasma membrane localization of PKC θ . Membrane localized PKC θ recruits the Carma1/Bcl10/MALT1 complex that is essential for activation of the IKK complex that leads to I κ B degradation. In resting cells, NF- κ B is present cytosolically bound to I κ B. This is an inhibitory molecule and prevents nuclear translocation of NF- κ B and resultant activity. DAG and the increase in intra cellular

calcium (described below) together activate the membrane proximal PKC θ molecules which phosphorylate I κ B on specific serine residues [13]. Phosphorylation targets I κ B for ubiquitinylation and proteosomal degradation. This releases the NF- κ B which then moves into the nucleus and contributes to gene transcription.

1.2.3 Costimulation during T cell stimulation

During TCR engagement, a number of accessory molecules interact with the APC and enhance stimulation. The CD28 T cell surface receptor is bound by the B7.1 and B7.2 molecules on the APC. This binding clusters the CD28 molecule into the immunological synapse formed at the T cell-APC interacting surface. Lck, bound to the cytosolic region of CD28, mediates phosphorylation of the tyrosine residues on the cytosolic portion of CD28, leading to important downstream signaling events including recruitment of PI-3K and the Grb-2 adaptor protein. The p85 regulatory subunit of PI-3K binds to CD28 while the p110 catalytic subunit of PI-3K phosphorylates inositol phospholipids to generate PIP2 and PIP3 [14], that induce recruitment of proteins with plekstrin homology (PH) domains such as PDK-1. PDK-1 activates the Akt kinase, which is important in mediating several functions including the regulation of glucose metabolism, inhibiting apoptosis and promoting cell survival by mediating upregulation of anti-apoptotic genes such as Bcl-2, and activating mTOR to aid in protein translocation [15]. PDK-1 also promotes the plasma membrane localization of PKC θ [16] that mediates NF- κ B activation as described above [17]. Activation of PI-3K also helps in recruitment of Rac1

and Cdc42 by SH3 domain dependent binding [18]. This event brings these proteins to the site of their interaction with the guanine nucleotide exchange factor Vav, which is recruited by Grb-2 binding, to the cytoplasmic tail of CD28. These events result in the conversion of the inactive GDP forms of Rac1 and Cdc42 to the active GTP conformation. These interactions further lead to WASP mediated actin cytoskeleton reorganization [19]. Signals downstream of these proteins have also been implicated in p38 and JNK activation and therefore, might contribute to the organization of the transcription factor AP-1. Studies have shown that JNK activity is dependent on CD28 mediated signaling events [20]. The correlation between CD28 mediated signaling and AP-1 activity has also been established by luciferase transcriptional activity assays[21].

Another key interaction downstream of CD28 engagement is the downregulation of cAMP accumulation. cAMP is inhibitory for T cells and has been shown to interfere with T cell activation and proliferation. CD28 stimulation has been shown to induce expression of a cAMP phosphodiesterase PDE7 that decreases levels of cellular cAMP [15, 22].

Costimulation is an integral component of T cell activation, as it is required for complete activation. The absence of costimulation leads to an impaired T cell response that will be described in detail later. Also the presence of inhibitory receptors such as CTLA-4 that bind the same ligands (B7.1, B7.2) as CD28 downregulate T cell activation signals and inhibit cell proliferation and cytokine production[23]. CD28 deficient mice also display defective cytokine production and effector T cell functions [24], underscoring the

importance of CD28 mediated signaling in obtaining an optimal T cell response. Thus the presence of positive a costimulatory signal is essential for complete T cell activation.

1.2.4 Calcium signaling – Intracellular store release

IP₃ that is generated from PLC γ mediated PIP₂ cleavage is an important second messenger molecule that initiates the intracellular influx of calcium into activated T cells. The mobilization of calcium within the cell is a key signaling process which dictates the downstream effectors. The calcium response to stimulation in T cells is a biphasic event divided into the initial intracellular store release of calcium and the subsequent global calcium influx from the plasma membrane channels [25] (Figure 1.3). In T cells, an initial peak in cytosolic calcium levels is followed by a sustained high concentration of calcium lower than the peak value. This sustained increase in calcium levels is essential for the transcriptional regulation which follows [26].

IP₃ is the best characterized second messenger involved in calcium signaling [27]. IP₃ binds to the IP₃ receptor on the surface of the ER, which is an important store for intracellular calcium [28]. The IP₃ receptor is also the channel supporting calcium movement on the ER membrane. The interaction between IP₃ and its receptor causes intracellular store release of calcium into the cytosol. Three subtypes of the receptor have been identified. Using knockdown and mutational studies in the Jurkat T cell lines, the IP₃R1 subtype has been implicated in T cell activation by Jayaraman et al.[29] Upon activation of T cells, the IP₃R1 has been shown to be phosphorylated at specific tyrosine

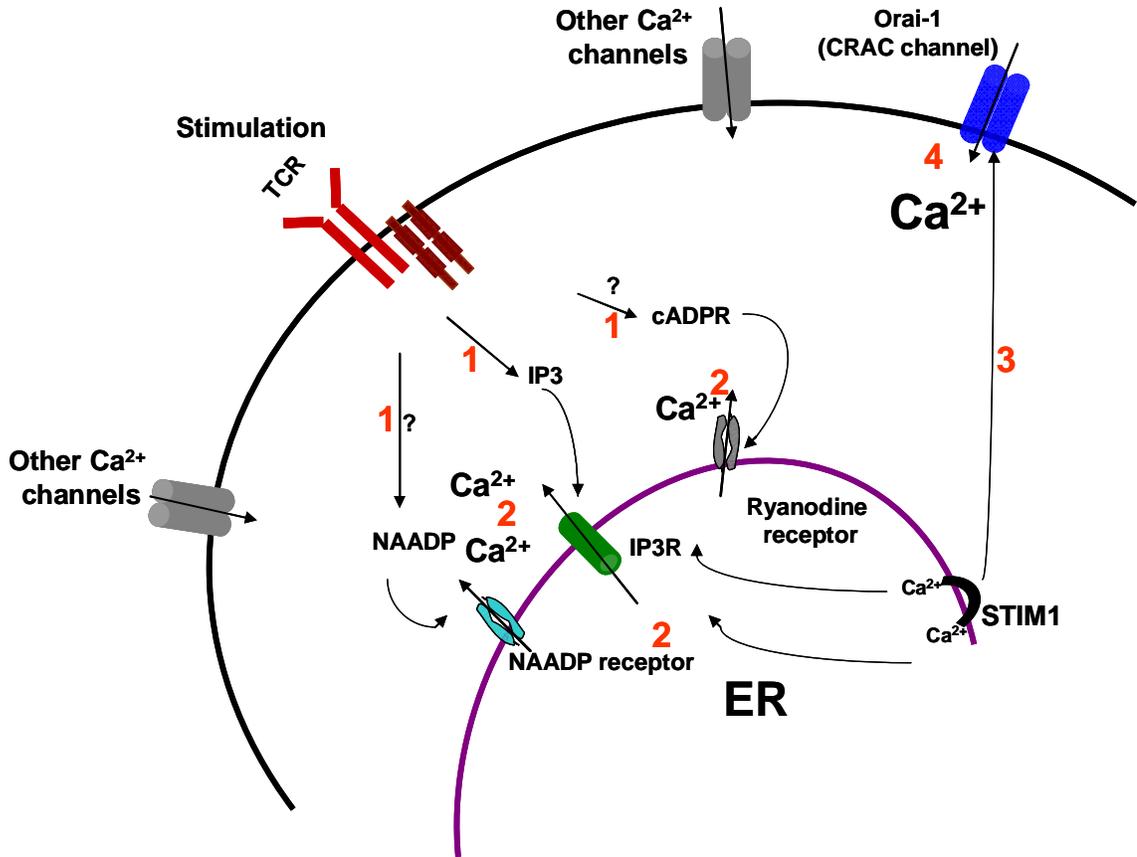


Figure 1.3: Calcium signaling in T cells. Intracellular mobilization of calcium in T cells after activation is a biphasic process, the first being the store release of calcium, leading to the second large scale influx of extracellular calcium. (1) Proximal signals downstream of TCR engagement lead to the generation of IP₃ from PIP₂. IP₃ binds to the IP₃ receptor (IP₃R) on the ER membrane, causing release of calcium from the ER stores (2). Two other second messengers cADPR and NAADP, have been implicated in release of calcium from the intracellular ER stores (2). cADPR binds to the ryanodine receptors on the ER membrane resulting in calcium release. The NAADP receptors have not been identified yet. The TCR related signals (1) that regulate cADPR and NAADP activity, are also not known. STIM1, an ER membrane protein is bound to calcium in the ER, in

resting T cells. Second messenger mediated calcium release from the ER leads to the aggregation of STIM1 molecules in close apposition to the plasma membrane CRAC channels (3). STIM1 migration to the plasma membrane leads to its interaction with Orai-1, a pore subunit of the CRAC channels, leading to the activation of the channels and influx of extracellular calcium (4). Other plasma membrane calcium channels such as the voltage gated calcium channels and the TRPM might also cause calcium influx.

residues [30]. However, the finding that the type I receptor is the predominant receptor expressed in T cells has been challenged over the years by other studies that show calcium signaling in the absence of the receptor type I [31]. It has been suggested that the three receptor subtypes exhibit functional redundancy and the role of the three isoforms still needs to be resolved completely in primary T cells [32].

There has been increasing evidence of at least two other second messengers implicated in calcium signaling [33]. In the Jurkat T cell line, microinjection of cADPR, a cyclic derivative of NAD produced by the catalytic action of cADP ribosyl cyclases [34], induces calcium flux [35], and addition of an antagonist of this molecule abolishes anti-CD3 induced free calcium increase in the cytosol [36]. These results suggested that cADPR could cause ER calcium release. cADPR binds to the ryanodine receptor, another receptor-calcium channel isolated on the ER membranes in T cells [37]. Similar to the IP₃ receptors, the ryanodine receptor is phosphorylated transiently after T cell activation [38].

Another calcium second messenger that has been identified in T cells is NAADP [39]. NAADP synthesis is also catalyzed by the cADP ribose-cyclase at acidic pH in the presence of nicotinic acid by a base exchange reaction using NADP as a substrate [40]. Similar to the cADPR experiments, microinjection of NAADP at nanomolar concentrations induced a calcium response in Jurkat T cells as visualized by single cell microscopy [41]. The ryanodine receptors have been implicated as potential targets for the NAADP ligand [42]. Significantly NAADP is self desensitizing at micromolar concentrations. Experiments where Jurkat T cells were microinjected with these self

desensitizing concentrations of NAADP showed abrogation of any calcium signals when co-injected with IP₃ or cADPR agonists. This shows that NAADP signaling maybe required for the activity of the other two second messengers.

ER store depletion is an essential step in the T cell activation cascade, and facilitates the second phase of calcium signaling. The large scale influx of calcium from the plasma membrane calcium channels is required for the activation of further downstream effectors [43]. Store Operated Calcium channels have been a subject of study for a long time, but the molecular identities of many proteins involved in the capacitative calcium entry directed by store release have only been identified in the past 2-3 years [44]. As determined by the electrophysiological and biophysical properties of the calcium current, the predominant calcium current in Jurkat T cells is the I_{CRAC} [45]. The electrophysiological properties of the CRAC current have been well established and are unique to the channels. CRAC channels display a characteristic I-V relationship with a high selectivity of calcium against monovalent ions such as sodium. The current shows inward rectification and has a dual specificity for the drug 2-aminoethoxydiphenyl borate (2-APB).

There have been a few hypotheses to explain the signal that transpires between the ER and the plasma membrane channels. One hypothesis states that the calcium released from the ER is essential for activation of the surface channels [46]. This has been disproved by a few studies where the presence of cytosolic calcium chelators after thapsigargin induced store release of calcium did not inhibit the opening of the plasma membrane

channels [25, 44]. Thapsigargin also induced calcium influx from the extracellular medium in IP₃ receptor knockout cells, which proves that the second messenger system is just a means of releasing the calcium from the store and is not required for the global phase of calcium signaling [32, 47]. The most prevalent hypothesis is that the emptying of the store calcium provides a signal to the plasma membrane calcium channels, which activates them and results in calcium influxing into the cytosol. This signal could be a soluble factor which translocates from the ER when the stores are emptied and causes SOC channel activation.

Recently, a number of studies using genome wide RNAi screens in the *Drosophila* S2 cells have yielded information about both the ER component that signals store depletion, and the molecular subunits of the CRAC channels. From a large scale suppression of genes that are involved in thapsigargin mediated calcium release, STIM1 was identified as an important gene regulating the SOC current [48]. Further studies identified that STIM1 resides in the endoplasmic reticulum in resting T cells and migrates to the plasma membrane when store depletion takes place [49]. This study also showed that EF hand mutants of STIM1 constitutively activated CRAC channels, providing information about the molecular mechanisms that might be involved in STIM 1 mediated SOC activation. The conserved calcium binding EF hand motif found in STIM1 is found inside the lumen of the ER, where STIM1 monitors the internal calcium concentration of the ER. The EF hand mutants constitutively localize to the plasma membrane, suggesting that store depletion leads to a downregulation of STIM1-calcium interaction that signals movement of STIM1 from the ER into the plasma membrane [50]. Later studies have identified that

STIM1 does not necessarily integrate into the plasma membrane. In resting cells, STIM1 has a diffused ER localization, while store depletion causes STIM1 to move into puncta or aggregates on the surface of the ER that is in close apposition with the plasma membrane. This localization has been analyzed by electron microscopy and has been found to be close enough for direct interactions with the plasma membrane or other transmembrane proteins integrated into the PM [51]. The direct correlation between STIM1 movement and activation of CRAC channels has been shown by Luik et al. who showed that STIM1 clustering near/at the plasma membrane preceded the large influx of calcium current [52].

Adopting a similar approach of genome wide RNAi screens, two other studies have identified another molecule that is involved in mediating the CRAC current. Vig et al. [53] identified two genes, that when suppressed with specific interfering RNA, inhibited store operated calcium current. These molecules were termed CRACM1 and CRACM2 (CRAC modulators), and a secondary patch clamping screen confirmed that these proteins were essential for normal CRAC current. The plasma membrane localization and the transmembrane domains present on these proteins also indicated that they either formed a part of the actual CRAC channel or regulate CRAC activity directly at the plasma membrane level.

Another study performed by Feske et al. [54] arrived at similar results. Using the high throughput RNAi screen in *Drosophila*, in parallel with a positional cloning approach, this study also identified the CRACM proteins as involved in the activation of the CRAC

channels. The CRACM1 protein was designated Orai1. Previously, this group had studied the signaling alterations in human peripheral T cells obtained from two patients suffering from severe combined immune deficiency (SCID) and had discovered that these T cells displayed grossly impaired intracellular calcium mobilization and resultant NFAT translocation and activity [55, 56]. The calcium defect in these cells were identified to be after ER release and therefore the mutation in these patients corresponded to a protein/proteins involved in the second phase of calcium influx. The mutation was tracked to Orai1 by complementation and sequencing the endogenous Orai1.

Further studies have identified that Orai1 and STIM1 interact with each other [57] and that the STIM1 puncta formed near the plasma membrane colocalize with Orai1 on the plasma membrane [51]. These two proteins have now been shown to traffic to the immunological synapse during T cell activation [58], leading to the hypothesis that proximal signaling after TCR engagement might be involved in the regulation of the CRAC channel assembly and function. Mutational studies have also established that Orai1 is the functional pore subunit of the CRAC channel.

There is still a lot of work to be done in characterizing the T cell CRAC channel. Although Orai1 and possibly STIM1 form part of the channel, there could be other possible channel components that are yet to be identified. Also, although STIM1 movement into localized puncta and interaction with Orai1 has been identified to activate the CRAC channel, there could be other signaling processes that synergize with this event. The mechanism by which STIM1 goes from a diffused state to its plasma

membrane proximal clustering state after store release is also at present unresolved. The contribution of the actin cytoskeleton in establishing the physical interaction between STIM1 and Orai1 could be a potential model. WAVE2, a Rac1 effector molecule has been shown to be required for actin reorganization and β -integrin mediated adhesion in activated T cells [59]. This protein has also been shown to regulate store operated calcium influx and could potentially act as a functional scaffold to maintain Orai1 and STIM1 interactions and the integrity of the CRAC channel.

Although CRAC channel mediated calcium influx and the discovery of the Orai and STIM proteins have taken centre stage in the study of calcium signaling in T cells, there have been studies on other type of calcium channels such as voltage gated calcium channels and the TRPM channels. T cells have been shown to express both the regulatory $\beta 4$ and pore forming Cav1 $\alpha 1$ subunits of the voltage gated calcium channels. The $\beta 4$ mutant T cells display impaired cytokine production. IFN γ and IL-4 expression were significantly downregulated, while IL-2 expression was moderately decreased [60]. The TRPM channels have also been shown to regulate calcium entry into T cells. The TRPM4 channel gets activated at the peak of a calcium oscillation leading to the depolarization of the membrane, reducing the drive for calcium entry. This activates voltage dependent K⁺ currents (Kv1.3) that repolarize the membrane potential, and reestablishes the drive for CRAC mediated calcium entry [61]. Thus the various calcium channels that are expressed in T cells could play a collective role in maintaining calcium oscillations and integrating the calcium signal that leads to downstream signaling events.

1.2.5 NFAT signaling

A critical downstream component of calcium signaling in T cells is the activation of the NFAT transcription factor family. Calcium, which enters the cell through the plasma membrane CRAC channels, binds to the EF hands of calmodulin. This leads to the activation of several calcium/calmodulin- dependent proteins including the phosphatase calcineurin. Calcineurin is involved in the systematic dephosphorylation of 20-21 serine residues on the NFAT proteins that are found in the cytosols of resting T cells. The dephosphorylation events mediated by calcineurin uncover the nuclear localization signal (NLS) on NFAT, which results in the nuclear translocation of NFAT. Nuclear NFAT then undergoes further activation and interacts with a multitude of binding partners such as AP-1, T-bet and GATA-3 which result in the coordinate induction of a variety of genes that enable productive immune responses and other regulatory mechanisms. NFAT regulates transcription of a wide range of genes, including the positive regulation of genes associated with a Th1 response such as IL-2, IFN γ and TNF α , genes such as IL-4 that are required for a Th2 mediated immune response, and also in negative regulatory genes such as Cbl-b and other ubiquitin ligases, which control the strength of activation of the T cell.

1.3 The NFAT family

Originally, NFAT was identified as the inducible nuclear factor which bound the antigen receptor response element-2 (ARRE-2) of the human IL-2 promoter. Although initially considered a single protein, it has now been identified that the NFAT family of transcription factors consists of multiple members that are differentially expressed in a

multitude of cell types and tissues [62]. Presently, five members of this family have been identified and have been termed NFAT1 (NFATp, NFAT-c2), NFAT2 (NFATc, NFAT-c1), NFAT3 (NFAT-c4), NFAT4 (NFATx, NFAT-c3) and NFAT5 (TonEBP). Of these, NFAT5 or TonEBP appears to have special characteristics and is involved in the regulation of cellular responses to osmotic stress. The various members of the NFAT family have been identified by their sequence similarity in the DNA binding rel-like homology domain. Additionally NFAT 1-4 have been defined as the classical members of this transcription factor family due to their shared ability to be activated by calcineurin mediated dephosphorylation, and to bind to AP-1, an important cooperating transcription factor for T cell activation.

These 4 members of the NFAT family have a highly conserved (~65%) Rel homology domain, and also comparably bind AP-1 proteins which are necessary for the transcriptional regulation of genes involved in immune activation such as IL-2 [63].

These proteins have all been shown to bind the distal ARRE-2 site on the IL-2 promoter in vitro and therefore all have the capability of regulating IL-2 transcription [62]. NFAT binding sites have been found in promoter sequences of a multitude of immune response genes such as IL-2, IL-4, GM-CSF, IFN γ , TNF- α and Foxp3 [64], and the number is a constantly growing one as more and more genes that are regulated by NFAT activation are being discovered. A systematic study to determine the individual family members that bind to gene specific promoter leading to transcriptional activation has not yet been undertaken. An important consideration when studying NFAT-mediated transcriptional activation is the composite interactions with other transcription factors, which enable

transcriptional activation or repression. Each of the NFAT family members might possess differential specificity for these cooperating proteins, leading to variability in gene expression. The similarities and differences of regulation of NFAT cellular localization and transcriptional activation, and also NFAT mediated regulation of transcriptional activity will be discussed in the relevant sections in this report.

1.4 NFAT mutants

Specific mutations of each of the isoforms of the NFAT proteins have helped to further characterize the functional similarities and differences between the different members of the NFAT family. Studies using knockout mice for each of the NFAT proteins have provided information about the distinct roles that these proteins play in physiology of various mammalian cell types during embryonic development. NFAT1 knockout mice display aberrant skeletal muscle growth and inhibition of chondrogenesis. NFAT2 knockout mice have shown defects in heart valve development and abnormalities in the cardiac septum. NFAT3 knockout mice are found to have defective formation of primary myofibers, that are the first multinucleated muscle cells. NFAT3 and NFAT4 double mutants have impaired organization of blood vessels. Thus these proteins play critical roles in the organization of different parenchymal tissue during embryonic development [65].

NFAT1 and NFAT2 are the two important NFAT family members expressed in peripheral T cells. Since these proteins regulate gene expression of an array of genes involved in various forms of immune response, knockout studies have yielded

information about the functional specificity of these family members and have helped in identifying redundant and non-redundant functions for these related proteins. Hodge et al.[66] generated NFAT1 knockout mice to look at functional phenotype in T cells. Thymocyte development was unimpaired in the knockout mice. NFAT1 knockout T cells displayed hyperproliferation and the cytokine profiles of these T cells after TCR stimulation was different from wild type cells. IL-4 production at early time points after stimulation was decreased in the mutant cells, while IL-2 expression remained unaltered. Paradoxically, IL-4 expression and Th2 development was increased at later time points, implying that initial early expression of IL-4 might be maintained by NFAT1, but other factors independently regulate IL-4 expression during sustained T cell activation. Unaltered IL-2 expression could probably be explained by the presence of other factors involved in transcriptional regulation of IL-2. Hyperproliferation of T cells in the absence of NFAT1 might mean that NFAT1 is important for the expression of genes which inhibit T cell activation.

NFAT2 knockouts are embryonically lethal and die by embryonic day 14.5 due to congestive heart failure. Therefore, mice with homozygous NFAT2 knockout cells were generated using a blastocyst complementation technique with NFAT2^{-/-} embryonic stem cells [67]. NFAT2 knockout T cells displayed impaired proliferation, but that did not correlate to a decreased production of IL-2. IL-2 levels produced by mutant cells were comparable to wildtype cells; therefore the proliferative defect appeared to be an intrinsic property of the absence of NFAT2. During an ongoing immune response, the absence of NFAT2 resulted in decreased production of Th2 cytokines such as IL-4 and IL-6, but

there was no difference in the expression levels of Th1 cytokines such as IFN γ and TNF α . Thus NFAT2 appears to be important for the establishment of a Th2 mediated immune response, and did not seem to have much of an effect on the expression of IL-2 or the other Th1 cytokines [67].

Peng et al. also investigated T lymphocytic properties which were doubly deficient in NFAT1 and NFAT2 [68]. Double knockout T cells were generated by fetal liver chimerization in irradiated RAG-2 deficient mice. The proliferative ability of the T cells was not markedly diminished, and these cells appeared to be spontaneously activated as seen by upregulation of activation markers. The double knockout T cells were severely impaired in the production of both Th1 and Th2 cytokines, reiterating the single knock out studies that NFAT1 and NFAT2 play important roles in determining the effector phenotype of the T cells [68].

These knockout studies have provided valuable information regarding the regulation of T cell phenotype after activation and the involvement of the NFAT proteins in the maintenance of T cell activation and immune response. NFAT1 therefore, appears to be the relevant transcription factor for the production of a Th1 mediated response, while NFAT2 skews the response toward a Th2 bias. Spontaneous activation of T cells in the absence of these factors implies that NFAT proteins are not only involved in the establishment of T cell functions, but also in the inhibition of T cell activity. Also, these studies reveal that these proteins have significant non-redundant functions and are important in establishing a balance in the manifestation of effector functions of T cells.

1.5 NFAT – domain structure

The classical members of NFAT (NFAT1-4) are all made up of three functional domains, the Rel-similarity domain (RSD), which is important for DNA binding activity and also for cooperative binding with other transcription factors, the regulatory domain or the NFAT homology region (NHR), and the transactivation domain (TAD) [62] (Figure 1.4).

The Rel Similarity domain has been named so due to its distant sequence similarity (~20%) with the Rel DNA binding domains of NF- κ B proteins. This domain is highly conserved (60-70%) between the various members of the NFAT family conferring some amount of overlap of DNA binding and gene regulation. NFAT binds with DNA in the major groove of the 5' half site of the conserved NFAT recognition sequence 5'GGAAAA 3'. The protein has another DNA binding site of about 20 amino acids referred to as the Rel-insert region (RIR) which contacts the 3' half site at the minor groove. These two interactions stabilize the DNA-NFAT contact and enable NFAT to bind promoter DNA as monomers, which NF- κ B is unable to do. The NFAT consensus recognition sequence is conserved in all the conventional members of the NFAT family, which explains the ability of all these proteins to bind the ARRE-2 site of the IL-2 promoter, at least in vitro. This domain is also required for the binding of NFAT to other transcription factor partners such as AP-1. The cooperative binding between these two proteins synergistically increases the affinity of the individual proteins for the DNA. AP-1 interacts with NFAT through hydrogen bonding and electrostatic interactions that constitute a weak interaction strengthened by the addition of the DNA.

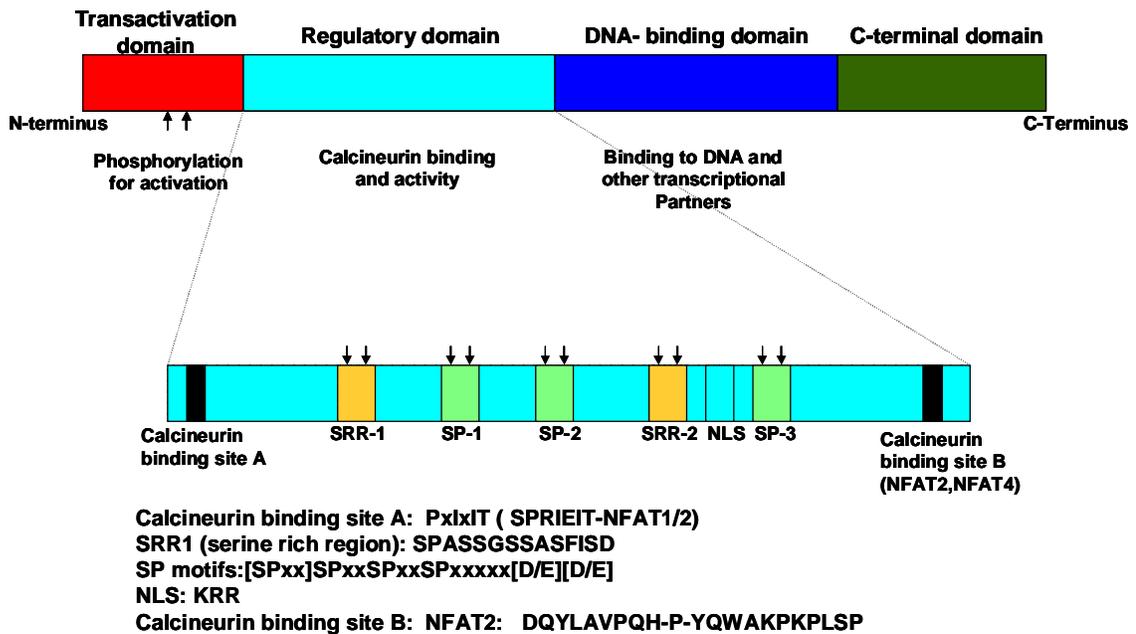


Figure 1.4: Domain Structure of NFAT proteins. NFAT proteins have three functionally important domains, the N terminal transactivation domain (TAD), the regulatory domain or the NFAT homology region (NHR) and the DNA binding domain or the Rel similarity domain (RSD). Specific serine residues in the N terminal TAD undergo phosphorylation reactions that are required for NFAT activation. The RSD is involved in binding to promoter/enhancer sequences of genes that are regulated by NFAT. Protein-protein interactions with other transcription factors such as AP-1 also take place in the DNA binding domain. The regulatory domain has calcineurin docking sites, and also has the serine residues that are dephosphorylated by calcineurin to result in nuclear translocation. Calcineurin binding site A (PxIxIT) is highly conserved among all the members of the NFAT family and is found toward the N terminal end of the domain.

The second calcineurin docking site is found only in NFAT2 and NFAT4 proteins. The regulatory domain also consists of serine rich sections that are termed SRR1 and SRR2 and three serine-proline repeat motifs (SPxx motifs). Calcineurin mediates dephosphorylation of 18-21 serine residues across these sections. The NLS or the nuclear localization sequence is also present in the regulatory domain. In the conformation of NFAT found in resting cells, the NLS is masked, but calcineurin mediated dephosphorylation results in the uncovering of the NLS.

The NFAT homology domain or the regulatory domain is about 320 amino acids long and is found toward the amino terminal end of the NFAT protein. This domain consists of regions that have a number of serine residues that are dephosphorylated by calcineurin. This domain also contains the calcineurin docking site (PxIxIT) which is highly conserved between all the NFAT family members. All the variable residues in the PxIxIT motif are made up of polar residues. In NFAT1 and NFAT2, the sequence is SPRIEIT, and in NFAT3, this sequence is CPSIQIT, and in NFAT4 it is CPSIRIT. Mutation of these polar residues to alanine decreased calcineurin-NFAT binding, which underscores the importance of this particular motif sequence for calcineurin- NFAT interactions [69]. This site is called Calcineurin binding site A and is present in all the members.

In NFAT2 and 4, the C terminal region of the NFAT homology domain contains another binding site called calcineurin binding site B [70]. This region is distinct from the conserved PxIxIT arrangement and has been found to be necessary for calcineurin interaction with NFAT2 and 4 in vivo. The peptide stretch corresponding to the second calcineurin binding region in NFAT1 did not mediate calcineurin binding in vitro. This provides evidence for an important regulatory event that is unique to NFAT2 and not NFAT1 that might prove important in defining the cellular and functional roles that these proteins play.

The regulatory region controls the cellular localization patterns of the NFAT protein by the conserved nuclear localization sequence (NLS) present in the domain. The domain can be broken down into different conserved motifs which are rich in serine residues; the

SRR1 (serine rich region) ¹⁷⁰SPASSGSSASFISD¹⁸³ and three serine proline repeat motifs [SPxx]SPxxSPxxSPxxxxx[D/E][D/E] [71]. The SRR motif is required for the maintenance of the NFAT proteins in the cytosol of resting cells, and deletion of this motif results in constitutively nuclear localization [72]. The NLS sequence is masked when the SRR1 motif is completely phosphorylated, and is uncovered upon calcineurin mediated dephosphorylation of serine residues in the SRR1 peptide region. The unmasking of the NLS then leads to translocation of the NFAT proteins into the nucleus via the cell's importin machinery [73].

The third important functional domain is the transcriptional activation domain. There are two transactivation domains in NFAT, one at the amino terminal end of the protein and the other at the carboxy terminal end. The N-terminal transactivation domain has been described as being more important in determining gene regulation. This domain is highly acidic, which is a hallmark of transactivation domains, and renders itself highly amenable to phosphorylation. This domain has a number of serine and threonine residues that are phosphorylated by a host of proteins leading to transcriptional activation

1.6 Calcineurin mediated dephosphorylation of NFAT

NFAT signaling in T cells is initiated by the dephosphorylation events mediated by the Ca⁺²/CaM-dependent serine/threonine phosphatase calcineurin [74]. Calcineurin consists of two functional domains, the catalytic calcineurin A (CnA) and the regulatory calcineurin B (CnB). The catalytic domain binds to the conserved calcineurin binding

site on NFAT proteins [75]. The calcium/calmodulin complex activates calcineurin by the displacement of an autoregulatory peptide that is found near its catalytic site [62]. The individual members of NFAT bind calcineurin with low affinity ($K_d = 10\text{-}30\mu\text{M}^{-1}$) needed to maintain sensitivity to activation signals and prevent constitutive binding and resultant dephosphorylation [69].

Calcineurin activity is downregulated by a host of cellular and pharmacological inhibitors which therefore interfere with NFAT dephosphorylation. The AKAP79 scaffold protein binds to calcineurin and prevents access to substrates such as NFAT [76]. Cain/Cabin 1 directly binds to calcineurin and inhibits enzymatic activity [77]. The calcineurin B homolog CHB binds calcineurin A but is unable to induce phosphatase activity. The MCIP1, 2 and 3 proteins, also termed DSCR proteins, are also able to block calcineurin activity. Thus, there are a number of negative regulatory proteins that can inhibit calcineurin mediated NFAT dephosphorylation, and that could therefore contribute to the overall regulation of cellular localization and activation of NFAT. The pharmacological inhibitors cyclosporin A (CSA) and FK506 are also widely used to downregulate calcineurin activity. These molecules are used as immunosuppressive agents as they potently inhibit calcineurin mediated dephosphorylation and NFAT transcriptional activity. CSA and FK506 are microbial products that bind to the intracellular proteins cyclophilin and FKBP respectively. These drug-protein composite surfaces bind the CnA-CnB complex, preventing substrate access. Various viral and bacterial proteins also downregulate calcineurin activity and prevent immune responses through NFAT transcriptional activation as an immune evasive strategy. Notably, the A238L protein of

the African swine fever virus binds the calcineurin complex much like CSA to prevent activity. Calcineurin is also targeted by pathogens like Trichoderma, Streptomyces and leukemia viruses, underscoring the importance of calcineurin activity and consequent NFAT activation in promoting productive immune responses [78].

1.7 NFAT mediated transcriptional regulation

The importance of the NFAT family of transcription factors can be explained by the vast array of immunologically relevant genes that are regulated by these proteins. An important consideration in studying transcriptional regulation by NFAT is the cooperative interactions between NFAT and other transcription factor proteins that are necessary to mediate gene expression. NFAT/AP-1 interactions have been studied extensively and have been a major focus due to their concerted action in the expression of important cytokine genes that define immune responses. NFAT/AP-1 composite sequences are present on the promoter/enhancer sites of these genes, and the cooperation between NFAT and AP-1 is required to stabilize DNA-protein complex and initiate transcription. AP-1 is composed of c-Fos and c-Jun heterodimers, Jun homodimers can also act as NFAT binding partners. The amino terminal region of the Rel homology domain of NFAT interacts with the leucine zipper regions of Fos and Jun to achieve cooperative association on the promoter DNA site [63].

Macian et al. [79] used an NFAT1 mutant protein that could not cooperate with AP-1 to identify genes that are dependent on NFAT/AP-1 cooperativity. IL-2 has four to five NFAT binding sites, and three of these are composite NFAT/AP-1 binding regions [80].

The distal NFAT site on the IL-2 promoter at -280 bp from the transcription start site (TSS) is an NFAT and AP-1 composite site, as is the proximal sequence at -135 bp [81]. GM-CSF is another gene with three NFAT/AP-1 cooperative sites. IL-3 is another gene that is regulated by NFAT and AP-1, although there exists an upstream enhancer site that contains NFAT/Oct binding elements. The requirement of other cooperative factors along with NFAT/AP-1 binding is a recurrent theme for genes such as IL-4, IL-5 and IFN γ . IL-4 has five NFAT binding sites, with three of them being NFAT/AP-1 composite sites, while NFAT/GATA3 binding sites are also found on the distal enhancer of IL-4. Cooperation between NFAT and other proteins such as c-Maf and NIP45 has also been reported. IFN γ , an important Th1 cytokine, has NFAT/AP-1 cooperative site and also has sites for cooperation between NFAT and T-Bet. Other genes that have been shown to contain NFAT/AP1 sites are FasL, MIP1 α , CD25 and Cox2, genes induced during T cell activation. In contrast, Macian et al, found that the expression of TNF α appears to be independent of NFAT/AP-1 cooperation [79]. The NFAT recognition sequence found on the TNF α promoter might accommodate NFAT dimers, or other NFAT binding partners might dictate expression. Interestingly, NFAT2 expression in T cells has been shown to be regulated by NFAT1 activity [82]. Based on this report, NFAT2 has been termed the inducible member of the NFAT family. It is not known if NFAT2 promoter sequences have AP-1 composite binding sites in them.

Among the genes that are upregulated independently of AP-1 are genes that are required for the regulation of the anergic phenotype. Macian et al.[83] studied the gene expression patterns in cells that were treated with ionomycin, which induces a large influx of

calcium and promotes calcium mediated downstream events, without inducing other signaling pathways activated by TCR and CD28 stimulation. NFAT nuclear translocation and activation in these cells resulted in the upregulation of genes such as Cbl-b, GRAIL and DGK α , which program the cells for future anergy or non-responsiveness.

Other significant NFAT cooperative factors such as Smad3 [84], and Foxp3 [85], implicate NFAT in the establishment of the T regulatory phenotype. Tone et al. [86] have identified NFAT and Smad3 binding sites in the enhancer regions of the Foxp3 gene. NFAT and Smad3 binding to these sites appears to promote histone acetylation in the enhancer region and positively regulate gene expression. Foxp3 binds regions in the regulatory domain of NFAT, at some regions that overlap with AP-1 cooperation. Foxp3 has also been found to bind AP-1 consensus sequences on the ARRE-2 site of the IL-2 promoter, and therefore appears to repress IL-2 production by forming cooperative complexes with NFAT and DNA, in active competition with NFAT/AP-1/DNA complexes. NFAT/Foxp3 cooperativity has been shown to positively regulate expression of CTLA-4 and CD25, proteins that are highly expressed by regulatory T cells [85]. Similar to Foxp3, the inducible cAMP early repressor (ICER) protein acts as a negative regulator of transcription of cytokines such as IL-2, IL-4 and GM-CSF. ICER/ NFAT complexes have been shown to bind NFAT/AP-1 composite sites on the promoters of these genes in human thymocytes [87]. p21SNFT is a leucine zipper protein that binds NFAT and Jun, and represses IL-2 production by competing with Fos and preventing the formation of functional AP-1 complexes [88].

The above examples provide an expanding number of genes that are regulated, either positively or negatively, by NFAT, based on its cooperating partner protein. Expression of genes such as IL-2 appears to be both induced and repressed by NFAT binding to promoter DNA, depending on the context of activation and the corresponding binding partner. The various signaling interplays that result from T cell engagement under different contexts are relayed to the NFAT proteins that, by their specific binding interactions dictate the phenotype of the responding T cells during a productive immune response as well as during a tolerant response [89].

1.8 T cell tolerance

The T cell receptor (TCR) is the product of somatic recombination of the V and the J germline gene segments in the alpha subunit and the V, D and J regions in the beta subunit. The enormous diversity of the TCRs is the result of the random combinations of these gene segments, where each combination leads to particular antigen specificity. VDJ recombination takes place in the thymus during development, and at this point the population consists of thymocytes specific for both foreign as well as self peptides. The TCR does not have the innate ability to distinguish between harmless self antigens and potentially inflammatory foreign ones. This reactivity and specificity is regulated by a series of processes together called tolerance. T cell tolerance is the failure to mount a response against an antigenic stimulus. Tolerance can be elicited by a number of mechanisms, the main aim of which is to prevent T cells from reacting to self antigens and causing unwarranted immunopathology. The T cell repertoire is subjected to tolerance at different levels, central and peripheral tolerance.

Central tolerance is the selection process that takes place in the thymus after the antigen receptor has been expressed on the surface of the thymocyte. Self proteins are circulated through the thymic blood flow or expressed by cells in the thymus and these are presented to the lymphocytes by the resident APCs. When there is a high affinity interaction between the TCR and MHC/antigen complexes, these antigen specific T cells undergo clonal deletion, as they correspond to self reactive T cells. This is called negative selection.

Though negative selection should theoretically delete all the self reactive T cells, there is still a percentage of mature peripheral T cells which are either autoreactive or are specific to harmless environmental antigens, such as food antigens. T cell responses mounted against these could lead to potential autoimmune disorders or unwarranted immunopathology. Peripheral tolerance mechanisms make sure these T cells are eliminated, or functionally inactivated.

The establishment of tolerance toward tissue antigens is particularly important for CD8⁺ T cells, due to the presence of MHC class I molecules on all nucleated cells. Even with efficient negative selection and removal of self-reactive T cells from the immunological pool, there is a necessity for mechanisms of peripheral tolerance to take place. Along with potentially pathogenic antigens, the immune system is being constantly exposed to a plethora of innocuous foreign proteins, such as food or other environmental antigens. These can be cross-presented on MHC class I by dendritic cells (DC) and macrophages, and might lead to unwanted immune responses. Peripheral tolerance of naïve CD8⁺ T

cells prevents uncontrolled immune responses toward these antigens. Furthermore, some self-antigens might not be expressed in the thymus at levels required for efficient negative selection, or have sub-optimal avidity towards antigen-specific T cell receptors (TCRs). If these conditions fall under the threshold required for deletion through negative selection, then the T cells mature and form part of the peripheral population [90].

Peripheral tolerance can be achieved by multiple means and can be classified into three main categories: clonal ignorance, death by deletion, and functional unresponsiveness. Low avidity self-antigens, which fail to initiate negative selection, are the most likely candidates for clonal ignorance. The TCR/MHC-Ag affinity requirement for normal peripheral T cell activation is higher than what results in thymic deletion [91]. Low avidity antigens, therefore, might never induce peripheral immune activation, and the population of Ag-specific naïve T cells could potentially remain untouched. Peripheral ignorance can also be achieved when the antigens are restricted to immune privileged sites, such as across the blood-brain barrier or across the fetal-maternal barrier [91]. However, this form of tolerance is not permanent, as there are situations where the antigen could be presented in the right kind of stimulatory conditions and might cause unwarranted immune responses. Alternatively, in conditions where there is uncontrolled expression of these self-antigens in concentrations much higher than normal, such as in tumor cells, the clonally ignorant T cells might become autoreactive.

1.8.1 Anergy in T cells

An important mechanism of peripheral tolerance in naïve T cells is the induction of anergy. Anergy is a form of functional unresponsiveness, believed to occur when a T cell has been subjected to MHC-Ag interaction in the absence of costimulation. An anergic T cell is rendered refractory to further stimulation, even in the presence of full costimulation [92]. Using this definition, many studies have been undertaken to elucidate the functional requirements for inducing anergy, and also the biochemical changes that are seen in anergic T cells. The hallmarks of anergic cells are defects in proliferation and IL-2 production, but other effector functions show variable (or sometimes no) reduction. Various models, both in vitro and in vivo, have been used to induce anergy in T cells. TCR engagement, either by agonist peptides in the absence of costimulation, or with partial agonist peptides in the presence of costimulation, was one of the first methods that was used to anergize T cells [93]. Anergy in T cells can also be achieved by stimulation with plate bound anti-CD3 antibodies [94] or by treating T cells with the calcium ionophore ionomycin [83]. The blockade of costimulation using CTLA-4 Ig during T cell stimulation using agonist peptides and splenic APCs is another common approach [95]. In vivo, T cells have been anergized by the administration of superantigens, which tolerize a portion of the V β T cell pool [96]. Administration of single or multiple doses of TCR-specific peptides to TCR Tg mice, or adoptively transferring these Tg T cells into mice that express the agonist peptide as self-antigen, are other favored techniques that results T cell hyporesponsiveness. Though the above models of induction of anergy all result in the corresponding T cells becoming refractory to further antigenic stimulation, the cellular mechanisms of anergy induction and the manifestation of the anergic

phenotype differ in the various models, prompting a more comprehensive study of the models of anergy to define the functional state of non-responsiveness.

Inhibition of cell cycle progression has been studied as a potential contributor to the induction of anergy. It has been shown that T cells from mice that lack p27^{KIP1}, a cell cycle progression inhibitor, are resistant to anergy [97]. p27^{KIP1} regulates tolerance by preventing the phosphorylation of Smad3, leading to the upregulation of the Cdk inhibitor p15, that results in blockade of cell cycle progression [98]. In contrast, A.E7 T cells that have been stimulated with anti-CD3 and anti-CD28 in the presence of sanglifehrin A, a cell cycle progression inhibitor, promoted cell cycle arrest, but did not program the T cells to become refractory to further stimulation [99]. Further studies with this model have identified that rapamycin, an inhibitor of mTOR, causes anergy in both A.E7 and primary mouse T cells when stimulated in the presence of both signal 1 (TCR engagement) and signal 2 (costimulation). mTOR integrates metabolic environmental cues, such as glucose and amino acid availability to dictate cell growth and proliferation. Therefore, the discovery that inhibition of mTOR by rapamycin can lead to anergy is interesting as it alludes to mTOR function as an integrator of costimulatory cues to direct T cells toward anergy or productive activation. Thus, cell cycle arrest might be a consequence rather than a cause of T cell anergy [100].

Anergy induction has been studied in great depth using a multitude of experimental methods of induction. Though these methods are all experimentally different from each other, an integrative paradigm for T cell hyporesponsiveness involves incomplete T cell

activation. Stimulation of the TCR (signal 1) in the absence of costimulation (signal 2), leads to a refractory state in T cells, and a blockade of cytokine expression (Figure 1.5). Initially, CD28 interaction with the B7 molecules on APCs was primarily considered as costimulation, but recent studies have spotlighted a number of both positive and inhibitory receptors that contribute to costimulation. Importantly, inhibitory molecules such as PD-1 and CTLA-4 have been shown to inhibit productive activation. The recent report by Zheng et al. [100] implicates mTOR as an integrator of costimulatory cues, both positive and negative, to determine the phenotype of the stimulated cell. Thus, if the signals from negative costimulatory molecules are more abundant than positive signals downstream of the surface receptors, then the cell may be programmed toward anergy.

The in vivo models represent the physiological conditions that potentially cause anergy. Therefore, they contribute toward better understanding of the intracellular and intercellular stimuli that can be manipulated therapeutically in using T cell anergy in treatment. One such area where mechanisms of T cell anergy or hyporesponsiveness could be used in treatment is in immunosuppressive regimens during allotransplantation. Cyclosporin and rapamycin are clinically used immune suppressive drugs. In vivo mouse models of T cell anergy, that use these drugs, or study molecules associated with these drugs, aid in better understanding of the molecular mechanisms that are targeted during active immune suppression.

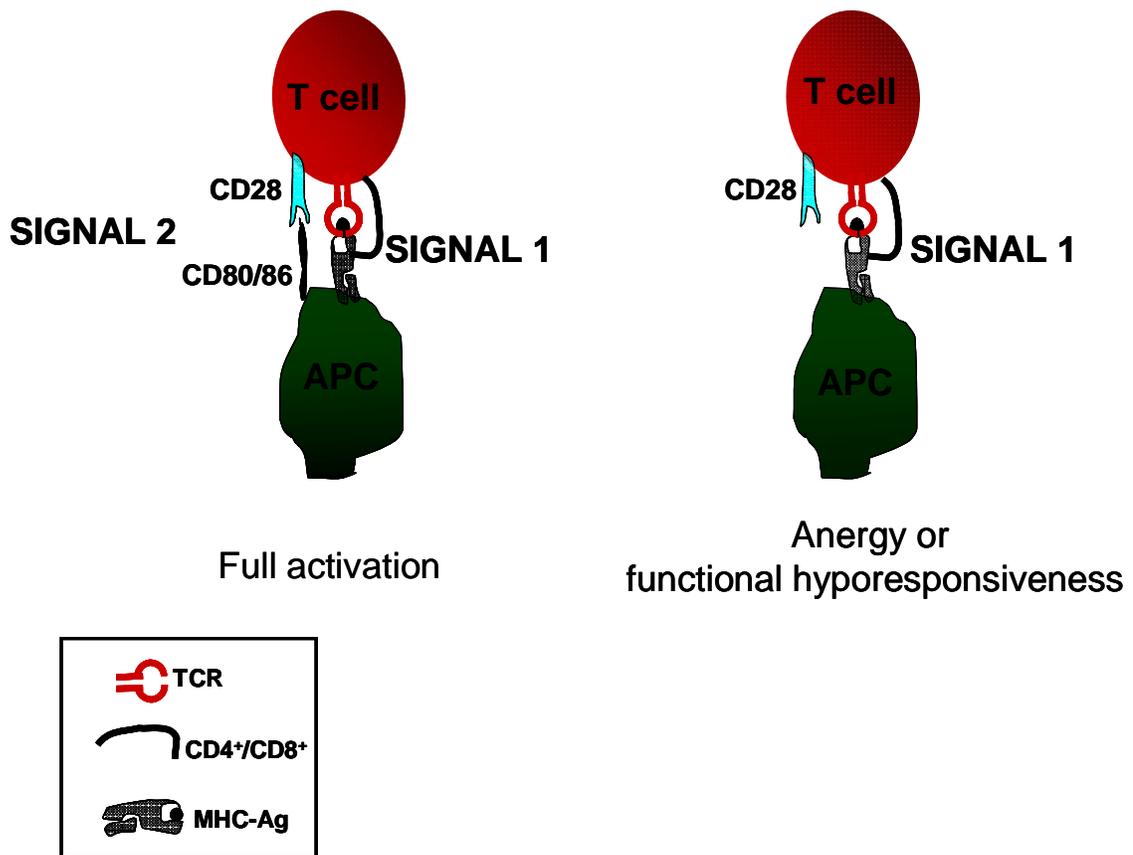


Figure 1.5: Two signal model of anergy induction. Productive activation of T cells required two signals. Signal 1 consist of MHC-peptide interactions with the TCR leading to downstream signaling events, while signal 2 refers to costimulation mediated by CD28 interactions with CD80/86 molecules on the surface of the antigen presenting cell (APC). T cell stimulation in the presence of signal 1 alone induces anergy or functional hyporesponsiveness. Anergic T cells are refractory to further stimulation.

1.8.2 Transcriptional activation during anergy induction

The conclusion that anergy is a programmed state of T cell differentiation, rather than just a blockade of a traditional productive immune response, is strengthened by results from a number of gene array studies that have determined the transcriptional pattern of genes during anergy induction in comparison with naïve and activated T cells [83, 101, 102]. A CD4⁺ in vivo anergy model, of TCR Tg T cells transferred into mice expressing the specific antigen constitutively, was used to study the gene expression patterns of anergic cells in comparison with naïve T cells and activated T cells [102]. 1176 candidate genes from gene array analysis of naïve, anergic and activated T cells, were considered and a distinct transcriptional pattern was identified for the anergic T cells. Among the genes that were differentially regulated were costimulatory molecules, proteins involved in signal transduction, and proteins involved in cell cycle progression. Notably, inhibitory surface receptors, such as PD-1 and CTLA-4, showed higher expression in anergic T cells than their naïve counterparts. Fyn, a protein kinase involved in proximal signaling after TCR engagement, that has been shown to be upregulated in certain models of anergy, shows upregulated expression as well. The expression of the cell cycle inhibitor p27^{KIP1} is also significantly upregulated in anergic T cells, providing support for the argument that cell cycle arrest is an important contributor to the anergic phenotype.

Macian et al. [83] studied the gene expression patterns of CD4⁺ T cells that were anergized by ionomycin treatment and found significant differences in the transcriptional patterns of these cells in comparison with cells that were activated with PMA+ ionomycin (complete signal). Using Affymetrix gene arrays, they have identified genes

such as Ikaros, DAGK α , CD98, FasL, 4-1BB-L as being upregulated in anergic T cells. Some of these molecules have been further studied to determine their role in anergy maintenance and definitive roles have emerged to explain the signaling alterations that have been seen in anergic T cells.

An important family of proteins that have been identified by these genetic screens of anergic cells is the E3 ubiquitin ligase family [103]. The upregulation of expression of ubiquitin ligases such as Cbl-B, GRAIL and Itch in anergic T cells has prompted the study of their role in the maintenance of the hyporesponsive phenotype. E3 ubiquitin ligases catalyze the transfer of ubiquitin to proteins that are then targeted by the proteosomal machinery to be degraded. Thus the E3 ligases might abrogate signaling pathways by targeting specific proteins for proteosomal degradation. Monoubiquitination mediated by the E3 ligases also serves another molecular purpose. Attachment of a single ubiquitin molecule to proteins alters the cellular trafficking patterns of proteins, with the ubiquitinated protein targeted to lysosomes. Thus the E3 ubiquitin ligase enzymes might negatively regulate signaling machinery by interfering with cellular localization of specific proteins [104].

GRAIL (gene related to anergy in lymphocytes) is one such E3 ubiquitin ligase that is upregulated in anergic T cells [105], and the forced expression of GRAIL in naïve T cells has been shown to induce anergy in these cells [106]. The molecular targets of GRAIL in anergic T cells have not been identified yet, but it has been seen that GRAIL colocalizes with the transferrin receptor in anergic T cells and that intact endocytic

trafficking is required for GRAIL mediated negative regulation of IL-2 expression [105]. Heissmeyer et al. [107] have shown that Cbl-b, Itch, and Nedd4 expression is upregulated in T cells anergized with ionomycin. These proteins were shown to bind to both PLC γ and PKC θ to target them for ubiquitination and subsequent degradation. This leads to the breakdown of the calcium signaling cascade and the PKC θ dependent MAP kinase pathway and NF- κ B activation, leading to hyporesponsiveness. Using Cbl-b knockout mice, Jeon et al. [108] have confirmed the role of Cbl-b in mediating the degradation of PLC γ in in vitro anergized T cells. T cells from the knockout mice were also resistant to in vivo induced anergy, both in a peptide injection and in a SEB administration model, although PLC γ degradation was not measured in the in vivo models.

Yet another gene chip analysis of anergic A.E7 T cells has identified the upregulation of expression of the transcription factors Egr-2 and Egr-3 [109]. Overexpression of these proteins resulted in upregulation of Cbl-b and conversely, Cbl-b levels were detectably lower in Egr knock out T cells. These transcription factors might therefore act in the positive regulation of expression of anergy related genes such as Cbl-b and therefore direct the anergic phenotype.

The Gajewski group has performed similar gene expression studies on anergic T cells and has identified that DGK α (diacyl glycerol kinase α) expression is upregulated in anergic CD8⁺ T cells [110], similar to the Macian study. DGK α phosphorylates DAG, generating phosphatidic acid and reducing the availability of DAG for further downstream signaling. Transduction of naïve T cells with adenovirus expressing DGK α caused the cells to

display an anergic phenotype. These cells produced decreased amounts of IL-2 in response to restimulation. DGK α expression also resulted in diminished recruitment of RasGRP1 to the plasma membrane. RasGRP1 has been identified as an important Ras guanine nucleotide exchange factor (GEF) that activates Ras signaling in activated T cells. The diminished membrane localization correlates with impaired Ras signaling that has been seen in anergic T cells. Conversely, reversal of anergy was seen in anergic T cells treated with a pharmacological inhibitor of DGK α . The causal role of DGK α in regulating the anergic phenotype was corroborated in a study using DGK α knockout mice in CD4⁺ anergy models [111]. DGK α deficient cells were resistant to anergy induction both in vitro and in vivo. Thus, inactivation of the Ras/MAPK pathway by DGK α appears to be an inhibitory mechanism used for anergy in both CD4⁺ and CD8⁺ T cells.

1.8.3 The role of NFAT in anergy induction

The requirement of signal 1, or TCR engagement, for the establishment of anergy suggests that signaling events downstream of TCR stimulation might be necessary for the expression of the anergy genes. Indeed, calcium signaling and NFAT activation has been shown to be indispensable for the upregulation of these genes. Using an ionomycin induced anergy model, Macian et al.[83] established the role of NFAT1 in the regulation of T cell anergy (Figure 1.6). The upregulation of the anergy related genes was abrogated in the presence of cyclosporin A, which inhibits calcineurin activity. In correlation with the in vitro data, NFAT1 knock out T cells were resistant to anergy induction and did not display upregulation of the appropriate anergy genes. The apparent paradox of

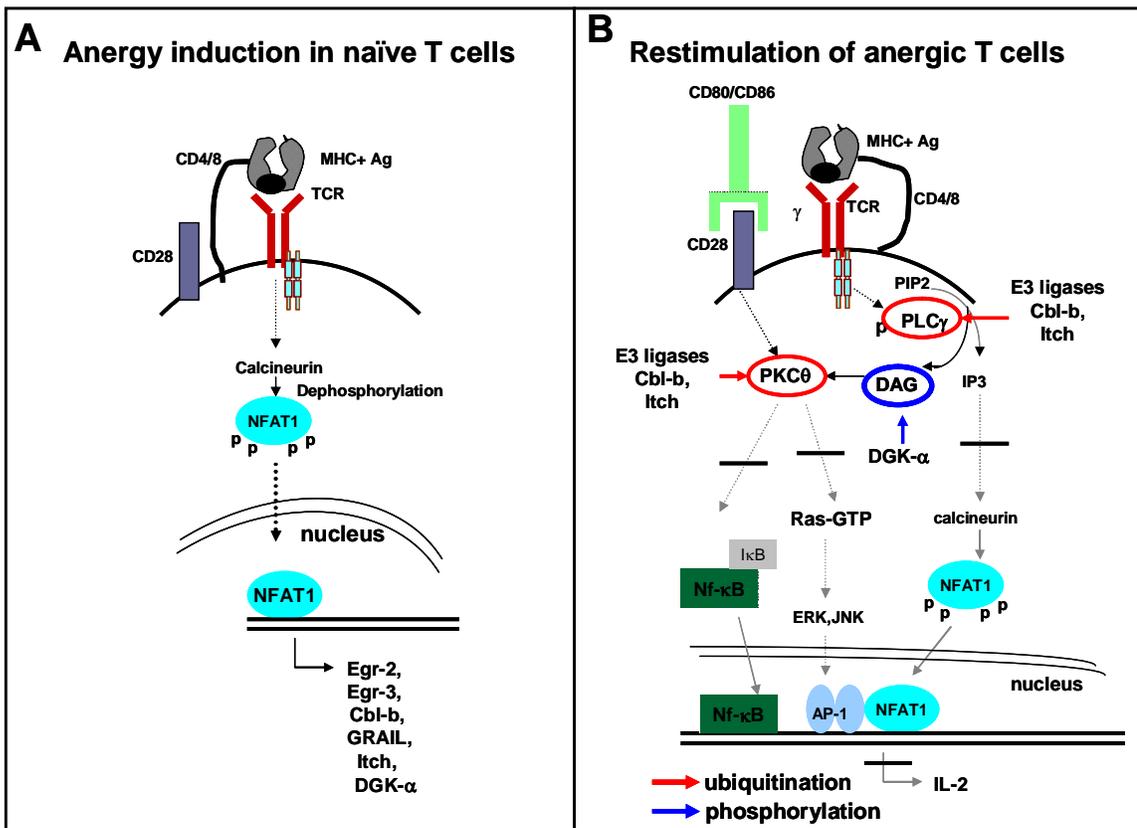


Figure 1.6: Transcriptional activation during anergy induction. T cell stimulation in the presence of signal 1 (TCR engagement) with no signal 2 (costimulation) leads to anergy induction. Signals downstream of TCR ligation result in the nuclear translocation and activation of the transcription factor NFAT1. NFAT1 binds to promoter/enhancer sequences of genes such as Egr2, Egr3, GRAIL, Cbl-b, Itch and DGK- α and upregulates transcription. When anergic T cells are subjected to complete stimulation (signal1+2), the E3 ligases Cbl-b and Itch mediate ubiquitination of substrates such as PLC γ and PKC θ . Polyubiquitination of proteins leads to subsequent proteosomal degradation, while monoubiquitination leads to alteration in cellular trafficking patterns of the proteins.

PLC γ and PKC θ have important functions during T cell activation leading to NFAT and NF- κ B mediated transcriptional activation. DGK- α is a kinase that phosphorylates diacylglycerol to form phosphatidic acid. This depletion in DAG concentrations in T cells leads to impaired Ras signaling and MAP kinase activation, as well as reduced NF- κ B activation. MAP kinase activity is required for the formation of the transcription factor AP-1. Thus the proteins, whose expression is upregulated during anergy induction, lead to the attenuation of T cell activation signals during restimulation, yielding a net result of decreased proliferation and IL-2 production.

transcription factor associated with promoting a productive immune response being necessary to induce a hyporesponsive phenotype was solved by the discovery that AP-1 cooperation is absent in T cells stimulated under anergizing conditions. Macian et al. demonstrated this elegantly, using an engineered NFAT1 derivative incapable of associating with AP-1. Cyclosporin treatment also abrogated the proteosomal degradation of PLC γ and PKC θ that were seen in anergic T cells [107].

Thus, NFAT1 appears to play a pivotal role in both transcriptional regulation of genes involved in a productive immune response and in establishing T cell non-responsiveness. This dual and antagonistic functionality of NFAT is achieved by differential association with binding partners. Thus AP-1 is indispensable for expression of genes such as IL-2, IFN γ , IL-4 and granzyme, while transcriptional activity of genes such as Cbl-b, Egr2, Egr3 and DGK α require only NFAT binding to promoter/enhancer sequences. Although, experimental evidence has been acquired only for NFAT1 requirement in anergy induction, other members of the NFAT family such as NFAT2 and NFAT 4, might also be involved. Also, NFAT could potentially cooperate with transcriptional partners other than AP-1 in regulating gene expression of the distinct set of genes expressed during anergy.

2. Methods and Materials

2.1 Antibodies and reagents

Anti-CD3 (mAb 145-2C11) and anti-CD28 (mAb 37.51) antibodies, control hamster IgG, PE-labeled anti-Thy1.2, anti-CD8 and anti-CD44 antibodies were purchased from eBioscience (San Diego, CA). Goat anti-hamster IgG was purchased from Pierce (Rockford, IL). Anti-NFAT1, anti-NFAT2 antibodies were from Affinity Bioreagents (Golden, CO). Anti-PLC γ , anti-phospho- PLC γ (Y783), anti-ERK1, anti-phospho-ERK1 (E10), and anti-c-Fos antibodies were from Cell Signaling Technology (Danvers, MA). Anti- α tubulin antibody was from Sigma- Aldrich (St.Louis, MO). Anti-c-Jun, anti-phospho c-Jun (KM-1) and anti-actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). HRP-conjugated anti-mouse IgG and anti-rabbit IgG were from Bio-Rad (Richmond, CA). The H-2K^b restricted 2C TCR- reactive peptide SIYRYYGL was purchased from NeoMPS (San Diego, CA). JNK inhibitor (SP600125) and the p38 inhibitor (SB203580) were purchased from BIOMOL International, L.P, (Plymouth Meeting, PA). Leptomycin B was purchased from Sigma-Aldrich.

2.2 Animals

2C TCR-Tg/RAG2^{-/-} mice express the 2C $\alpha\beta$ T cell receptor specific for the L^d MHC class I receptor [112] and were maintained on a mixed 129/C57BL/6 background. C57BL/6J mice (6-8 weeks old) were purchased from The Jackson laboratory (Bar

Harbor, ME). All mice were maintained in ventilated M.I.C.E. microisolator cages (Animal Care Systems, Littleton, CO) in the University of Maryland animal facility (College Park, MD). Animals received humane care in compliance with the “Guide for the Care and Use of laboratory Animals” published from the National Institute of Health (Bethesda, MD).

2.3 In vivo anergy induction

Mice were injected intraperitoneally (i.p.) with 25-50 nmol of the 2C peptide (SIYRYYGL) in sterile PBS or PBS alone. For single injections, spleens were harvested seven days after administration. For double injections, mice were injected on day 1 and day 5 and spleens were harvested on day 12. T cells were purified and tested for proliferation and cytokine secretion.

2.4 Cell culture

All cells were maintained in RPMI1640 medium (Mediatech, Herndon, VA) supplemented with 10% FBS (Hyclone, Logan UT), 2mM glutamine, penicillin/streptomycin, 10mM HEPES buffer, MEM non-essential amino acids, and 55 μ M 2-mercaptoethanol at 37°C in a 5% CO₂ atmosphere.

2.5 T cell purification

Murine T cells were purified from the spleens using the SpinSep or EasySep negative-selection system (Stem Cell Technologies (Vancouver, British Columbia, Canada)).

Spleens were harvested from mice and macerated in PBS+2% FBS. For some experiments, the brachial, inguinal and mesenteric lymph nodes were also extracted for purification and combined with spleens. The cell suspension was then filtered through a nylon mesh and centrifuged at 1500 rpm for 5 minutes at room temperature and the pellet after centrifugation was resuspended at a concentration of 100×10^6 cells/ml in PBS + 2% FBS + 5% rat serum. Appropriate volumes of T cell enrichment cocktail were added to the cell suspension as per manufacturer's protocol and incubated for 15 minutes at 4°C. The enrichment cocktail contains a combination of biotinylated monoclonal antibodies against cell surface antigens on mouse cells of hematopoietic cells that are not of T cell origin (CD11b, CD19, CD45R, CD49b, and TER119). The cells were then incubated with Biotin selection cocktail for 15 minutes at 4°C. The Biotin cocktail contains two mouse antibodies against biotin and dextran respectively. These two antibodies are held in a tetrameric antibody complex by a rat anti mouse IgG1. The cells were then incubated for another 15 minutes at 4°C with a suspension of magnetic dextran iron particles that can bind to the biotin selection cocktail. The T cells were then purified from this suspension by centrifugation over a density medium (SpinSep) or by using magnetic separation of the unwanted magnetically labeled non-T cells (EasySep).

2.6 Estimation of T cell purity by flow cytometry

100µl of the enriched T cell suspension was incubated with 1µl of anti-Thy1.2-PE (20µg/ml) or 1µl of Rat IgG-PE, which was used as isotype control. After a 15-30 minute incubation at room temperature, 300µl of FACS buffer (1X PBS+1% FBS) was added to

each sample. The samples were then read for PE fluorescence on a FACScalibur flow cytometer. A successful T cell purification yielded ~90% pure T cells.

2.7 Estimation of CD44 surface expression

Anergic T cells and the control T cells from PBS injected mice were analyzed for CD44 surface expression levels. 100µl of purified T cells were incubated with either 1µl of anti-CD44-PE (200µg/ml) or 1µl of Rat IgG-PE. After incubation at room temperature for 15-30 minutes and PE fluorescence was measured using the flow cytometer.

2.8 Proliferation and cytokine assays using peptide and APC stimulation

T cell proliferation was measured by stimulating the T cells with TCR specific 2C peptide and splenic cells that function as APCs. 2C peptide was resuspended in RPMI at a concentration of 400nM and titrated down the rows of a 96 well plate. The final concentration of 2C peptide antigen ranged from 200nM to 0.05nM. The last row contained no peptide and served as a control. Spleen cells were obtained as described above, and resuspended in RPMI at a concentration of 4×10^6 cells/ml. 50µl of splenic cells (APCs) were added to each row of the 96 well plates. The purified T cells were resuspended at a concentration of 1×10^6 cells/ml. 50µl of T cells (either from a PBS injected or a peptide injected mouse) was added to all the rows of the plate. The assay was done in triplicates for each peptide concentration. The cells were incubated at 37°C in a 5% CO₂ atmosphere. Proliferation after 72 hours of stimulation in vitro was determined by [³H] thymidine incorporation. Cells were pulsed for the last 6-8h with 1

$\mu\text{Ci/well}$ of [^3H] thymidine (MP Biomedicals, Solon, OH) and incubated at 37°C . The cells were then transferred to glass fiber filters with a 96-well cell harvester (Tomtec, Hamden, CT), and analyzed by liquid scintillation using a 1450 Microbeta Trilux scintillation counter (Wallac, Turku, Finland). The counts per minute (c.p.m.s) obtained for each well were then averaged for the triplicate samples and plotted against peptide concentrations to obtain a proliferation curve. Standard deviations were also determined for each sample using standard calculations.

Cytokine assays were organized in parallel to proliferation assays. $500\mu\text{l}$ of peptide (400nM), $250\mu\text{l}$ of splenic cells ($4 \times 10^6/\text{ml}$) and $250\mu\text{l}$ of purified T cells (1×10^6 cells/ml) were incubated together at 37°C in a 5% CO_2 atmosphere for 24-36 hrs. The supernatants from these stimulations were stored and cytokine concentrations were analyzed by ELISA.

2.8.1 Proliferation assays using splenic cells

Splenic cells were isolated from mice as described above. The spleen consists of both T cells and other antigen presenting cells such as dendritic cells that provide costimulatory cues for T cell activation. The cells were resuspended in complete RPMI with added EGTA ($600\mu\text{M}$) or CSA (300nM). The proliferation assay set up was similar to the one described above using 2C peptide and spleen cells. Anti-CD3 antibody was titrated across the rows of a 96 well plate instead of the 2C antigenic peptide. Anti-CD3 concentrations ranged from 6.4ng/ml to $1\mu\text{g/ml}$ and were serially titrated down the rows. $100\mu\text{l}$ of 2.5×10^6 spleen cells were added to each well and incubated at 37°C for 72 hours. The cells were pulsed with [^3H] thymidine at a concentration of 1mCi/well for the last 8

hours of incubation. The proliferative ability of the cells were then measured as the c.p.m generated by the radioactive thymidine integrated into the DNA of the dividing cells and measured as described above.

2.9 Enzyme linked immunosorbent assay (ELISA)

IL-2 and IFN- γ levels in 24 or 36 hour stimulation supernatants were determined using a sandwich ELISA. Primary and biotinylated secondary anti-cytokine antibodies and recombinant cytokine standards were purchased from eBioscience (San Diego, CA) and used at the concentrations recommended by the manufacturer (Table 1). Alkaline phosphatase-conjugated avidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and used at a 1:3000 dilution. Colorimetric alkaline phosphatase substrate was purchased from Sigma-Aldrich (St.Louis, MO) and used at 1 mg/ml in 10% diethanolamine buffer. Quantification was performed on a Versamax spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were analyzed using Softmax Pro software (Molecular Devices). Data points for all analyses are presented as the mean of triplicate wells \pm S.D.

	IL-2	IFNγ
Standards	20ng/ml – 0.3125ng/ml	100ng/ml – 1.5625ng/ml
Primary antibody	1 μ g/ml	2 μ g/ml
Secondary biotinylated antibody	1 μ g/ml	1 μ g/ml

Table1: Concentrations of standards, primary and secondary antibodies used for detection of IL-2 and IFN γ secretion by ELISA.

2.10 T cell stimulation

T cells were stimulated with anti-CD3 and anti-CD28 antibodies. The cells were first incubated with anti-CD3 and anti-CD28 antibodies (10 μ g/ml) on ice for 30 minutes. For unstimulated controls, cells were incubated with Syrian hamster IgG and Armenian hamster IgG control antibodies. 5ml ice cold PBS was added to the samples and centrifuged for 5 minutes at 1500 rpm at 4°C. The supernatant was discarded and the pellets were resuspended in complete RPMI. The samples were then stimulated for appropriate time points with goat anti-hamster IgG as a secondary cross linking antibody (10 μ g/ml) at 37°C. The stimulation was stopped at the different time points by addition of ice cold 1X PBS to the samples and transferring the samples to 4°C. The samples were then centrifuged at 4°C for 5 minutes at 1500 rpm and the pellet used for further analysis.

2.10.1 T cell stimulation with anti-CD3 and anti-CD28 coated beads

For some experiments T cells were stimulated with magnetic beads that were conjugated with anti-CD3 and anti-CD28 antibodies. Purified T cells were mixed with beads in the ratio of 1:3. The beads are magnetic and therefore can be separated from the solvent using a magnet. The beads were washed with 1X PBS and resuspended in RPMI. Once the cells were mixed with the beads, they were centrifuged at 1500 rpm for 5 minutes at 4°C. Without removing the supernatant, the samples were then stimulated at 37°C for appropriate time points. The stimulation was stopped by transferring the samples to 4°C and adding 5ml of ice cold PBS. The cells were then centrifuged again, for 5 minutes, at 1500 rpm at 4°C and the pellet was used for further analysis. Unstimulated samples were

generated by incubating T cells with beads coated with Syrian and Armenian hamster IgG antibodies.

2.10.2 Linkage of anti-CD3 and anti-CD28 antibodies to tosyl activated beads

Anti-CD3 and anti-CD28 antibodies were coupled to tosyl activated magnetic beads as a means of crosslinking the antibodies when they bind to CD3 and CD28 molecules on the T cell surface. Beads or antibody coated beads can be separated from the suspension buffer by magnetic separation. 75µg/ml anti-CD3 and anti-CD28 antibodies were added to 4×10^8 beads/ml and incubated at 37°C for 24 hours in 0.1M borate buffer (pH 9.5). The beads were then washed twice at 4°C with 1X PBS+ 0.1% BSA for 5 minutes each at 4°C and the washed in 0.2% Tris (pH 8.5) with 0.1% BSA for 24 hours at room temperature. The Tris buffer is used to inactivate the unoccupied tosyl groups that have not bound to the antibodies. Antibody coated beads were then separated from the buffer by magnetic separation and resuspended at a concentration of 4×10^7 beads/ml in 1X PBS + 0.1% BSA. 0.02% NaN₃ is added to prevent bacterial contamination. Beads coupled to Syrian and Armenian hamster IgG were generated similarly.

2.11 Western blots

The T cells were stimulated and lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na₃VO₄ in PBS) The lysates were resolved on a 8% or a 12% SDS PAGE gel ($\sim 1.25 \times 10^6$ cells/sample) and transferred onto nitrocellulose membrane. The blots were blocked with 5% non-fat dry milk in PBS/0.1% Tween-20 (PBS-T) and probed with primary antibody (in PBS-T) at specified

concentrations, followed by HRP conjugated anti rabbit and anti mouse IgG respectively. The bands were visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce).

2.12 Measurement of intracellular calcium

The intracellular calcium flux was measured by flow cytometry using the calcium sensitive dyes Fluo3 and Fura Red (Molecular Probes). Fluo-3 fluorescence at 530 nm increases with increasing Ca^{+2} binding, whereas Fura Red fluorescence at 670 nm decreases with increasing Ca^{+2} binding, allowing ratiometric measurement of calcium [113]. Purified T cells were resuspended in RPMI 1640 medium supplemented with 1% FBS at a concentration of 5×10^6 /ml. Cells were stained with 4 μM Fluo3, 10 μM Fura red and co-incubated with 10 $\mu\text{g}/\text{ml}$ anti-CD3 antibody and PE-labeled anti-CD8 for 30 minutes at 30°C. The cells were then washed in RPMI with no additives and centrifuged at room temperature for 5 minutes at 1200 rpm. The pellet was then resuspended in incomplete RPMI at a concentration of 2×10^6 cells/ml. The Fluo3/Fura red fluorescence values were then read on a FACSCalibur flow cytometer (Beckton Dickinson, Mountain View, CA). 300 μl cell suspension was added to 900 μl of prewarmed RPMI and the basal Fluo-3 and Fura Red fluorescence levels were measured for 60 seconds, after which 300 μl of cells were transferred to pre-warmed (37°C) RPMI (900 μl) containing 7.5 $\mu\text{g}/\text{ml}$ goat anti-hamster IgG to crosslink the TCR/CD3 complex. The increase (Fluo3) and decrease (Fura Red) in fluorescence levels were then recorded for at least 500 seconds, collecting approximately 100 events per data point using CellQuest software (Beckton

Dickinson) The relative calcium concentration inside the cell was then plotted as a ratio of Fluo3 to Fura Red emission values using FlowJo software (Treestar, Ashland, OR).

2.12.1 Measurement of calcium in the presence of EGTA

Cells were stained as described above and resuspended in incomplete RPMI at a concentration of 2×10^6 cells/ml. The basal Fluo3 and Fura red fluorescence values were measured for 60 seconds, then the cells were transferred to pre-warmed RPMI that contained 7.5 $\mu\text{g/ml}$ secondary crosslinking antibody and required concentrations of EGTA for the total volume. The fluorescence values after activation were then measured in the presence of EGTA

2.12.2 Measurement of calcium in the presence of thapsigargin

Cells were stained and Fluo3 and Fura red fluorescence levels were determined, as described above. After measuring basal fluorescence, cells were stimulated by the addition of 1 μM thapsigargin, instead of secondary crosslinking antibody. Calcium flux was determined as before.

2.13 Immunofluorescence

T cells were stimulated with anti-CD3 and anti-CD28 antibodies. 1×10^6 cells/sample were incubated with anti-CD3 plus anti-CD28 (10 $\mu\text{g/ml}$) in complete RPMI1640 medium on ice for 30 minutes. Cells were centrifuged at 4°C for 5 minutes at 1500 rpm and the pellet was resuspended in complete RPMI. Cells were stimulated using Goat anti-hamster IgG

(10 μ g/ml) at 37°C for appropriate times. Stimulations were stopped by addition of 5 ml of ice cold PBS. Cells were centrifuged for 5 minutes at 4°C at 1500 rpm and samples were resuspended in 100 μ l of RPMI/BSA (RPMI1640, 10 mM HEPES, pen/strep. 0.6% BSA). Cells were plated on to the poly-L-lysine-coated microscope slides (Polysciences, Warrington, PA) and incubated on ice for 40 minutes. After aspirating the supernatant, the adhered cells were fixed with ice cold methanol for 15 minutes and washed once with ice cold 1X PBS. Cells were then blocked and permeablized with PB buffer (RPMI 1640, 10%FBS, 0.05% saponin, 10 mM glycine, 10 mM HEPES) for 20 minutes. Cells were incubated with anti-NFAT1 or anti-NFAT2 antibody at 2 μ g/ml for one hour at 4°C. Cells were washed three times with PB buffer and incubated with 2 μ g/ml Alexa Fluor 594-linked anti-IgG (Molecular Probes, Eugene, OR) in PB buffer for one hour in the dark. After three washes in PB, cells were incubated in 1 μ M SYTO13 (Molecular Probes, Eugene, OR) in PB buffer for 20 minutes in the dark. Cells were post fixed in 4% paraformaldehyde and coverslips were then fixed on to the slides. The cells were viewed using a LSM 510 microscope (Carl Zeiss Microimaging, Thronwood, NY). For experiments done with specific treatments, cells were incubated with specific concentrations of EGTA, BAPTA, CSA, SP600125 (200 μ g/ml), SB203580 (20 μ g/ml) during the initial 30 minute incubation at 4°C with anti-CD3 and anti-CD28 antibodies. The cells were also maintained in RPMI-BSA containing the appropriate concentrations of the reagents during stimulation at 37°C. For leptomycin treatment experiments, cells were incubated with 200nM leptomycin for 30 minutes at 4°C before addition of anti-CD3 and anti-CD28. Cells were maintained in 200nM leptomycin containing RPMI-BSA during stimulation for appropriate time points.

Quantification of nuclear localization was done using the ImageJ software package from NIH, using the colocalization macro. Two points were considered colocalized on the overlay, if their respective individual intensities were higher than the threshold and if the ratio of intensity is higher than the ratio calculated for the two channels of detection of fluorescence. The relative colocalization quantification for an individual cell was the cumulative score of all its colocalization points. The relative concentration of NFAT in the nucleus was then correlated with the strength of the intensity

2.14 Intracellular staining for phospho c-Jun

Naïve T cells were stimulated for appropriate time points by first incubating them with anti-CD3/anti-CD28 antibodies and then secondary crosslinking antibodies in the presence or absence of varying concentrations of the JNK inhibitor SP600125. After stimulation, each sample of 1×10^6 cells was fixed in 100 μ l of 2% formaldehyde in PBS for 20 minutes at room temperature. The cells were then washed twice with 500 μ l of 0.03% saponin in PBS by centrifuging at 1500 rpm for 5 minutes at 4°C. The pelleted cells were then incubated with primary anti-phospho c-Jun antibody in 100 μ l of 1XPBS containing 10% normal rat serum (NRS) and 0.3% saponin. NRS or normal rat serum functions as a blocking agent, while saponin permeabilizes the cell membrane. After 30 minutes incubation at 4°C, the cells were washed as indicated above and incubated with secondary biotinylated anti-mouse IgG. All antibodies were resuspended in 1X PBS that contained 10% NRS and 0.3% saponin and incubations were for 30 minutes at 4°C. The cells were washed twice by centrifugation, as described above, between every incubation. After incubation with secondary antibody, the cells were incubated with PE conjugated

streptavidin. After another 30 minute incubation, the cells were washed once in PBS + 0.03% saponin and once in FACS buffer (1X PBS + 1% BSA), and resuspended in 300µl FACS buffer. The PE fluorescence that corresponds to phosphorylated c-Jun was then measured by flow cytometry.

2.15 RNA isolation

T cells were stimulated for appropriate times (resting, 3, 8 and 24 hours) with anti-CD3/anti-CD28 conjugated beads. After stimulation the cells were centrifuged at 1500 rpm for 5 minutes at 4°C and the pellet stored at -80°C. RNA from the cell pellets was purified using the Total RNA isolation Nucleospin RNA II kit from Macherey-Nagel (Bethlehem, PA) following manufacturer's instructions. The purified RNA was quantified and 150-200ng of RNA from each sample was used to generate cDNA using reverse transcriptase using the iSCRIPT cDNA synthesis kit from Biorad (Hercules, CA). 150-200ng of RNA from each sample was added to 4µl 5X iScript reaction mix and 1µl iScript reverse transcriptase and DEPC RNase free water to make up a final volume of 20µl. The reverse transcription reaction was performed at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. cDNA was then used in regular PCR using primers specific for IL-2 (forward primer: 5'TGCTCCTTGTC AACAGCG 3', reverse primer: 5'TCATCATCGAATTGGCACTC 3') at a melting temperature of 58°C and 18sRNA (forward primer: 5' ATGCGGCGGCGTTATTCC 3', reverse primer: 5'GCTATCAATCTGTCAATCCTGTCC 3') at a melting temperature of 55°C. Reactions were performed for 35 cycles. The PCR DNA products were then analyzed on 1-2% agarose gels.

2.16 Statistics

All statistical analyses were performed using GraphPad Prism version 5. The minimal level of confidence at which experimental results were considered significant was $p < 0.05$. Statistical significance between proliferation of responsive and anergic T cells or cells stimulated in the presence of various treatments, over concentration of 2C antigenic peptide or concentration of anti-CD3 antibodies was determined by two-way ANOVA followed by the Bonferroni post test analysis. Statistical significance between cytokine production of responsive and anergic T cells, or cells stimulated in the presence of various treatments was determined by the non-parametric two-tailed T test, followed by the Bonferroni post test analysis. Statistical significance between nuclear colocalization of NFAT1 and NFAT2, or between responsive and anergic T cells was determined by two-way ANOVA followed by the Bonferroni post test analysis.

3. In vivo anergized CD8⁺ T cells display impaired calcium mobilization.

3.1 Introduction

Anergy, or T cell hyporesponsiveness, is a mechanism of peripheral tolerance in response to self-antigens that is seen in both CD4⁺ and CD8⁺ T cells. Reduced proliferative capability and decrease in IL-2 production are the hallmarks of an anergic phenotype.

There is significantly less information about the effects of anergy induction on the other effector functions of tolerant T cells. IFN γ production is found to be variable in different models of anergy. In CD8⁺ T cells, the anergic T cells appear to retain some cytolytic ability, even though proliferation is affected [114]. T cell anergy is essentially a two-phase process. Initially, an tolerogenic stimulus results in the programming of the naïve T cells to become anergic, which is then manifested when these T cells are introduced to a secondary stimulus. Incomplete activation of T cells or TCR stimulation in the absence of accompanying positive costimulatory cues is the most prevalent and accepted model of anergy induction [115]. The upregulation of certain genes during TCR stimulation alone, is responsible for the establishment of the hyporesponsiveness to restimulation [83]. The second phase or the establishment of the anergic phenotype is characterized by a number of signaling alterations downstream of TCR engagement. This dichotomy in the induction phase and the manifestation phase has been an area of intensive study, in both CD4⁺ and CD8⁺ T cell models.

Many models have been used to mimic the incomplete activation of naïve T cells in a tolerogenic setting [116]. It has been difficult to categorize these models of anergy into distinct classifications, as the various anergy induction regimes appear to result in slightly different phenotypes. A broad categorization has been attempted by Shwartz et al, wherein the in vitro models of incomplete T cell activation leading to hyporesponsiveness have been termed clonal anergy, while the establishment of a pool of antigen specific anergic T cells in vivo by antigenic persistence leading to desensitization has been termed adaptive tolerance [92]. These include models where TCR transgenic T cells are adoptively transferred into animals that express their cognate antigen endogenously, or when cognate peptide is administered to animals that are transgenic for the peptide-specific TCR. Anergic cells generated in response to either set of tolerizing stimuli, both in vitro and in vivo, display notable signaling alterations in proximal signaling events immediately after TCR engagement, that leads to a distorted immune response [117].

In an adoptive transfer model of anergy induction in T cells, where pigeon cytochrome C (PCC) specific T cells were transferred into mice expressing PCC under the control of a MHC class I promoter, Chiodetti et al. [118] reported a decrease in the tyrosine phosphorylation levels in tolerant T cells that are restimulated in vitro. Specifically, ZAP70 and PLC γ , proteins that are activated immediately downstream of TCR engagement, display impaired phosphorylation. Corresponding to the decreased phosphorylation of PLC γ , tolerant T cells displayed an impaired calcium response. The MAP kinase pathway appeared to be only moderately affected in these cells. In another peptide injection model of in vivo anergy [119] where PCC TCR transgenic mice

were immunized with PCC peptide, impaired phosphorylation of the adaptor protein LAT was the main defect detected. LAT is an adaptor protein that is phosphorylated by ZAP70 and acts as a scaffold for other critical signaling molecules such as PLC γ , Grb2 and PI-3K. Anergized cells displayed reduced localization of LAT to the immune synapse that resulted from a decrease in palmitoylation and phosphorylation. Palmitoylation is required for localization of LAT to the lipid rafts of the immunological synapse, and a decrease in overall palmitoylation was reflected in impaired downstream signaling. Anergic cells also displayed reduced PLC γ phosphorylation. In an in vitro model, T cells treated with the calcium ionophore ionomycin display an unresponsive phenotype to further restimulation [120], and are defective in IL-2 production. This method of anergy induction has been used by a number of studies to identify the signaling alterations in the anergized cells. The signaling alterations described above were also detectable in T cells anergized in vitro using ionomycin [119].

The in vivo administration of the superantigen staphylococcal enterotoxinB (SEB) has also been reported to induce CD4⁺ T cell hyporesponsiveness [96]. SEB administration leads to an initial large scale clonal expansion of the SEB reactive T cells bearing V β 8 containing TCR. This is followed by death of most of the T cells by apoptosis, resulting in a stable pool of anergic T cells. These surviving hyporesponsive T cells display defective phosphorylation of PLC γ and a corresponding defect in intracellular calcium mobilization. The MAP kinase pathway appears to be intact in these anergic T cells, suggesting that superantigen induced anergy was regulated mainly by a blockade of calcium signaling. These results were strengthened by the observation that SEB

administration did not lead to anergy in T cells which expressed a constitutively active form of calcineurin. Zu et al. further identified that anergic T cells generated by SEB administration had increased expression levels of CTLA-4 that might contribute to the anergic phenotype[121].

The above studies demonstrate that anergic T cells display significant defects in proximal signaling events that result in defective calcium signaling spanning both in vitro and in vivo models. There are a number of studies that have found no detectable defects in events that regulate calcium influx, but instead have shown significant MAP kinase and Ras signaling defects in anergic T cells. In vitro anergy induction of A.E7 T cells (CD4⁺ T cell clone) using plate bound anti-TCR β displayed marked decrease in ERK phosphorylation and defective NF- κ B mobilization to the nucleus, while the calcium signal cascade was unaffected [118]. Fields et al.[122] also reported defective ERK signaling corresponding to altered Ras signaling in pGL10 T cells (another CD4⁺ T cell clone), when anergized by plate bound anti-CD3 antibodies. Yet another model of in vitro anergy, where A.E7 cells were tolerized by antigen pulsed splenic APCs, also reported that the anergic cells displayed a decrease in the inducible expression of the components of AP-1, that is regulated by MAP kinase activation [123].

Oral tolerance is a systemic antigen induced T cell anergic state achieved by oral administration of the antigen in tolerizing conditions. This system is highly physiologically relevant, as this is a mechanism by which innocuous food antigens are tolerated by the immune system. The signaling alterations in orally tolerized T cells were

studied in an OVA specific TCR Tg model, where the mice were fed OVA protein as part of the diet [124]. The anergic CD4⁺ T cells obtained from these mice displayed overall decreased tyrosine phosphorylation, compared to naïve T cells. Specifically, ZAP-70, TCR- ζ , LAT and PLC γ were found to be hypo-phosphorylated after in vitro stimulation. This corresponded with impaired calcium mobilization and decreased NFAT1 nuclear localization. The MAP kinase pathway was unaffected. Interestingly, it was found that orally tolerized cells displayed impaired degradation of the cell cycle inhibitor p27^{KIP1}, compared to naïve T cells. T cell anergy induced by oral administration of antigen therefore displays a combination of characteristics of other types of in vitro and in vivo anergy described here.

The above examples illustrate the various signaling defects that have been reported in anergic CD4⁺ T cells (Figure 3.1). CD4⁺ and CD8⁺ T cells share the basic signaling machinery that is activated by stimulation. Also, both peripheral CD4⁺ and CD8⁺ T cells require IL-2 as their cytokine stimulus for proliferation and expansion. However, a strict extrapolation of CD4⁺ based results into the CD8⁺ system might miss CD8⁺-specific phenomena. CD8⁺ T cells are known to have more stringent requirements for activation, and do not appear to be as dependent on CTLA-4 for anergy induction [125].

Nevertheless, results garnered from CD4⁺ trials of anergy provide an important comparison for analyzing features of CD8⁺ T cell anergy. A number of groups have focused specifically on CD8⁺ T cell anergy. Just as the study of the various models of CD4⁺ anergy induction has yielded different combinations of signaling defects, there is

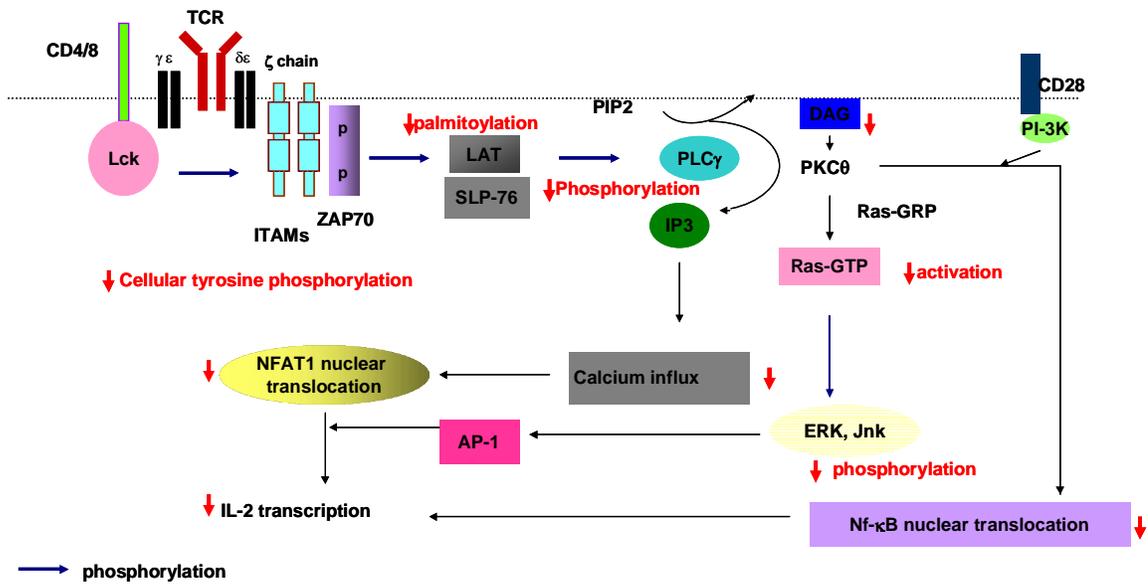


Figure 3.1: Signaling alterations in anergic T cells upon restimulation. Induction of energy in primary murine T cells results in multiple signaling modulations in the cells, upon secondary stimulation, consisting of both signal1 (TCR engagement) and signal2 (positive costimulation). Anergic cells display a decrease in cellular tyrosine phosphorylation levels, specifically, the ITAM motifs in the cytosolic regions of the ζ chain component of the TCR, ZAP-70, the adaptor protein LAT, and PLCγ phosphorylation are affected. Palmitoylation of LAT, required for membrane recruitment is also decreased. Intracellular mobilization of calcium has also been found to be detectably impaired in anergic T cells. NFAT1 nuclear translocation that is mediated by calcium signaling is also impaired in these cells. Decrease in DAG levels leading to impaired Ras signaling has been reported in some models. Defective Ras signaling results in the impaired organization of the transcription factor AP-1 that participates in IL-2

transcription. NF- κ B nuclear translocation mediated by events downstream of PI-3K and PKC θ signaling is also affected in certain models of anergy. This is a composite look at the possible signaling alterations that define an anergic phenotype. As has been described, anergic T cells that have been tolerized by different modes of induction show some or all of these modulations in signal transduction following T cell stimulation.

variability in the signaling changes reported for the various anergy models in CD8⁺ T cells.

Dubois et al. [126] characterized the proliferative and signaling features of anergic T cells using the F5 (influenza NP366-374/D^b) TCR transgenic mice in a RAG knockout background. In vivo anergy was established by peptide injections delivered in the absence of adjuvants. They observed that the anergized cells displayed a partial block in the proximal tyrosine phosphorylation events, including ZAP70 and PLC γ . These cells also displayed reduced calcium flux. The MAP kinase pathway and phosphorylation of important molecules such as ERK was not examined in this model. A study with a similar model of in vivo anergy, using the 2C TCR Tg T cells, found that defective Ras signaling and resultant impaired MAP kinase activation were the main signaling defects displayed by the anergic T cells. The transduction of constitutively active Ras rescued MAP kinase activation and IL-2 production in these cells, preventing them from undergoing anergy [127].

Frauwirth et al. have previously described a model of *in vivo* induction of anergy in CD8⁺ TCR transgenic mice, using intraperitoneal (i.p.) injection of the 2C antigenic peptide in the absence of adjuvants to tolerize the T cells [125]. T cells recovered from peptide injected mice showed reduced proliferation in response to restimulation with 2C antigenic peptide and a corresponding reduction in expression of IL-2. These cells were antigen experienced and had undergone at least one round of cell cycle progression, as seen by upregulation of CD44 surface expression and increased cyclin D and E

expression (K. A. Frauwirth, unpublished observations). I investigated the signaling alterations in these anergic cells upon ex vivo restimulation, with special emphasis on calcium signaling.

3.2 Results

3.2.1 Intraperitoneal administration of 2C peptide induces anergy

2C TCR transgenic mice on a RAG deficient background were used to obtain homogenous pools of transgenic T cells. Mice were injected with 2C antigenic peptide resuspended in sterile PBS. The control animals were injected with sterile PBS alone. Seven days after the final injection, the T cells were purified from the spleens of the test and control animals. CD44 is a T cell surface protein upregulated upon TCR engagement; therefore CD44 surface expression was determined in T cells from peptide injected mice. Surface expression of CD44 was elevated in the T cells from peptide injected mice (Figure 3.2A), confirming that the T cells from peptide injected T cells were antigen experienced and not naïve. T cell anergy has been characterized by reduced proliferation and cell expansion to restimulation. To determine the proliferative ability of the T cells from peptide injected mice, cells from both the test pool and control pool were stimulated in vitro for 72 hours in the presence of splenic cells (APCs) and graded concentrations of TCR specific 2C peptide. Proliferation was determined by the amount of [³H] thymidine present in the cells. T cells from peptide injected mice displayed strongly reduced proliferation compared to the control cells (Figure 3.2 B).

Anergic cells also display an altered cytokine profile compared to completely activated T cells (Figure 3.3). Expression of IL-2, the cytokine that regulates proliferation and clonal

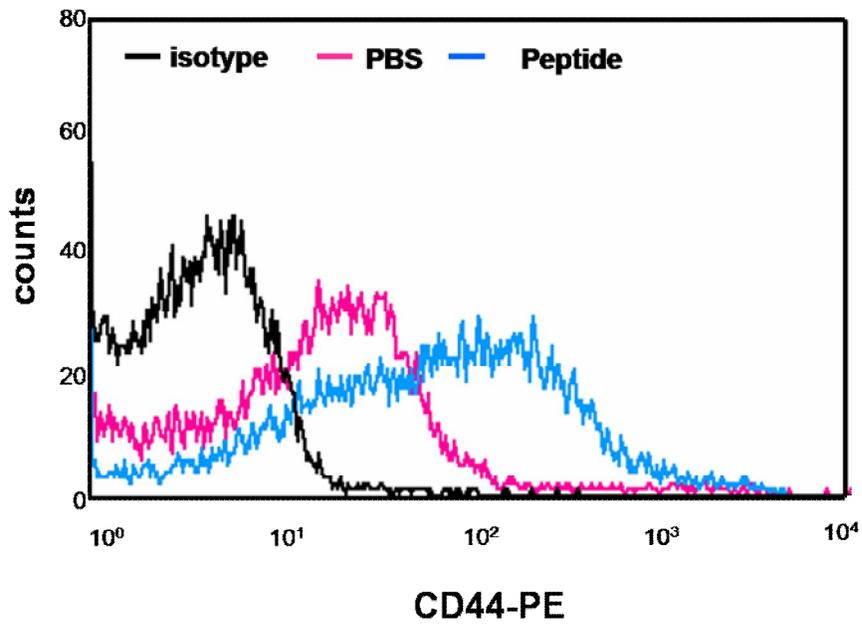
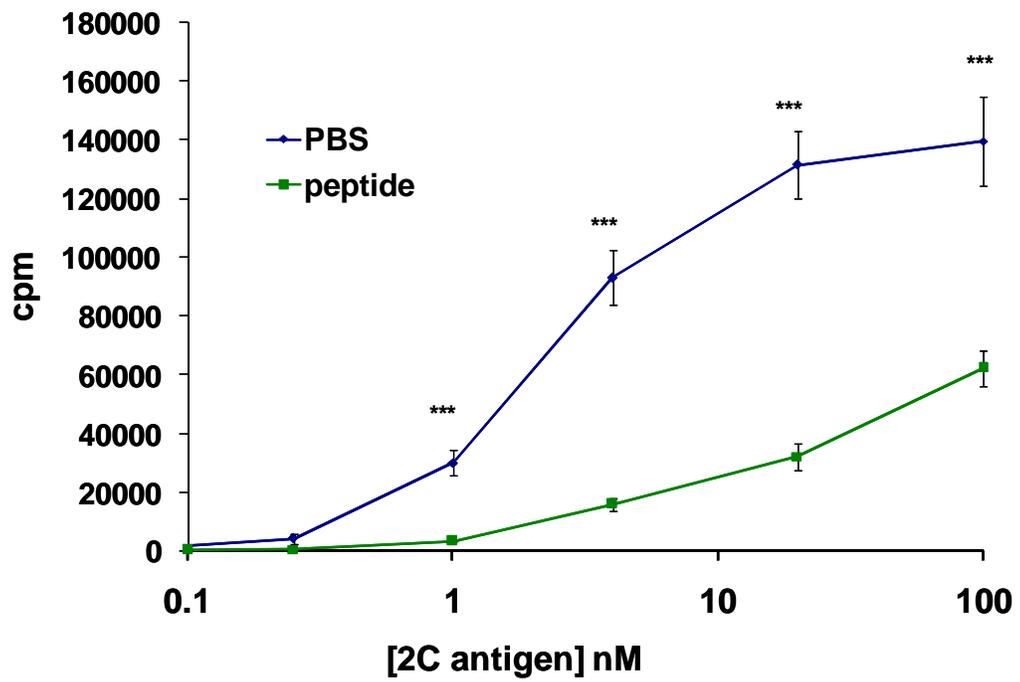
A**B**

Figure 3.2: Figure 1. T cells from peptide injected mice are anergic. T cells were purified from PBS and peptide injected mice. **A.** Analysis of surface expression of CD44 for T cells from peptide and control mice. 1×10^6 cells were incubated with anti-CD44-PE antibody and surface expression was determined by flow cytometry. As negative control, cells were also incubated separately with Rat IgG-PE antibody (isotype control). **B.** *In vitro* proliferation measurements for splenic T cells purified from PBS and peptide injected mice. T cells (5×10^4) were stimulated with graded concentrations of peptide in the presence of splenic APCs for 72 hours at 37°C. [^3H] Thymidine was added to the cells during the last 8 hours of stimulation. Proliferation was assayed by [^3H] thymidine uptake in the cells. Shown are combined averages (\pm s.d.) from three independent experiments. Each individual experiment was done in triplicates. ***, $p < 0.001$

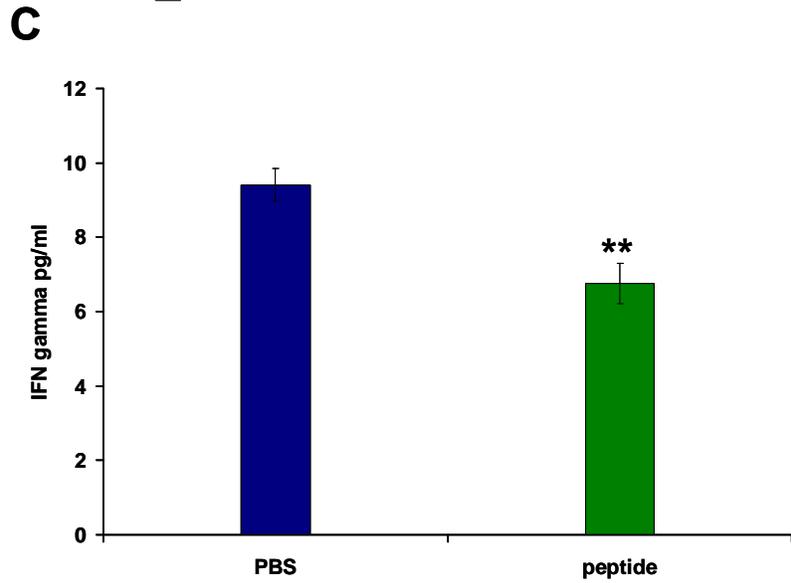
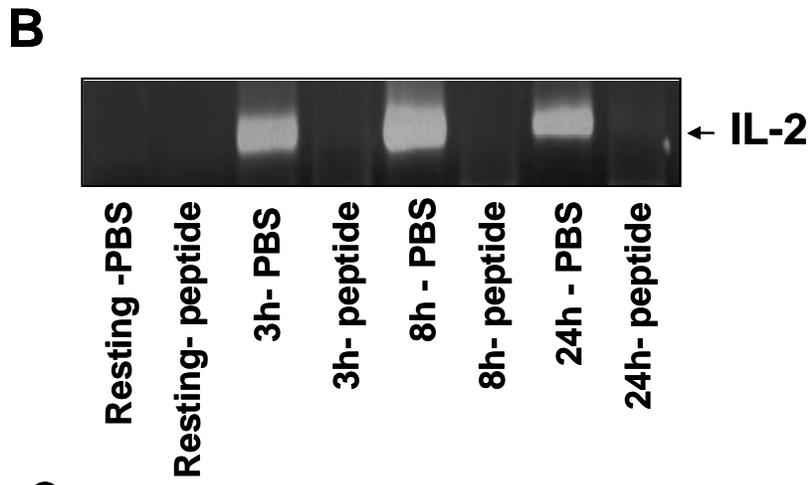
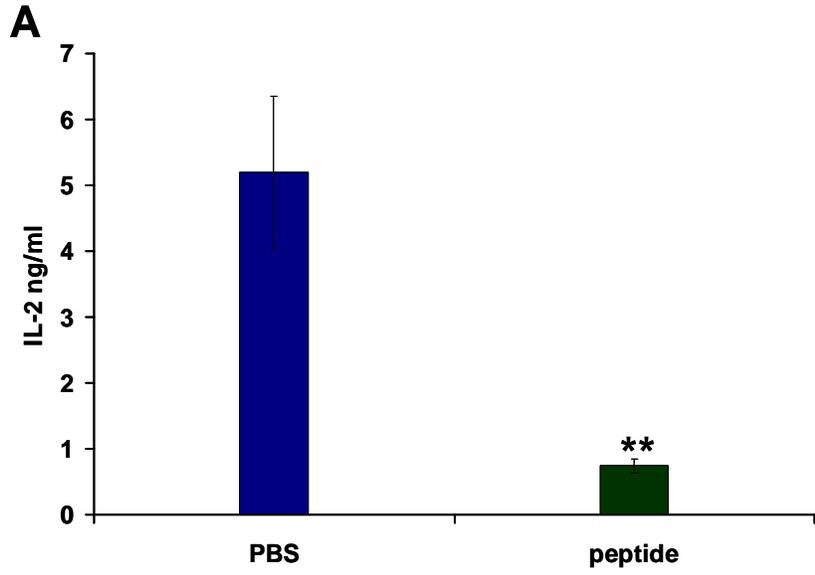


Figure 3.3: IL-2 production is decreased in T cells from peptide injected mice. T

cells were purified from PBS and peptide injected mice. Cytokine production in these cells was determined by ELISA and mRNA analysis. **A.** IL-2 secretion in purified T cells was determined by sandwich ELISA. 1×10^6 T cells were stimulated with 100nM 2C peptide and 4×10^6 splenic APCs for 24 hours, and the supernatants were used for ELISA analysis. Displayed are combined averages (\pm s.d.) from three independent experiments conducted in triplicate. **, $p < 0.01$ **B.** IL-2 expression was also analyzed by mRNA analysis. Purified T cells (5×10^6 cells) were stimulated with anti-CD3/anti-CD28 coupled beads for 3, 8 and 24 hours, or left unstimulated. Total RNA from these samples were then reverse transcribed to generate cDNA, which was then analyzed by PCR for IL-2 expression using IL-2 specific primers. Shown is a representative result of two independent experiments. **C.** IFN γ secretion from T cells purified from PBS and peptide injected mice was analysed similar to IL-2. Shown are combined averages (\pm s.d) from three independent experiments conducted in triplicate. **, $p < 0.01$

expansion in activated T cells, is upregulated after complete stimulation. T cells from peptide injected mice display ~80% reduction in IL-2 secretion (as determined by ELISA), upon in vitro stimulation for 24 hours. The expression profile of IL-2 mRNA also mirrored secretion, with cells from peptide injected mice expressing minimal to no IL-2 mRNA, upto 24 hours after stimulation. Thus, the systemic administration of TCR-specific, sterile antigenic peptide induces anergy in 2C TCR transgenic CD8⁺ T cells.

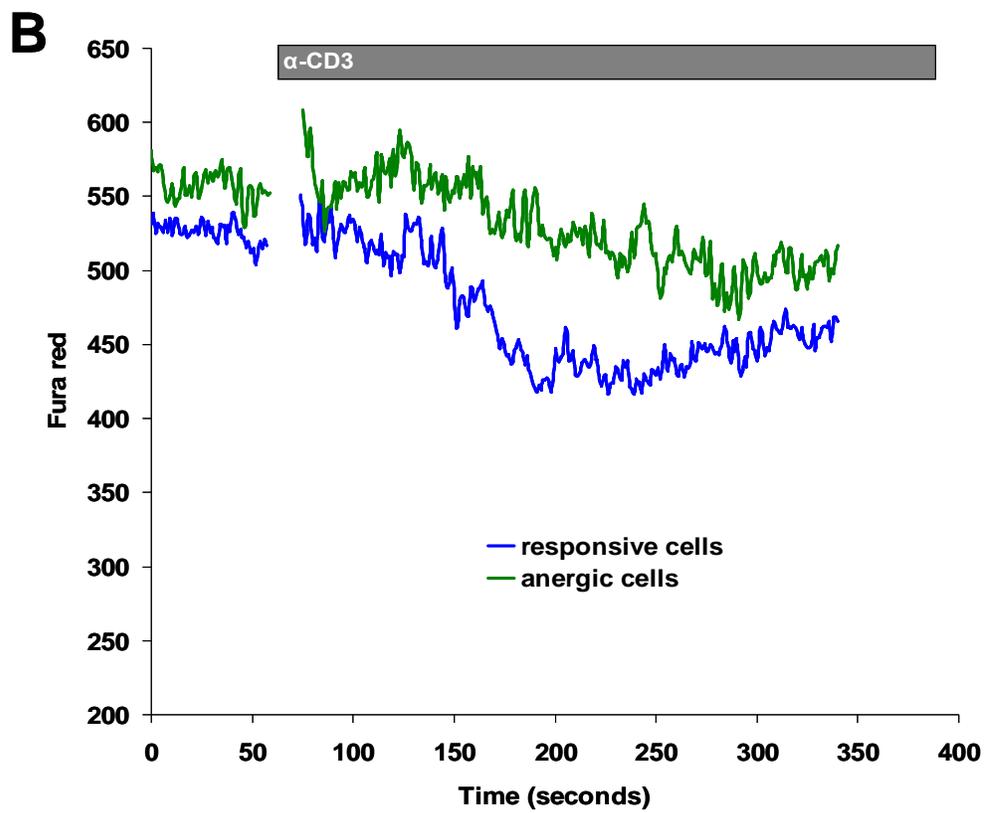
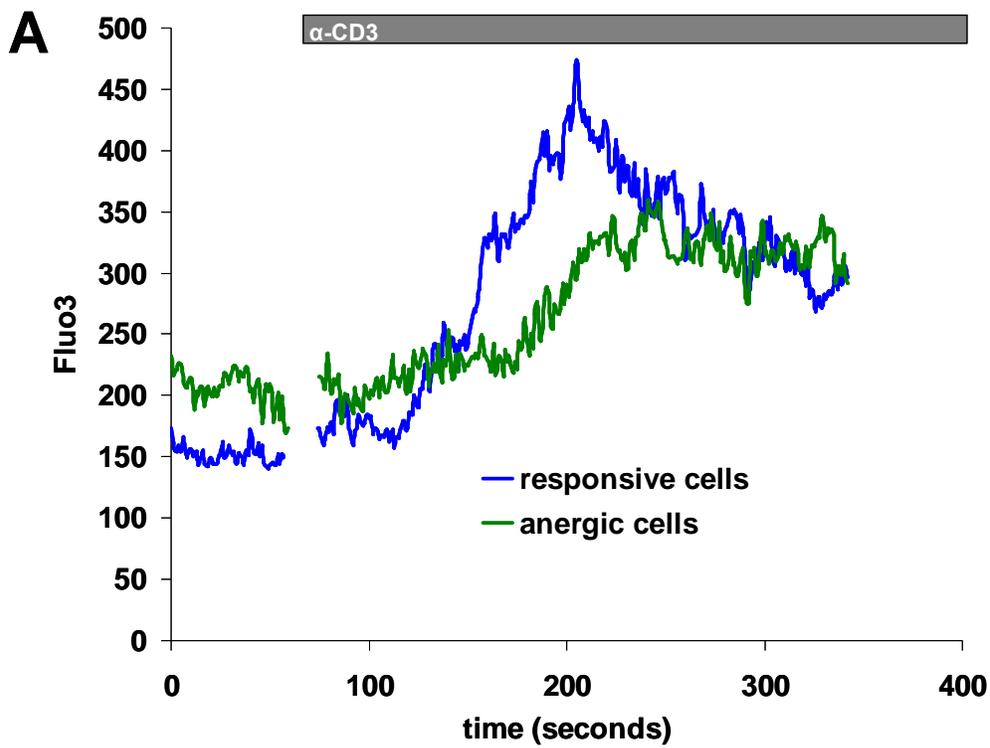
IFN γ is an important effector cytokine that is upregulated in activated CD8⁺ T cells. This cytokine is responsible for the differentiation of naïve CD8⁺ T cells into effector cytolytic cells (CTLs) and also has anti-viral properties. Impaired upregulation of IFN γ has been seen in some, but not all models of anergy [2]. 2C T cells from peptide injected mice showed a moderate decrease in IFN γ levels as determined in cell supernatants obtained after stimulation for 24 hours. This indicates that anergy induced by i.p. injection of peptide differs from studies of anergy, in which T cells show markedly impaired IFN- γ responses [1, 92].

3.2.2 Intracellular mobilization of calcium is impaired in anergic T cells

Anergic T cells have been found to display significant signaling defects, including impaired calcium signals. I determined the intracellular mobilization of calcium in anergic cells by measuring the fluorescence levels of the two calcium sensitive dyes Fluo3 and Fura red. Fluo-3 fluorescence at 530 nm increases with an increase in Ca⁺² binding, whereas Fura Red fluorescence at 670 nm decreases with increasing Ca⁺²

binding, allowing ratiometric measurement of calcium [113]. Cells were loaded with these dyes and the fluorescence emission values were measured after stimulating the cells. Upon stimulation by crosslinking of CD3, the tolerant cells displayed a reduced calcium flux, although it was not completely abrogated (Figure 3.4 and Appendix). The anergic cells appeared to be less competently influxing calcium. The calcium response curve looked similar to naïve cells, but at a lower scale. Thus the anergic cells were not completely deficient in intracellular calcium, but fluxed reduced concentration of calcium that might inhibit or modify downstream signaling events. This is consistent with the observation of Dubois et al. in a similar peptide injection system, but using the F5 TCR transgene [126]. Thus, defective calcium signaling may be an important component of peptide-induced *in vivo* anergy in CD8⁺ T cells.

Calcium signaling in T cells is biphasic. The release of endoplasmic reticulum (ER) calcium activates surface calcium release-activated calcium (CRAC) channels, causing a large influx of extracellular calcium. These phases occur in rapid succession, but can be resolved by chelating extracellular calcium with EGTA. Crosslinking the TCR of naïve T cells in the presence of EGTA produced a small rise in intracellular calcium (Figure 3.5), a result of ER calcium release. The rise in anergic T cells was comparable to that in naïve cells, but occurred more slowly. When extracellular calcium was restored, I still saw a reduced calcium influx in anergic T cells, possibly due to the delayed kinetics in ER release.



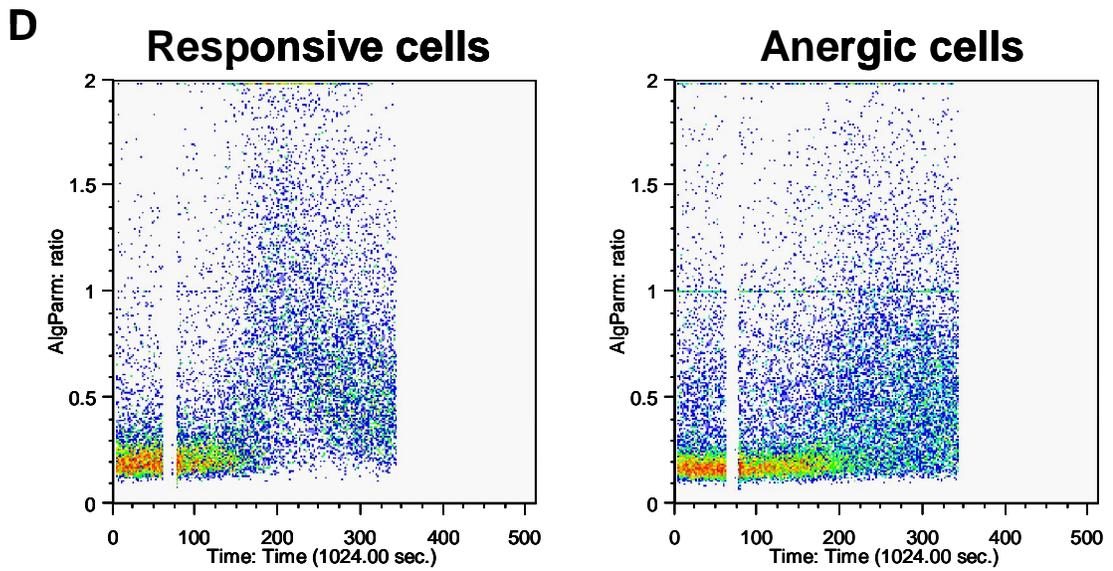
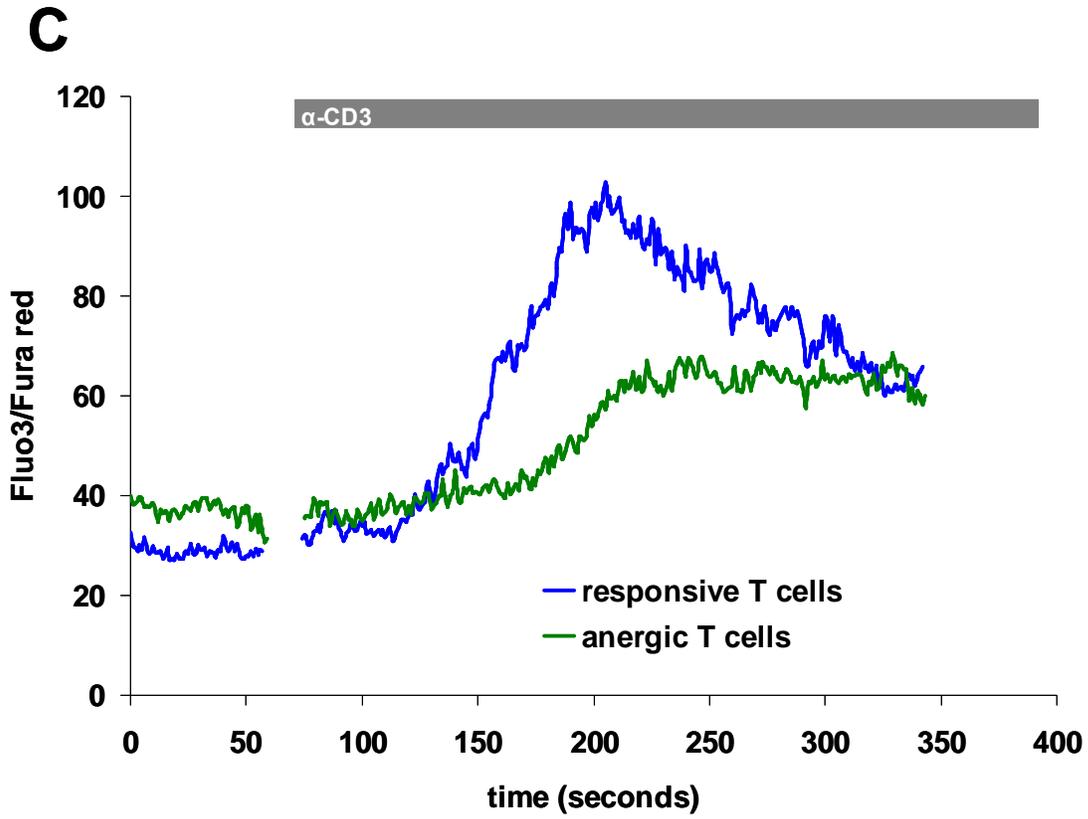


Figure 3.4: Anergic CD8+ T cells display a calcium signaling defect. Anergic and naïve T cells were loaded with Fluo3 and Fura Red, and intracellular calcium responses to anti-CD3 and secondary crosslinking antibody stimulation were determined by flow cytometry. The basal fluorescence levels of Fluo3 and Fura red in the cells were measured for the first 60 seconds, before addition of the stimulatory crosslinking anti-hamster IgG antibody. **A.** The increase in Fluo3 fluorescence levels upon stimulation was measured. **B.** The decrease in Fura red fluorescence levels upon stimulation was measured. **C.** Intracellular calcium responses to anti-CD3 stimulation were depicted as a ratio of Fluo3/ Fura Red fluorescence. Shown above are the averages of the Fluo3/Fura red ratio for all the cells at each time point. **D.** Dot plot of Fluo3/Fura red ratio for responsive and anergic T cells from the experiment shown in **C.** Shown are representative results of three independent experimental trials.

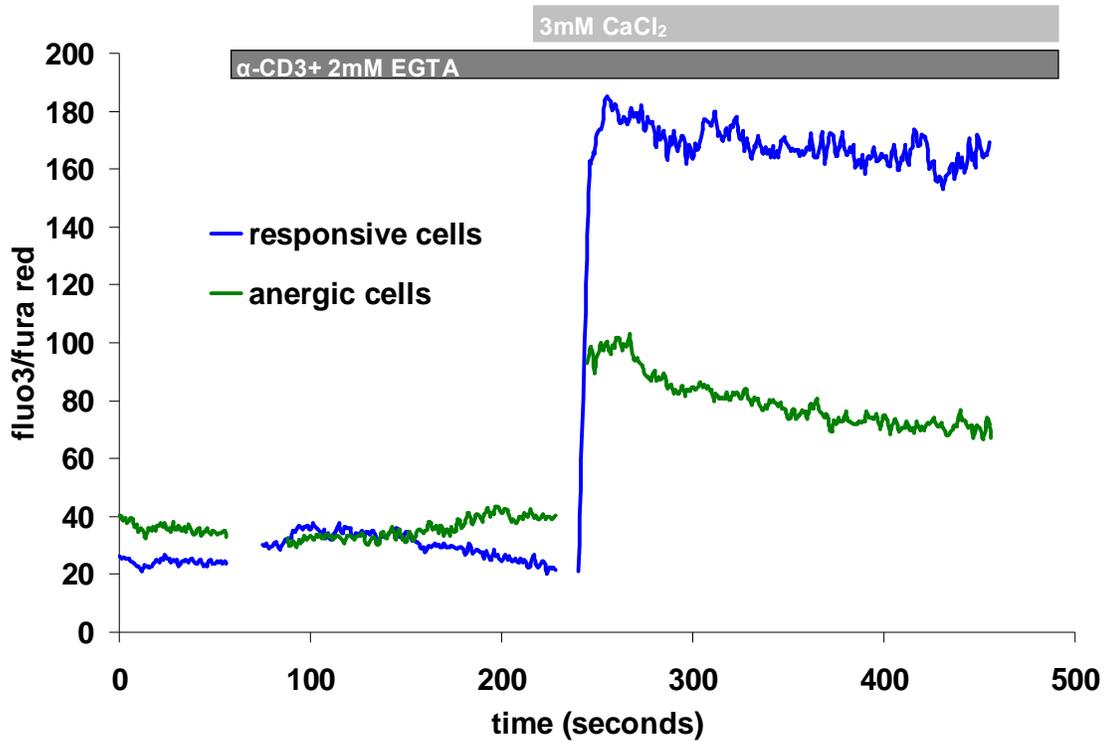


Figure 3.5: Anergic T cells display defective intracellular calcium mobilization. ER store release of calcium in anergic T cells was measured by stimulation in RPMI containing 2 mM EGTA. After 60 seconds of measuring the basal fluorescence levels of Fluo3 and Fura red, cells were stimulated by anti-CD3 and secondary crosslinking antibody stimulation. After ~ 200 seconds of stimulation, 3 mM CaCl₂ was added to replenish the extracellular calcium source. Calcium flux was measured as in Figure 3.4. Shown is a representative result of three independent experimental trials.

3.2.3 Defective phosphorylation of PLC γ in anergic cells.

Since ER calcium release was altered in anergic T cells, we examined the activation of PLC γ , an upstream inducer of calcium release. The activation of PLC γ is an important event following T cell stimulation, as it enables calcium signals and resultant NFAT activation, which contributes to the transcriptional regulation of the activated T cells. We determined the activation of PLC γ in CD8⁺ anergic cells, to determine if the defect in calcium signaling seen, can be attributed to PLC γ based signaling alterations. Complete activation of PLC γ requires phosphorylation at Y783, which was detected using a phospho-specific antibody. As shown in Figure 3.6 A, TCR-induced phosphorylation of PLC γ in anergic T cells was impaired compared to naïve T cells. PLC γ phosphorylation is a proximal signaling event following TCR engagement and has been shown to peak within the first fifteen minutes after stimulation. Total PLC γ levels remained stable for at least 30 minutes in both responsive and anergic cell lysates (Figure 3.6 B), indicating that the impaired phosphorylation is not a result of proteolytic degradation of PLC γ .

3.2.4 Calcium signaling in anergic cells is altered upstream of intracellular store release

The modulations in calcium signaling in anergic cells could be either at the stage of release of calcium from ER, or during the subsequent capacitative influx of calcium through the plasma membrane. The reduced calcium signal generated in anergic cells

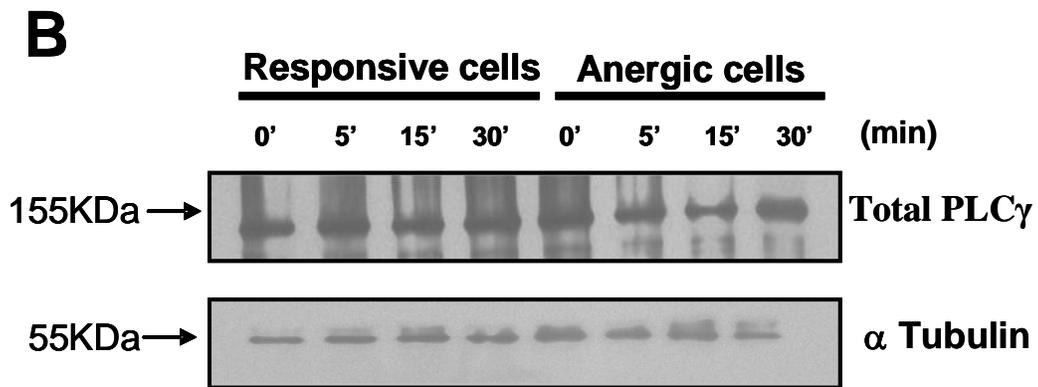
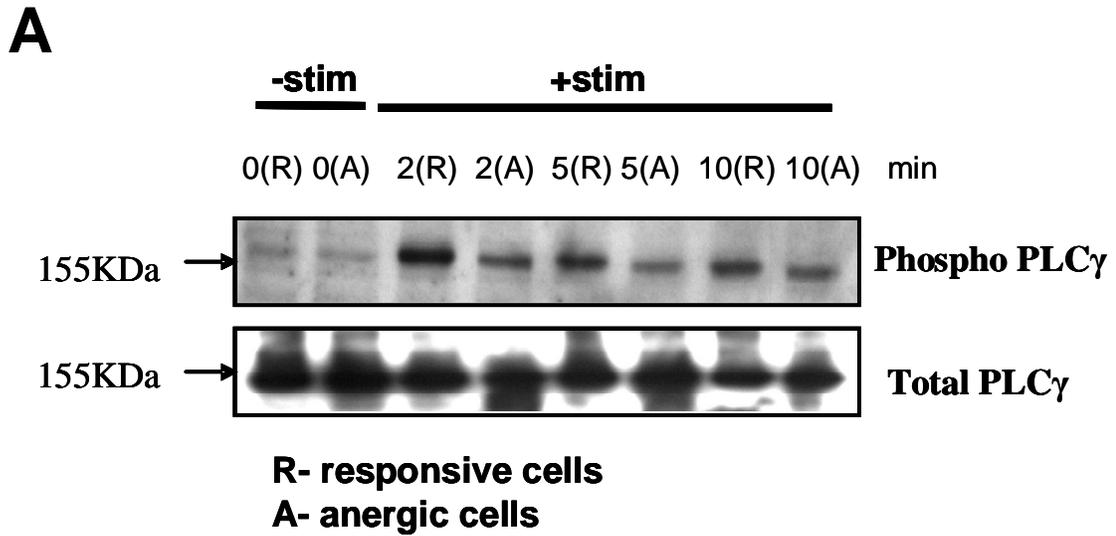


Figure 3.6: Anergic T cells display reduced phosphorylation of PLCγ. Responsive and anergic T cells were stimulated for the appropriate time points with anti-CD3/anti-CD28 and secondary crosslinking anti-hamster IgG antibodies. The cells were then lysed using RIPA buffer, and lysates from equivalent cell numbers ($\sim 1 \times 10^6$ cells/sample) were

used for western blotting analysis. **A.** *In vitro* stimulated anergic and naïve T cell lysates were assayed for activation of PLC γ by Western blot for Y783 phosphorylation. As loading controls, the lysates were also assayed for total PLC γ levels. Shown are representative results of three independent experiments. **B.** Whole cell lysates from anergic and responsive T cells were assayed for total PLC γ levels using western blot analysis. As loading controls, lysates were also assayed for α -tubulin levels in each sample. Shown are representative results from three independent experiments.

could be entirely due to the proximal signaling defects or could be the cumulative effect of a number of defectively functioning signaling modules within the calcium signaling pathway. Recently it was observed that human peripheral T cells isolated from patients afflicted with an immune deficiency displayed a strong post ER defect in calcium mobilization, which affected the proliferation and cytokine production of these cells [55]. Since the phenotype of these cells resembles anergic T cells, we looked to see if CD8⁺ anergic T cells also displayed reduced calcium signaling after ER store release. To delineate between defects upstream of store release and post ER defects of calcium signaling, we measured calcium flux in anergic cells that were treated with thapsigargin. Thapsigargin is an inhibitor of the Sarco/ endoplasmic reticulum calcium ATPase (SERCA) that functions as a calcium pump on the ER [128]. Thapsigargin treatment depletes calcium stores in the ER, and inhibits refilling of the stores once calcium release is complete. This pharmacological inhibitor has often been used to trigger store release of calcium, bypassing TCR engagement and the initial proximal signaling events associated with TCR stimulation. Calcium signaling was initiated by treating anergic and control cells with thapsigargin in the presence of EGTA to chelate extracellular calcium. This resulted in complete emptying of the intracellular stores. When the calcium in the medium was replenished, there was an immediate elevation in the intracellular calcium levels due to the intense signal generated by thapsigargin treatment. Anergic cells and naïve T cells responded similarly to thapsigargin and displayed comparable calcium levels during the sustained response (Figure 3.7). Thus, defective calcium signaling in anergic CD8⁺ T cells can be attributed entirely to the defects in proximal signaling that attenuates store release.

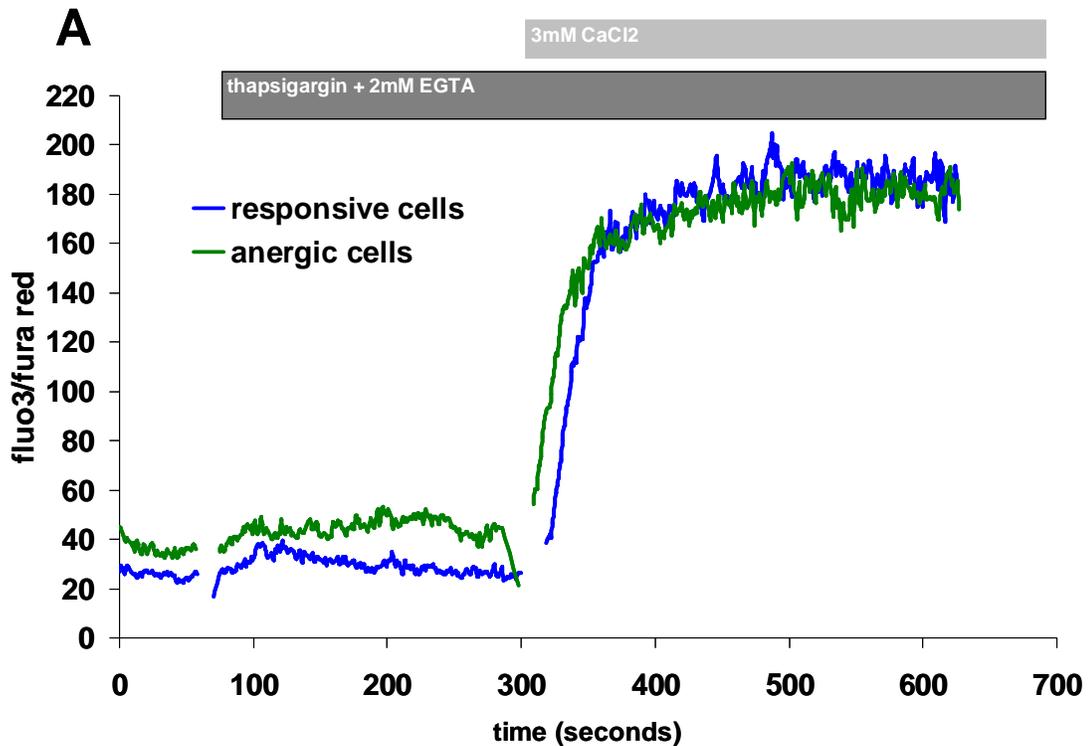


Figure 3.7: Defective calcium influx in anergic T cells is determined by signaling events upstream of ER release. A. Intracellular calcium influx after total ER release was measured by stimulating anergic T cells with thapsigargin. Fluo3 and Fura red loaded T cells were resuspended in RPMI supplemented with 2mM EGTA and stimulated with 1 μ M thapsigargin. After ~200 seconds of measuring Fluo3 and Fura red fluorescence values, 3mM CaCl₂ was added to replenish extracellular calcium. As described above, calcium flux is depicted as a ratio of Fluo3/Fura red values. Shown is a representative result from three independent experiments.

3.3 Discussion

Systemic administration of an antigenic peptide in the absence of any adjuvants results in the antigen specific T cells becoming refractory to further stimulation, even in the presence of complete costimulation. Abrogated production of the proliferative cytokine IL-2 leads to a decrease in large-scale clonal expansion in response to stimulation. These cells are not completely non responsive, however, as IFN γ production, is only partially reduced. This suggests that these cells still retain functional capability and could perform effector functions. These results were similar to another study, where anergy induction in CD8⁺ murine clones elicited normal expression of IFN γ [114]. Other models of *in vivo* induction of anergy in CD4⁺ cells correlated with a significant reduction of IFN γ in the anergic cells [92], therefore it would be informative to determine if this characteristic is unique to CD8⁺ T cells and their functions.

In order to better understand the induction of hyporesponsiveness in anergic T cells, I studied the signaling alterations of *in vivo* tolerized CD8⁺ 2C TCR-transgenic T cells. Two major signaling pathways frequently found to be altered in anergic T cells are the ras/MAPK cascade and calcium signaling [92]. Although I could not detect consistent defects in MAPK activation in our model of *in vivo* anergy induction, I found that calcium responses in anergic cells were blunted relative to naïve cells. This is consistent with multiple other *in vivo* anergy models, spanning different induction methods and both CD4⁺ and CD8⁺ T cells [96, 118, 126], suggesting that the calcium limitation is a common feature of *in vivo* T cell anergy. It is important to note that the Gajewski group works with the same transgenic mouse model (2CTCR Tg), and yet they have

consistently reported significant Ras-MAP kinase defects in cells anergized in vivo under similar conditions [127]. These paradoxical results might reflect the complexity of studying a physiological process such as anergy, which is a functional consequence of various alterations in the vast network of signaling cascades that interact with each other after T cell stimulation.

An important initiating event of T cell calcium signaling is the activation of PLC γ [7, 129]. In anergic 2C T cells, phosphorylation of PLC γ was partially inhibited, indicating reduced activation correlating with a slower release of ER calcium. It has been reported that degradation of PLC γ mediated by ubiquitin ligases, such as Cbl-b and gene related to anergy in lymphocytes (GRAIL), accounts for the reduction of calcium flux and subsequent functional defects in anergic T cells [107]. Examination of total PLC γ in anergic T cells from peptide injected 2C TCR-transgenic mice did not show degradation for at least 30 minutes after restimulation. Thus, reduction in PLC γ protein levels does not appear to be a significant contributing factor to the reduced calcium flux in this system. The degradation of PLC γ and PKC θ has been observed in cells that were anergized by ionomycin treatment and might be a peculiarity of that system. Also, expression analyses of these ubiquitin ligases have all been done in CD4⁺ models of anergy. It will be important to ascertain the expression profile of these anergy related genes in CD8⁺ T cells, to determine their role in the maintenance of the anergic phenotype.

The defect in mobilizing calcium in anergic cells has been solely attributed to the proximal signaling events that precede calcium entry. The decrease in PLC γ phosphorylation suggests a causal role in the impaired calcium mobilization into the cells. However, reduction in phosphorylation is not complete and the decrease in phosphorylation might not entirely explain the calcium signaling defect. IP $_3$ generated by PLC γ activity binds to its receptor on the ER membrane, the IP $_3$ R, which also functions as the active calcium channel through which ER calcium is released. The tyrosine kinase Fyn, has been shown to phosphorylate the IP $_3$ R, and tyrosine phosphorylation on Fyn is upregulated upon T cell stimulation [30]. Phosphorylation of the receptor increases the open probability of the channel, and also aids in keeping the channel open, once cytosolic calcium levels start increasing, as the IP $_3$ R becomes inactive when it senses high concentrations of cytosolic calcium. Thus Fyn dependent phosphorylation of the receptor of IP $_3$ might be an important regulatory mechanism to ensure sustained elevated calcium levels in activated T cells [130]. Experiments done to determine the phosphorylation patterns of the IP $_3$ R1 in anergic T cells were inconclusive due to technical difficulties. More sensitive assays such as microscopy to determine phosphorylation patterns of the receptor [130] might yield more information about the role of IP $_3$ -IP $_3$ R interactions in regulating calcium influx.

The multitude of altered signaling profiles that have been described by various studies highlights the variable biological states that T cells can exist in under different stimulation conditions. Antigenic conditions and the mode of induction of anergy therefore, play an important role in determining the constitution of the anergic state. This

will be highly relevant in physiological situations such as transplantation which use induction of anergy as a means for tolerizing alloreactive T cells.

4. Reciprocal NFAT1 and NFAT2 nuclear localization in anergic T cells is determined by sub optimal calcium signaling.

4.1 Introduction

An important downstream target of calcium signaling in T cells is the activation of the NFAT family of transcription factors. The large influx of calcium activates the serine/threonine phosphatase calcineurin that dephosphorylates serine residues on the regulatory domain of NFAT, leading to the movement of cytosolic NFAT into the nucleus, where activated NFAT enables transcription of the various NFAT- dependent genes [131]. NFAT also functions as a transcription repressor for certain genes [132]. In tandem, kinase molecules collectively referred to as the NFAT kinases rephosphorylate the serine residues to restore the cytosolic resting conformation of NFAT. The cellular localization and the activation state of NFAT are therefore mediated by a balance between these two events that are regulated by T cell activation.

4.1.1 Calcineurin mediated dephosphorylation of NFAT

Multiple serine residues are dephosphorylated in the NFAT regulatory domain by calcineurin. An important consequence of this is the exposure of the NLS, which leads to nuclear accumulation of NFAT [72]. This mechanism is common to NFAT 1-4 and has been widely targeted during immunosuppressive regimens by the use of CSA or FK506. Okamura et al.[73] determined that the serine residues were dephosphorylated in a specific order using a mass spectrometry based strategy, where phosphorylation sites in

an enzymatic digest of a protein are identified by mass spectrometry by the change in mass after dephosphorylation. NFAT1 was shown to be phosphorylated at ~ 21 serine residues in resting cells. Of these, 18 were located in the regulatory region, and 14 of them are found in sequences conserved through the NFAT family. Upon stimulation, 13 of these residues were dephosphorylated to result in complete exposure of the NLS and masking of the nuclear export sequence (NES). They showed that the SRR1 region is dephosphorylated first, resulting in partial unmasking of the NLS and in enhanced accessibility of calcineurin to the SPxx repeat motifs.

Okamura et al.[73] also predicted a conformational switch model for the effect of dephosphorylation on the active and inactive conformations of NFAT. The active conformation is defined by complete dephosphorylation of the 13 serine residues and total uncovering of the NLS in all the NFAT molecules. On the other end of the spectrum, NFAT in the inactive conformation consists of totally phosphorylated serine residues and a masked NLS. Progressive dephosphorylation therefore marks the intermediate stages which are associated with an increasing probability that any molecule of NFAT will be in active conformation.

4.1.2 Transactivation

The phosphorylation of residues in the transactivation domain has been shown to be necessary for the induction of transcriptional activity of NFAT. This domain has binding sites for co-activators, such as p300 and cAMP response element-binding protein (CREB)-binding protein (CBP), that enhance transcriptional activity of NFAT. These

proteins act as histone acetyltransferases and regulate chromatin assembly, and therefore influence gene transcription. These proteins also recruit RNA polymerase and other components of the basal transcriptional machinery to aid in transcription [133]. Various proteins such as p38 [134], PKC ζ [135], Cot/Tpl2 [136] and Pim1 [137] can bind to the transactivation domain and initiate phosphorylation events that lead to higher transcriptional activity. In Jurkat T cells, p38 has also been shown to promote the binding of CBP to sites in the transactivation domain of NFAT2, an event required for NFAT activity [138]. These signaling events provide a potentially calcineurin-independent component of NFAT activation that acts in concert with the calcineurin mediated dephosphorylation processes to result in gene expression. It has not been entirely resolved if these events take place in the nucleus or in the cytoplasm and if the activating phosphorylation reactions precede or follow the dephosphorylation of residues in the regulatory domain. Also, the possibility of interdependence between these two processes needs to be investigated to obtain a complete picture of regulation of translocation and activation of NFAT in T cells.

4.1.3 NFAT kinases

The desphosphorylation and subsequent nuclear localization of NFAT is not irreversible. Intracellular kinase proteins collectively referred to as NFAT kinases rephosphorylate the serine residues that have undergone calcineurin phosphatase activity in order to restore NFAT to its resting conformation (phosphorylated). Therefore, the cellular location of NFAT in resting and activated cells is maintained by a balance between the phosphatase activity of calcineurin and the rephosphorylating activity of the NFAT kinases (Figure

4.1). These kinases act either in the cytosol, immediately after calcineurin activity, or in the nucleus to promote nuclear export. Some of these kinases also function as maintenance kinases, which retain NFAT in the cytosol in resting T cells. These kinases can be pan-NFAT kinases that phosphorylate all members of the NFAT family, or can be member-specific, conferring distinct patterns of regulation. Casein kinase1 (CK1) is involved in the phosphorylation of the SRR-1 region of the regulatory domain of NFAT. CK1 binds NFAT at the amino terminal end at a sequence conserved in all the NFAT proteins. CK1 is bound to NFAT in resting cells and therefore maintains the cytosolic localization of NFAT in unactivated T cells [139]. Glycogen synthase kinase 3 (GSK3), another cytosolic NFAT kinase, requires priming of NFAT by the cAMP-dependent protein kinase (PKA). PKA phosphorylates both NFAT1 [139] and NFAT2 [140] at serine residues in the SP2 motif, and the NLS, providing the priming phosphates for subsequent phosphorylation by GSK-3 at the SP2 and SP3 repeat motifs. GSK-3 also functions as a maintenance kinase, retaining NFAT in the cytosol in resting T cells. GSK-3 activity is inhibited by Akt, which is activated downstream of CD28 signaling, and is therefore downregulated during T cell activation, tilting the balance toward calcineurin activity and nuclear accumulation of NFAT [141].

Another family of NFAT kinases are the dual-specificity tyrosine-phosphorylation regulated kinases (DYRK) family proteins that phosphorylate the SP-3 motif of NFAT1 [142]. These kinases were identified as part of a genome wide drosophila RNAi screen for NFAT regulators. DYRK2 is cytoplasmic and serves to maintain NFAT1 in the cytosol, while DYRK1A is found in the nucleus and promotes nuclear export [143]. The

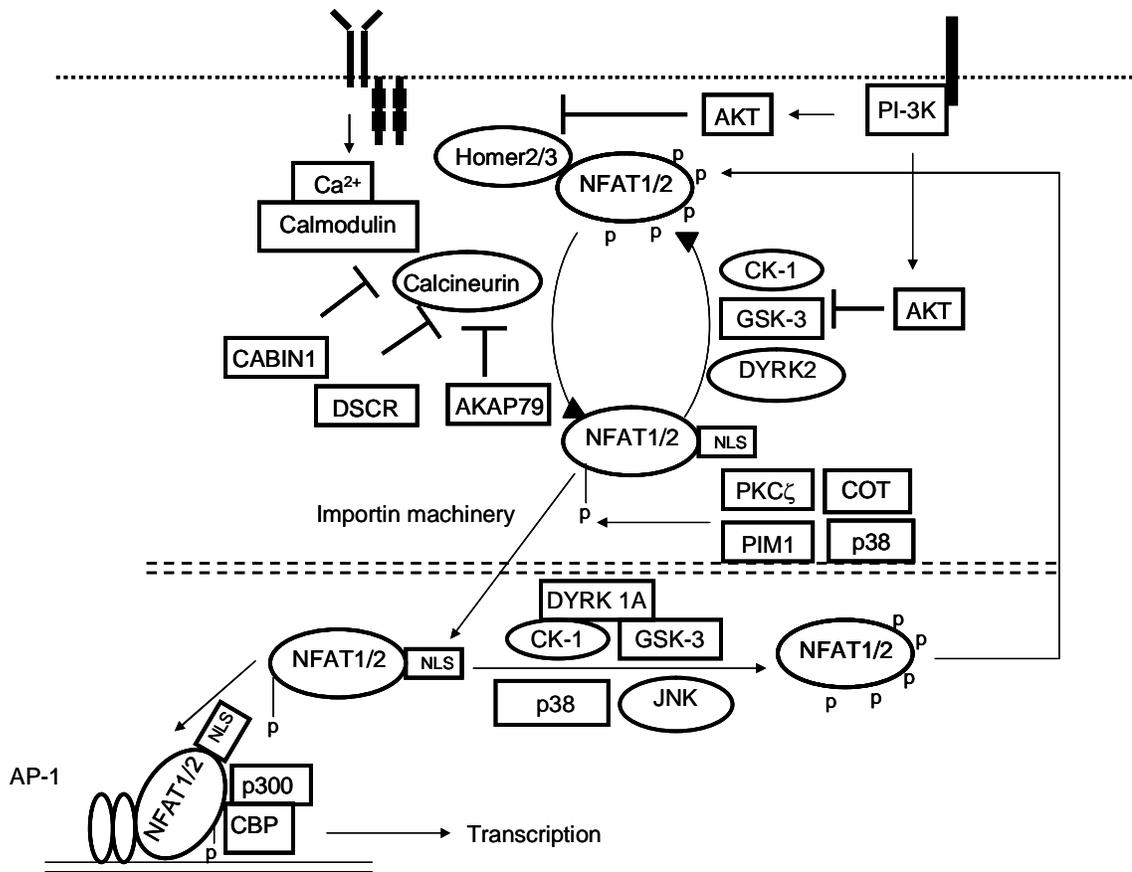


Figure 4.1: Regulation of NFAT activation. Signals downstream of TCR ligation lead to the large influx of extracellular calcium. Calcium binds calmodulin and activates calcineurin. Activated calcineurin dephosphorylates NFAT, exposing the nuclear localization signal (NLS) on these proteins. This leads to the nuclear trafficking of these proteins via the importin machinery of the cell. Other molecules such as PKC ζ , COT, PIM-1 and p38 (specific for NFAT2) phosphorylate specific residues in the transactivation domain of NFAT to further activate them. In the nucleus, activated NFAT proteins bind to promoter/enhancer sequence of DNA to initiate transcription of various genes, either alone or by forming composite interactions with other transcription factors, such as AP-1. Coactivators of transcription such as CBP and p300 also bind the

transactivation domain of NFAT to aid in transcriptional initiation. NFAT nuclear localization is negatively regulated at various stages. The activity of calcineurin is negatively regulated by proteins such as CABIN-1, AKAP79 and the DSCR proteins, which bind calcineurin to inhibit enzymatic activity. Homer2/3 binds to NFAT at calcineurin recognition sites, opposing calcineurin binding. The NFAT kinases CK-1 and GSK-3 aid in maintaining NFAT in the phosphorylated state in the cytosol, or rephosphorylate serine residues in nuclear NFAT to expose the nuclear export sequence and aid in translocation out of the nucleus. DYRK-2 is a NFAT1-specific cytosolic NFAT kinase, while DYRK-1 promotes nuclear export. The MAP kinases p38 and JNK specifically phosphorylate nuclear NFAT1 and NFAT2 respectively, promoting export. NFAT regulation by signals downstream of CD28 activation is mediated by the negative regulation of Akt on Homer2/3 and GSK-3. NFAT 1/2 cellular localization and transcriptional activity is thus regulated by a network of signaling events, that are activated downstream of TCR and CD28 engagement.

MAP kinases p38 [144] and JNK [145] act as NFAT1 and NFAT2 specific kinases, respectively. These proteins act on the SRR-1 region of the NFAT proteins and mediate nuclear export. JNK has also been shown to act on specific residues in the PxIxIT calcineurin docking motif of NFAT2 and prevents calcineurin binding to NFAT2 [146]. The presence of multiple pan-NFAT, and protein-specific NFAT kinases, reinforces the idea that regulation of cellular localization and transcriptional activation of the NFAT proteins is a complex network of signaling events that can mediate specificity and non-redundancy.

The cytosolic localization of NFAT in resting cells is maintained by a number of scaffolding proteins which act in tethering the NFAT proteins in the cytosol. Recently, the Homer proteins have been identified as inhibitors of calcineurin binding. These proteins interact with NFAT at regions corresponding to the calcineurin docking site, preventing calcineurin docking. Homer 2 and 3 have been shown to bind both NFAT1 and NFAT2 in vitro in primary murine T cells, and act as negative regulators of NFAT dephosphorylation [147]. Upon T cell activation, these proteins are negatively regulated by AKT to allow calcineurin binding and nuclear translocation. These scaffolding components, thus introduce another layer of complexity in the regulation of cellular trafficking of the NFAT proteins.

4.1.4 NFAT signaling networks

Although NFAT and MAP kinase signaling are usually presented as parallel and separate pathways, more and more proteins have been identified with multiple roles that have integrated these two cascades into a complex network of regulation of activation. The

large influx of calcium mediated by TCR signaling, leading to calcineurin activity, plays the central role in NFAT translocation and activity. However, both the MAP kinase pathway and signals downstream of the CD28 activation pathway have been implicated in the regulation of these events. The NFAT kinase GSK3 and the scaffolding proteins Homer2 and 3 are negatively regulated by Akt, a kinase activated downstream of CD28 stimulation. The MAP kinases p38 and JNK play important roles as NFAT kinases that enhance nuclear export of NFAT proteins. CD28 and Grb-2 cooperate with Vav1 to upregulate NFAT/AP-1 mediated transcription [148]. PKC ζ and Pim1, proteins that are activated by PI-3 kinase signaling, and p38, have also been implicated in phosphorylation of the transactivation domain to enhance transcriptional activity of NFAT. Thus the regulation of NFAT proteins in T cells is a complex network of positive and negative regulation, both in resting and activated T cells.

NFAT proteins are early initiators of transcription, and therefore also contribute to regulation by transcribing proteins that feed back into the network. Carabin is one such negative feedback regulatory protein that binds to calcineurin and inhibits NFAT dephosphorylation [149]. The study of NFAT regulation, therefore, involves more than just the study of the role of calcium-calcineurin activity. Also, these regulatory mechanisms have been studied either using NFAT1 or NFAT2 as a representative of the NFAT family of proteins. Therefore, it is not known if these signaling events affect all the NFAT family members that are expressed in the cells that are studied, or if the effect is specific for the particular NFAT family member. Direct comparisons between NFAT1 and NFAT2 to ascertain the result of any signaling modification is required to obtain a

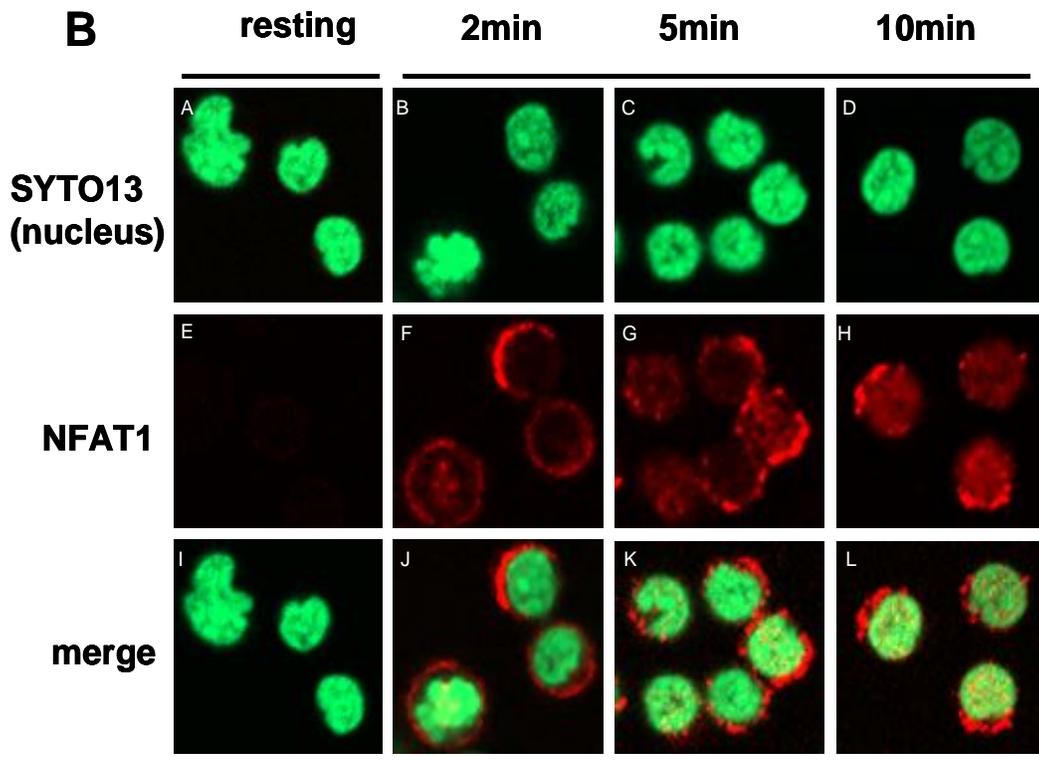
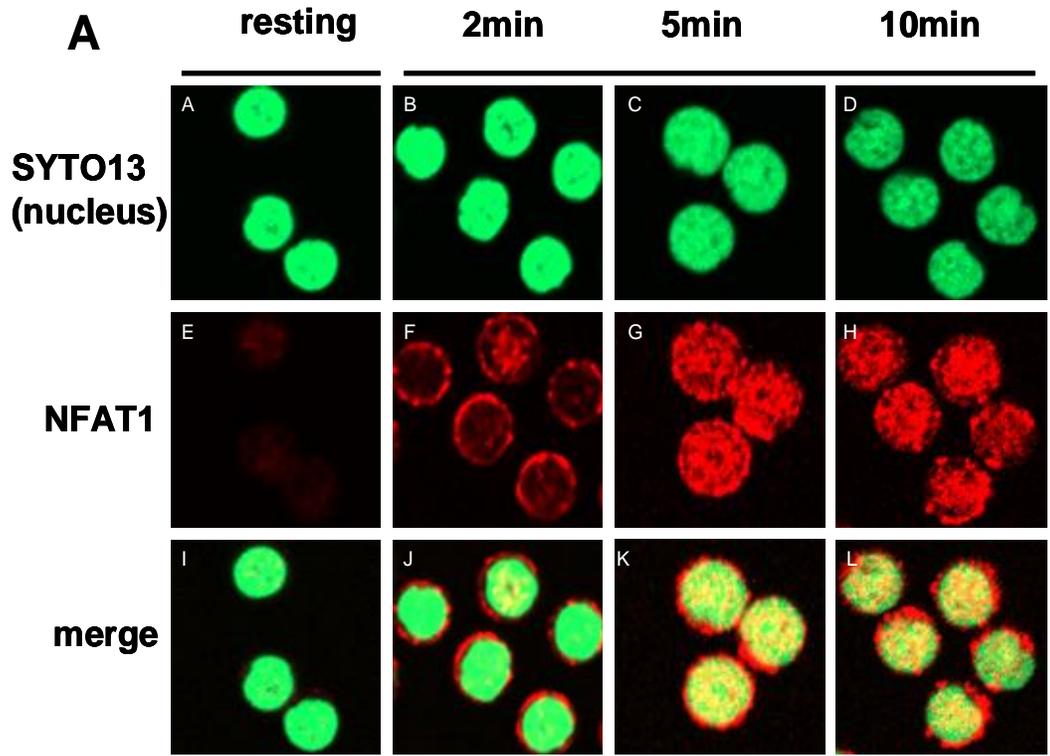
more comprehensive measure of the regulation of NFAT, and the individual roles NFAT1 and NFAT2 play in T cell activation and anergy.

Various studies have looked at defective NFAT nuclear translocation as a product of the impaired calcium response in anergic T cells [126, 150]. These studies have focused only on NFAT1 nuclear movement. In order to determine the cellular localization pattern of the NFAT family members in my model of anergy I analyzed the nuclear translocation of both NFAT1 and NFAT2 as a response to restimulation in CD8⁺ anergic T cells. NFAT1 translocation into the nucleus was blocked in anergic T cells. Unlike NFAT1, NFAT2 was rapidly translocated into the nuclei of anergic, but not responsive T cells. I also found that directly limiting extracellular calcium or calcineurin activity in normal T cells was sufficient to replicate the anergic T cell pattern of NFAT localization. This indicates that NFAT1 and NFAT2 are responsive to different cytosolic calcium levels, an observation that was previously unreported. The reciprocal regulation of NFAT1 and NFAT2 suggests that while NFAT1 is the predominant isoform involved in a productive immune response, NFAT2 might be important for controlling genes involved in T cell tolerance.

4.2 Results

4.2.1 NFAT 1 translocation into the nucleus is inhibited in tolerant cells

Calcium entry into T cells activates the calcium/calmodulin-dependent phosphatase calcineurin, leading to NFAT dephosphorylation and translocation into the nucleus. NFAT activity has been correlated with the induction of IL-2 expression in activated T cells [79]. Since anergic cells displayed a defective calcium response, I investigated whether there was a corresponding defect in NFAT regulation that corresponds to the reduced expression of IL-2 in anergic T cells. The translocation of the cytosolically distributed NFAT into the nucleus precedes transcriptional regulation. The role of NFAT1 has been extensively studied in T cell activation and altered regulation has been reported in some models of CD4⁺ T cell anergy [150]. Therefore I looked at the cellular distribution patterns of NFAT1 in anergic T cells after stimulation. After stimulating T cells for different time points, the localization of NFAT1 in anergic cells was analyzed by immunofluorescence microscopy. In non-anergic cells, NFAT1 was seen as a cytosolic ring at two minutes after stimulation, and translocation into the nucleus was detected within five minutes of stimulation (Figure 4.2 A). By 30 minutes, NFAT1 had largely returned to the cytosol. In anergic T cells, NFAT1 was also detected in the cytosol after 2 minutes of stimulation, but translocation into the nucleus was strongly reduced compared to naïve cells (Figure 4.2B). The level of NFAT1 nuclear localization was quantified and is represented in Figure 4.2 C. This indicates that the NFAT1 nuclear translocation is blocked in CD8⁺ anergic T cells upon restimulation.



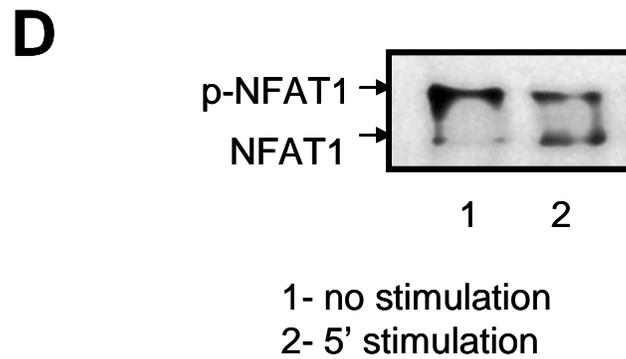
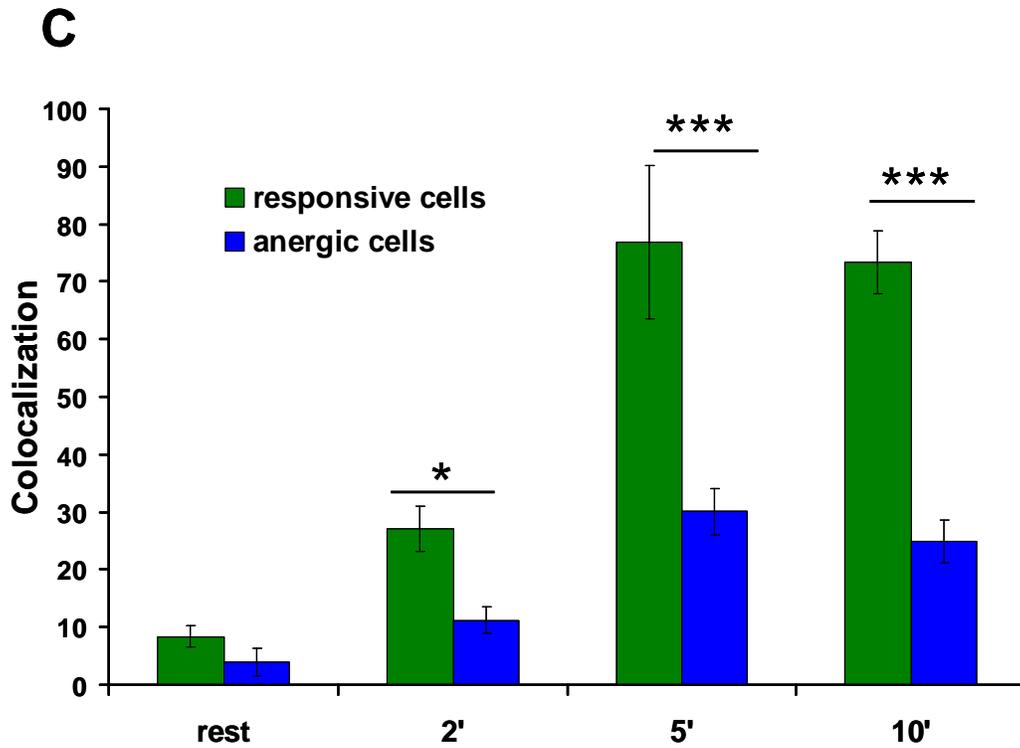


Figure 4.2: NFAT1 translocation into the nucleus is impaired in anergic T cells. A.

Naïve (responsive) T cells were stimulated for 2, 5 and 10 minutes with anti-CD3 and

anti-CD28 and secondary crosslinking anti-hamster IgG antibodies, or left unstimulated. Cells were incubated with anti-NFAT1 antibody followed by Alexa Fluor 594 anti-mouse IgG antibody. Nuclei were stained with SYTO13 (green, panels A-D), and NFAT1 nuclear localization (red, panels E-H) was measured by immunofluorescence microscopy. Colocalization is shown in yellow in the merge images (yellow, panels I-L). **B.** Anergic T cells were stimulated similar to naïve T cells, and NFAT1 nuclear translocation was measured as in A. **C.** Quantification of NFAT1 nuclear localization. Shown are combined averages (\pm s.d.) from three independent experiments. Colocalization was quantified from 25 cells per individual experiment. *, $p < 0.05$. ***, $p < 0.001$ **D.** Western blot of NFAT1 in whole-cell lysates from resting (lane 1) and 5-minute stimulated (lane 2) naïve T cells. 5×10^6 cells per sample were stimulated similar to immunofluorescence assays, and subjected to RIPA lysis prior to western blot analysis.

Surprisingly, we were unable to detect NFAT1 in resting naïve or anergic T cells, although the same antibody was able to detect NFAT1 in lysates from both resting and stimulated naïve T cells by Western blots (Figure 4.2 D). This suggests that the epitope (which is distinct from the nuclear localization signal) might be hidden prior to stimulation, either by protein conformation or by a binding partner. Stimulation presumably induces a conformational change in the protein that precedes nuclear translocation by minutes, allowing its detection in the cytosol. Interestingly, 2 minutes after stimulation, cytosolic distribution of NFAT1 was similar in anergic and naïve T cells. This suggests that NFAT1 in anergic cells also starts to undergo conformational changes due to dephosphorylation of the serine residues of the regulatory domain. The impaired ability of NFAT1 in anergic cells might be due to the incomplete dephosphorylation of enough serine residues to result in movement into the nucleus.

A second explanation for the impaired nuclear NFAT localization in anergic cells could be that anergic cells might import NFAT1 into the nucleus at a rate equivalent to naïve T cells, but the simultaneous nuclear export of the protein is accelerated. To address this possibility, tolerized T cells were stimulated in the presence or absence of leptomycin B, a nuclear export inhibitor [151]. As shown in Figure 4.3, leptomycin-treated anergic T cells displayed increased NFAT1 nuclear localization after stimulation, compared to leptomycin-untreated cells. Although this result indicated that some NFAT1 was getting into the nuclei of anergic T cells, only to be quickly exported, the amount of nuclear localization in leptomycin-treated anergic cells after 30 minutes of stimulation was still lower than that of control naïve T cells after five minutes of stimulation. This suggests

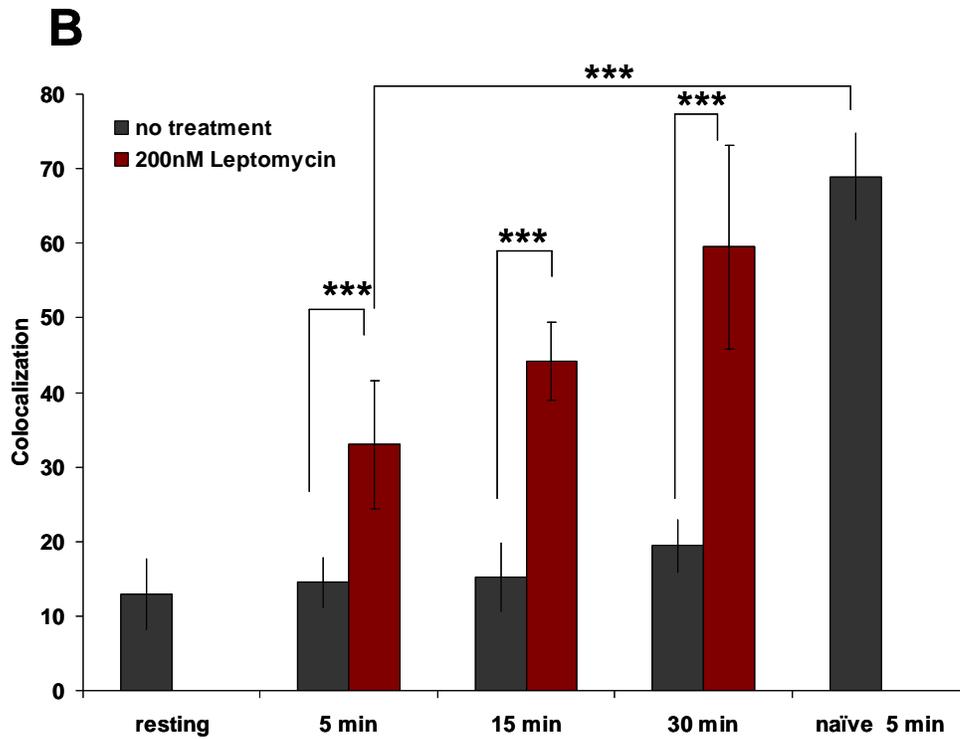
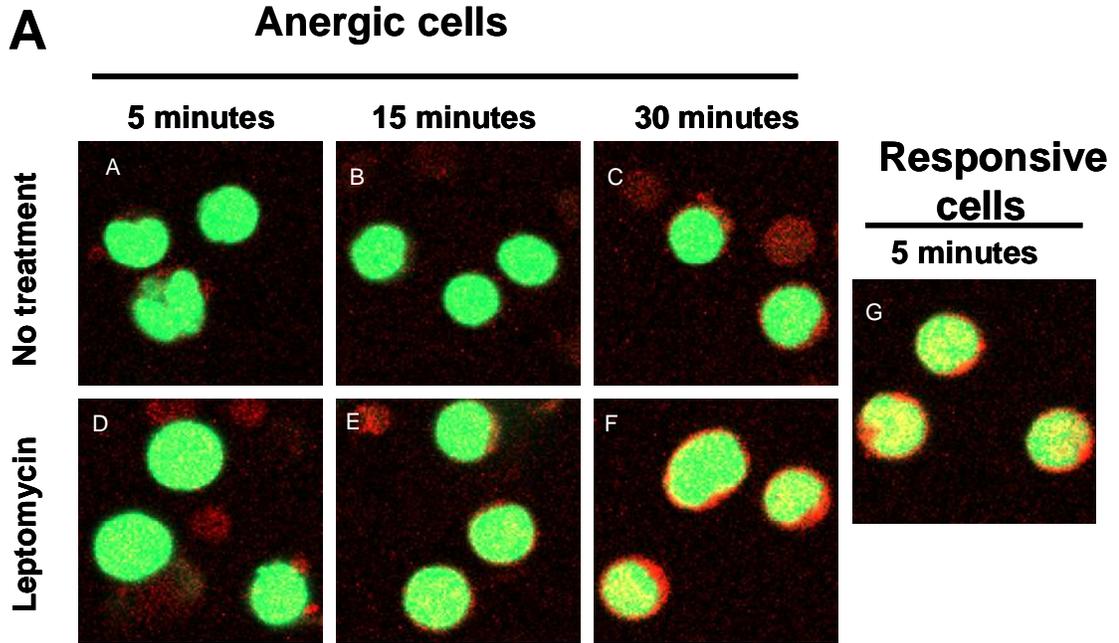
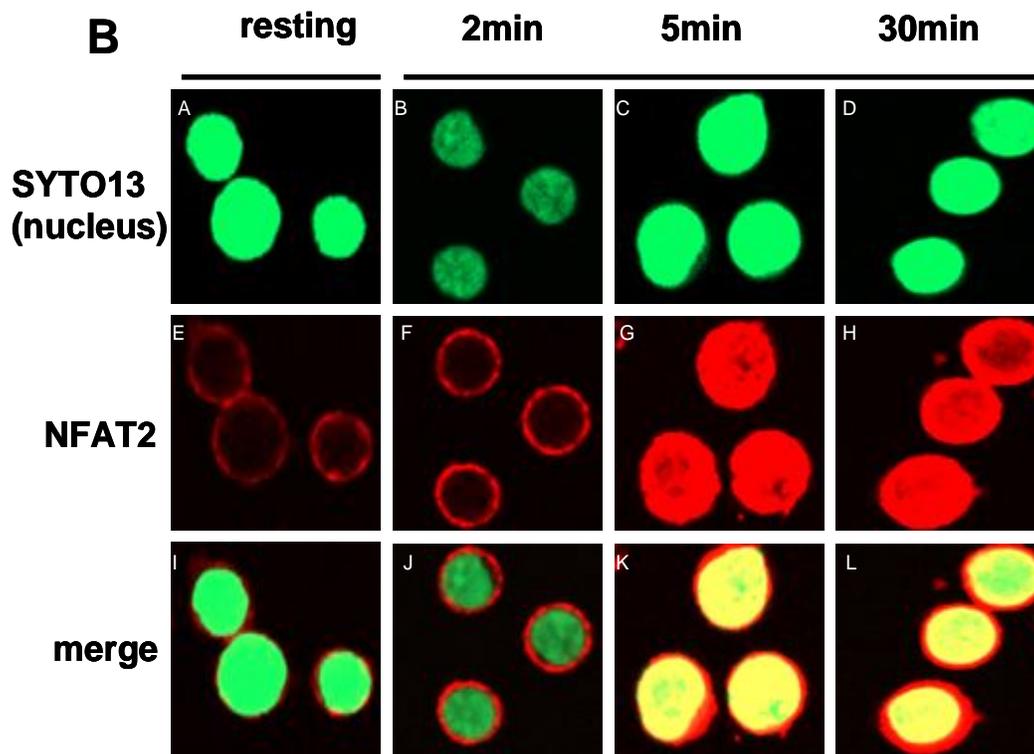
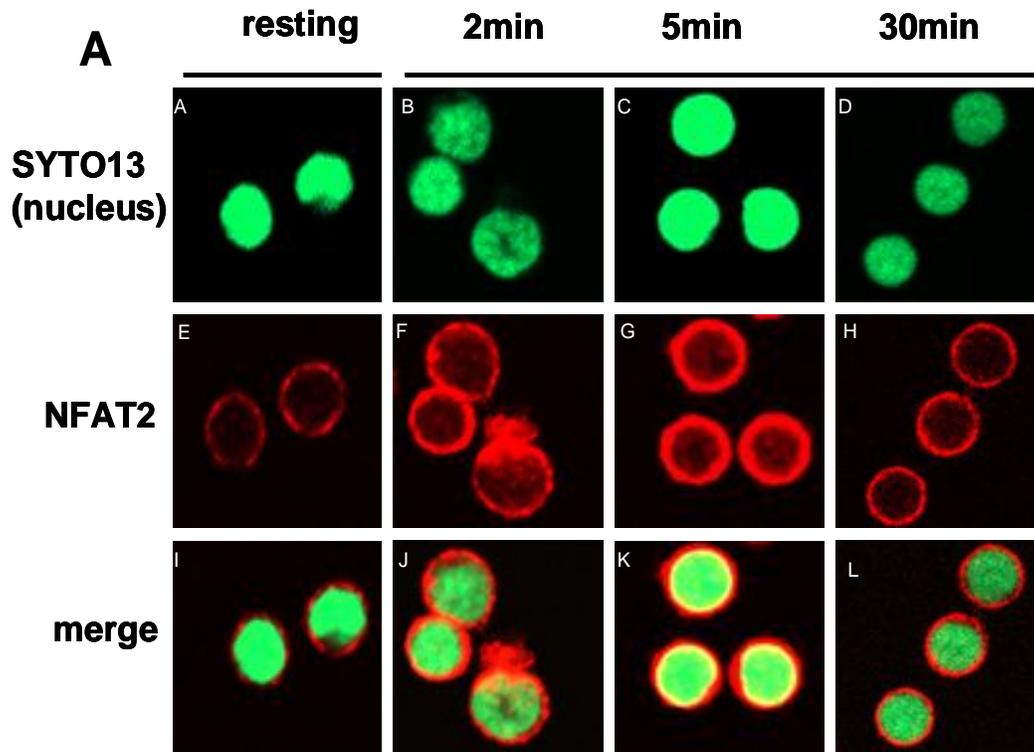


Figure 4.3: Leptomycin treatment of anergic T cells moderately enhances NFAT1 nuclear accumulation. **A. B.**, Anergic T cells were treated with 200 μ M Leptomycin B pre stimulation (panels D-F), or left untreated (panels A-C). Cells were stimulated as described above, for 5 minutes, 15 minutes and 30 minutes. T cells from control mice were also stimulated as described for 5 minutes (panel G). The nuclei were stained with SYTO 13. NFAT1 nuclear localization was determined by immunofluorescence and represented as colocalization of NFAT1 and SYTO 13 (merge, yellow). Colocalization was quantified for 25 cells per sample using ImageJ. ***, $p < 0.001$

that a lowered rate of import is the major component of the reduced nuclear localization of NFAT1 in anergic cells.

4.2.2 NFAT2 is regulated differently from NFAT 1 in anergic cells

NFAT2 is another member of the NFAT family of proteins that is expressed in peripheral T lymphocytes. Previous studies using in vitro gel shift assays have shown that NFAT2 binds the IL-2 promoter similar to NFAT1, and is also capable of interacting with AP-1 to form productive transcriptionally active complexes [152]. Both isoforms appear able to activate transcription of many of the same genes [64]. However, most previous studies on NFAT localization have focused only on NFAT1. We therefore investigated if NFAT2 displayed a similar pattern of nuclear translocation. In resting naïve cells, we readily detected cytosolic NFAT2 (Figure 4.4A). However, unlike NFAT1, there was little translocation to the nucleus after stimulation, even after 30 minutes. Resting anergic T cells also showed cytosolic NFAT2, but displayed NFAT2 localization into the nucleus within five minutes of stimulation (Figure 4.4 B), when NFAT1 in these cells was largely cytosolic. We could detect nuclear localization of NFAT2 for at least 30 minutes after stimulation. NFAT2 nuclear colocalization was quantified and represented in Figure 4.4 C. Thus, NFAT1 and NFAT2 are reciprocally regulated in naïve and anergic T cells. In naïve T cells, NFAT1, but not NFAT2, responds to TCR ligation by rapidly translocating to the nucleus, and this pattern is reversed in anergic T cells.



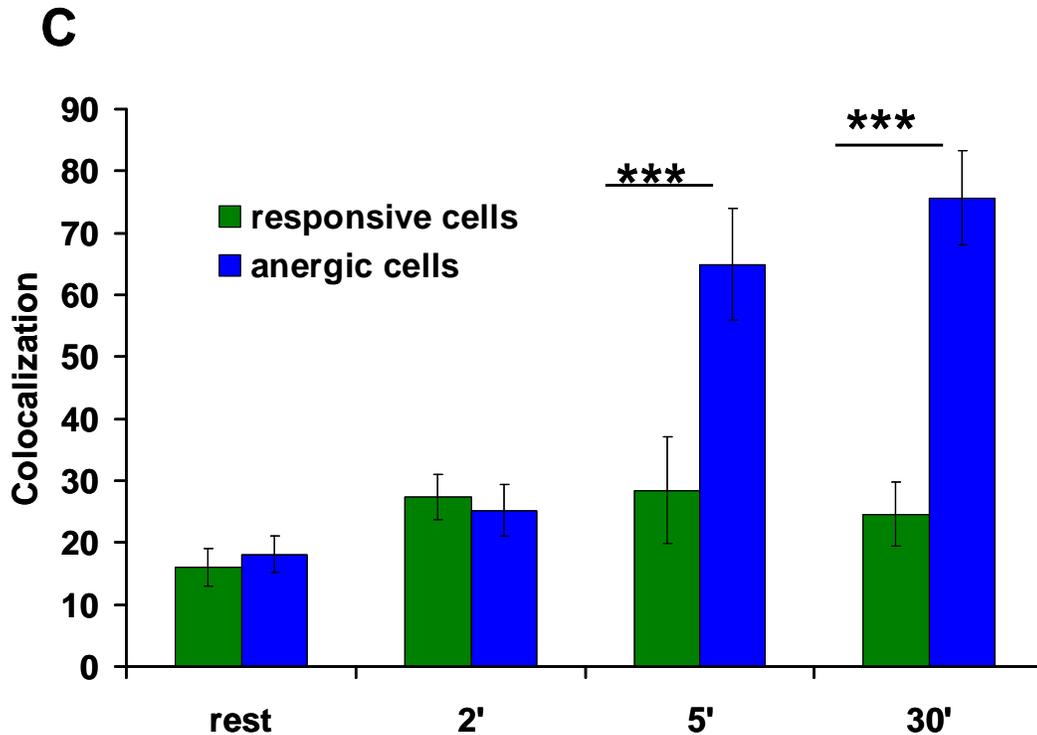
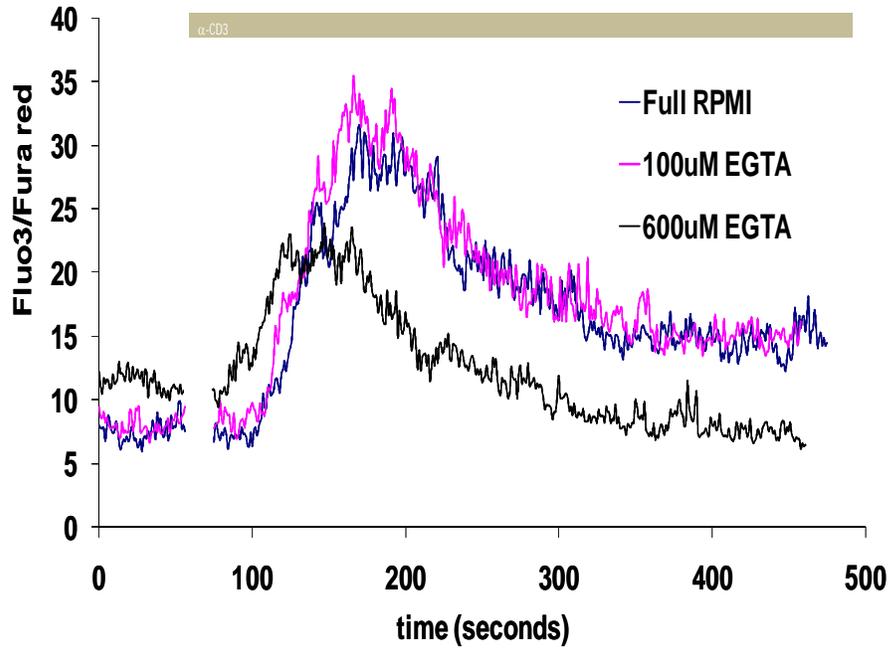
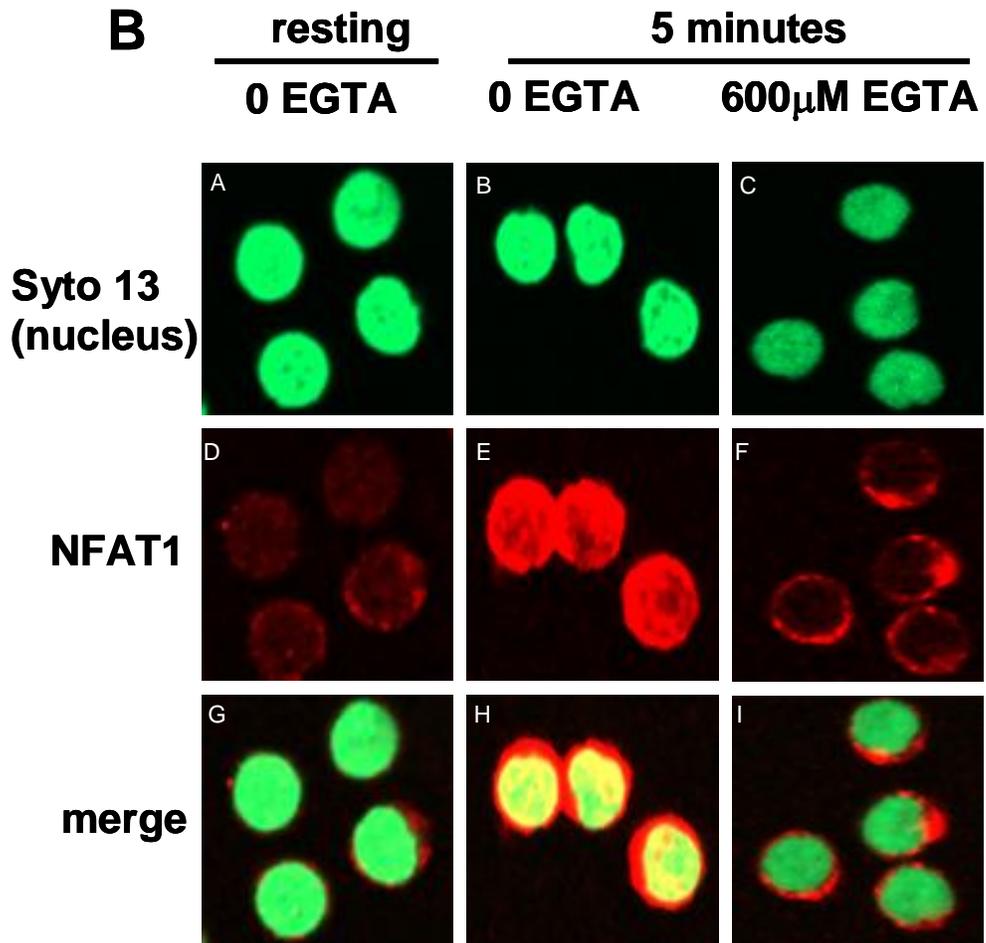


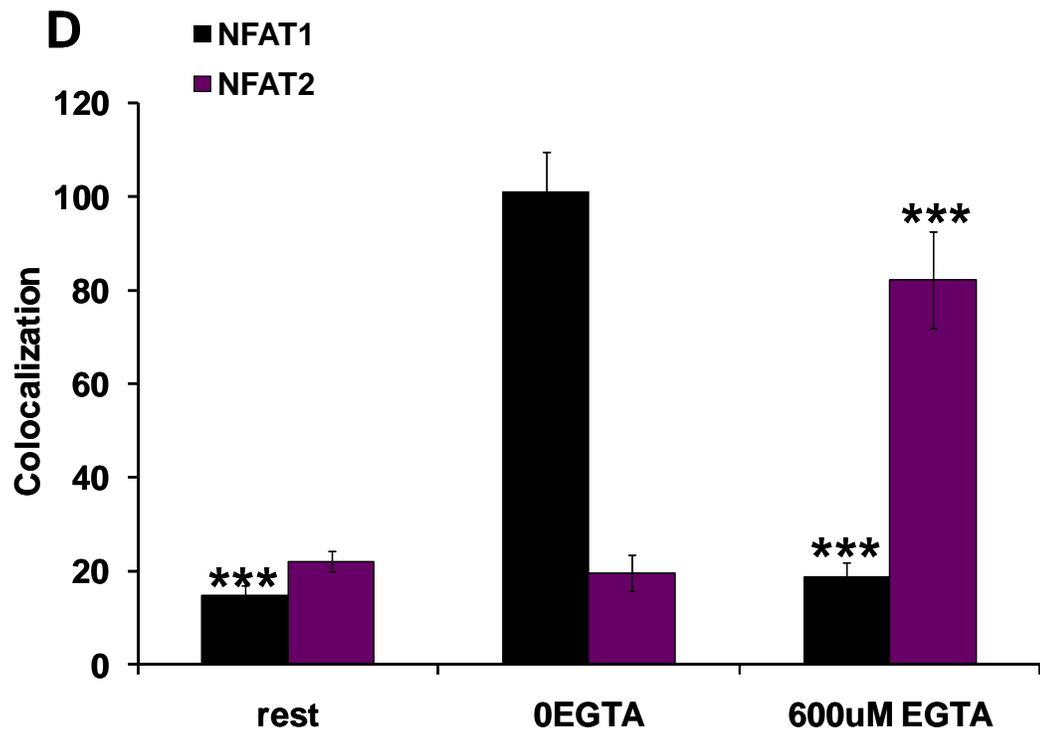
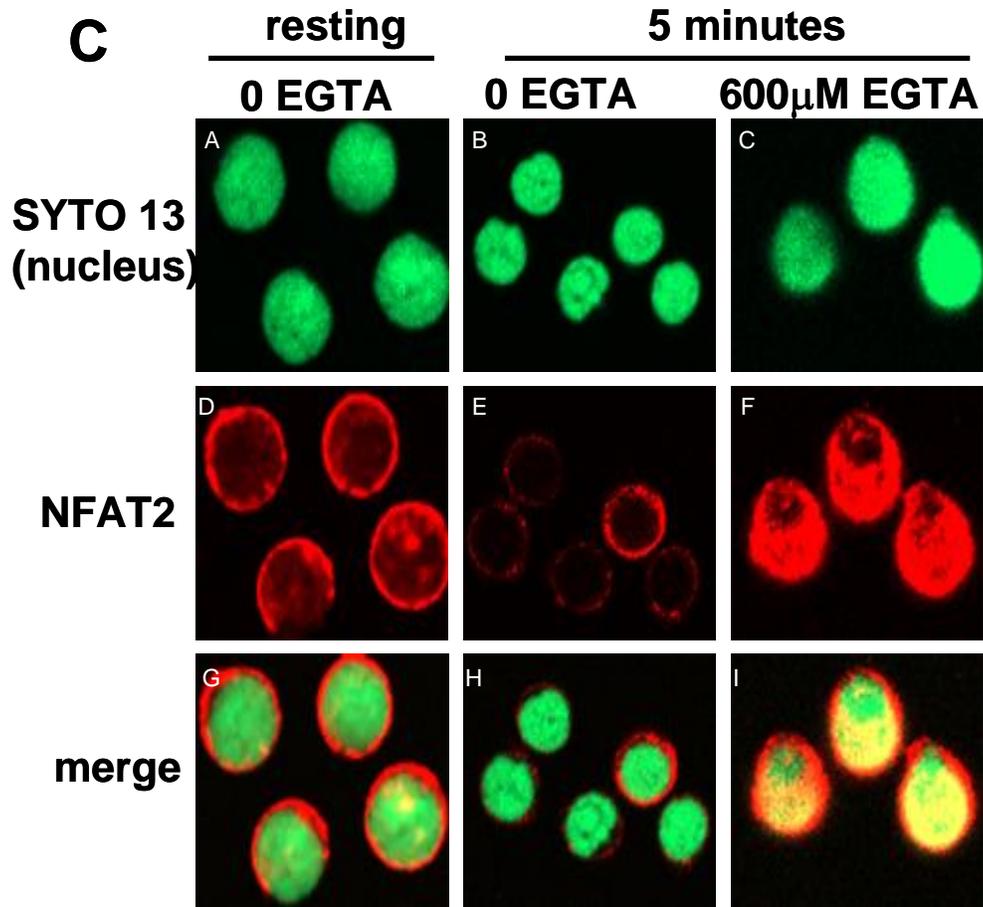
Figure 4.4: NFAT2 nuclear translocation is enhanced in anergic T cells. **A**, Naïve (responsive) T cells were stimulated as in Figure 4.2 for 2, 5 and 30 minutes, and NFAT2 nuclear localization was determined by IF microscopy. Nuclei (green) are shown in the panels A-D, NFAT2 (red) in the panels E-H and colocalization (yellow) is shown in the panels I-L. **B**, Anergic T cells were stimulated as described and NFAT2 localization was measured as in A. Nuclear colocalization is represented as the merge images (panels I-L). **C**, Quantification of NFAT2 nuclear localization using ImageJ. 25 cells from each individual experiment were quantified. Shown are combined averages (\pm s.d.) from three independent experiments. ***, $p < 0.001$

4.2.3 NFAT1/2 translocation is controlled by extracellular calcium availability

The correlation between the defective calcium signaling and the switch to NFAT2 nuclear translocation in anergic cells suggested a causal connection. However, considering the complex network of signaling events that occur during T cell activation, anergic cells might be defective in other signaling modules. To determine if reduced calcium flux could account for the pattern of NFAT localization in anergic cells, I examined the translocation patterns of NFAT1 and NFAT2 in naïve T cells under conditions of calcium limitation.

The calcium available to T cells was varied by using EGTA to chelate extracellular calcium. By titrating EGTA and measuring calcium flux by flow cytometry, I determined that the addition of 600-700 μM EGTA to the T cell medium produced calcium response curves similar to those seen in anergic T cells (Figure 4.5 A). Cells stimulated in medium with no added EGTA displayed NFAT1 nuclear localization (Figure 4.5 B, D, E) while NFAT2 remained cytosolic (Figure 4.5 C, D, E), as previously observed. When 600 μM EGTA was added to the medium, this pattern was reversed, with NFAT1 cytosolic and NFAT2 in the nucleus (Figure 4.5). In excess EGTA there was no NFAT nuclear localization, confirming that both NFAT1 and NFAT2 are dependent on the influx of extracellular calcium for activation. Thus, decreasing calcium signaling in T cells is sufficient to account for the altered NFAT1/2 regulation observed in anergic cells.

A**B**



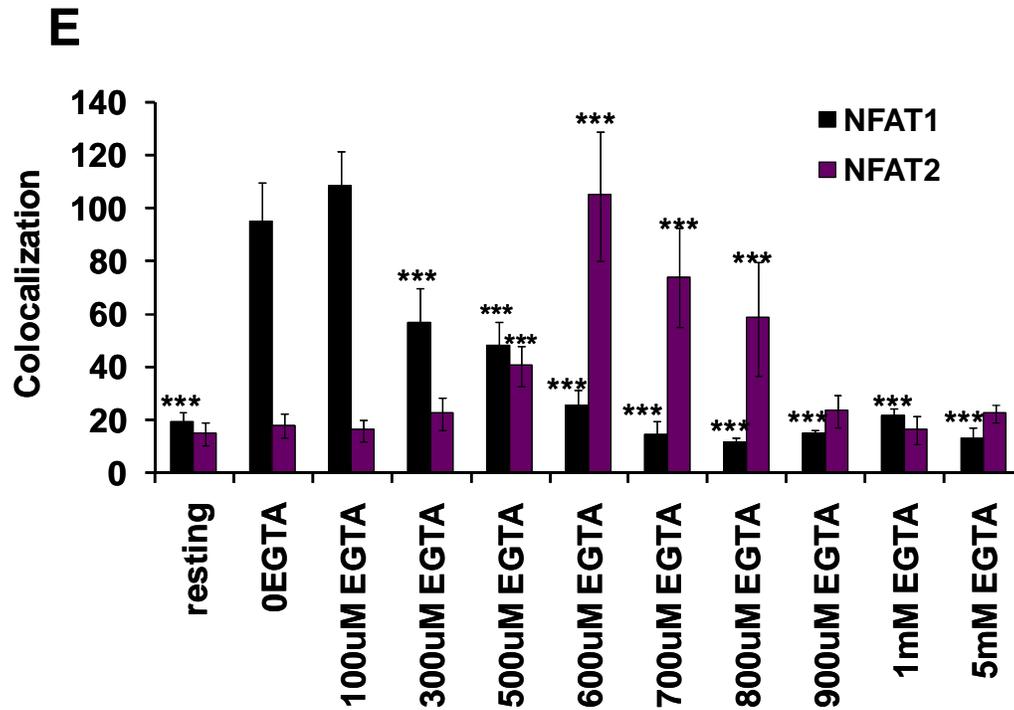


Figure 4.5: Responsive T cells display the anergic NFAT1/2 translocation pattern when extracellular calcium availability is limited by the calcium chelator EGTA. A. Measurement of calcium flux in naive T cells stimulated in the presence of 600 μ M EGTA. Naïve T cells were loaded with Fluo3 and Fura red, and calcium flux was measured as the ratio of Fluo3/Fura red fluorescence levels by flow cytometry. Basal fluorescence levels were measured for ~60 seconds, followed by stimulation of the cells with anti-CD3 and crosslinking anti-hamster IgG antibodies in the presence of 600 μ M EGTA. NFAT1 (B), and NFAT2 (C) localization was visualized using IF microscopy in resting cells (panels A,D,G), and in cells stimulated for 5 minutes in the absence (panels B,E,H) or in the presence of 600 μ M EGTA (C,F,I). Cells were stimulated as described above, and nuclear localization of NFAT1 and NFAT2 is represented as merge images

(yellow, panels G-I). **D.** Quantification of NFAT1 and NFAT2 nuclear localization in unstimulated cells (rest), and cells stimulated in the absence or presence of 600 μ M EGTA. Displayed are combined averages (\pm s.d.) from three independent experiments. 25 cells per sample were quantified for each individual experiment. Statistical significance was determined between colocalization in the presence of 600 μ M EGTA versus colocalization in the absence of EGTA, or between colocalization in unstimulated cells versus colocalization in cells stimulated in the absence of EGTA. NFAT1 colocalization between various samples, and NFAT2 colocalization analysis between samples were done independently. ***, $p < 0.001$ **E.** Quantification of NFAT1 and NFAT2 nuclear localization in cells treated with 100 μ M, 300 μ M, 500 μ M, 600 μ M, 700 μ M, 800 μ M, 900 μ M, 1mM and 5 mM EGTA and stimulated for 5 minutes. Graphs were generated with data from colocalization merge images from 25 cells per sample using ImageJ from one experiment. Statistical significance was determined between colocalization in the presence of various concentrations of EGTA versus colocalization in the absence of EGTA, and colocalization in unstimulated cells versus colocalization in cells stimulated in the absence of EGTA. Comparisons were done between independently for NFAT1 colocalization in the various samples and NFAT2 colocalization for the samples. ***, $p < 0.001$

In order to confirm that the effects of restricting calcium were not an artifact of using EGTA, the NFAT1 and NFAT2 translocation patterns were also determined by titrating BAPTA, another calcium chelator, into the medium. The calcium response curves obtained from titrating a range of concentrations of BAPTA corresponded to distribution obtained with EGTA, with increasing higher concentrations of BAPTA attenuating the peak influx of calcium after TCR stimulation. NFAT1 and NFAT2 showed normal distribution in naïve stimulated cells at lower range of BAPTA concentrations used. NFAT1 was preferentially localized in the nucleus while NFAT2 remained cytosolic. At 300 μ M BAPTA, NFAT2 was detected in the nucleus, while NFAT1 localization was predominantly cytosolic, while both NFAT1 and 2 remained cytosolic in cells treated with higher concentrations of BAPTA (Figure 4.6). There was a higher degree of variability concerning the BAPTA concentrations that induced the pattern shift, compared to the consistent shift seen when cells were treated with 600 μ M EGTA. However, the nuclear localization patterns of NFAT2 were always similar to the pattern shift that was seen with EGTA treatment, although the concentrations of the chelator used were shifted.

As a more direct approach I also looked at NFAT1 and 2 cellular distributions in naïve T cells resuspended in medium with defined calcium concentrations. Pharmacological compounds such as EGTA and BAPTA might perturb the cells in ways unrelated to the calcium signals themselves, whereas directly limiting calcium would circumvent any of

5 minutes

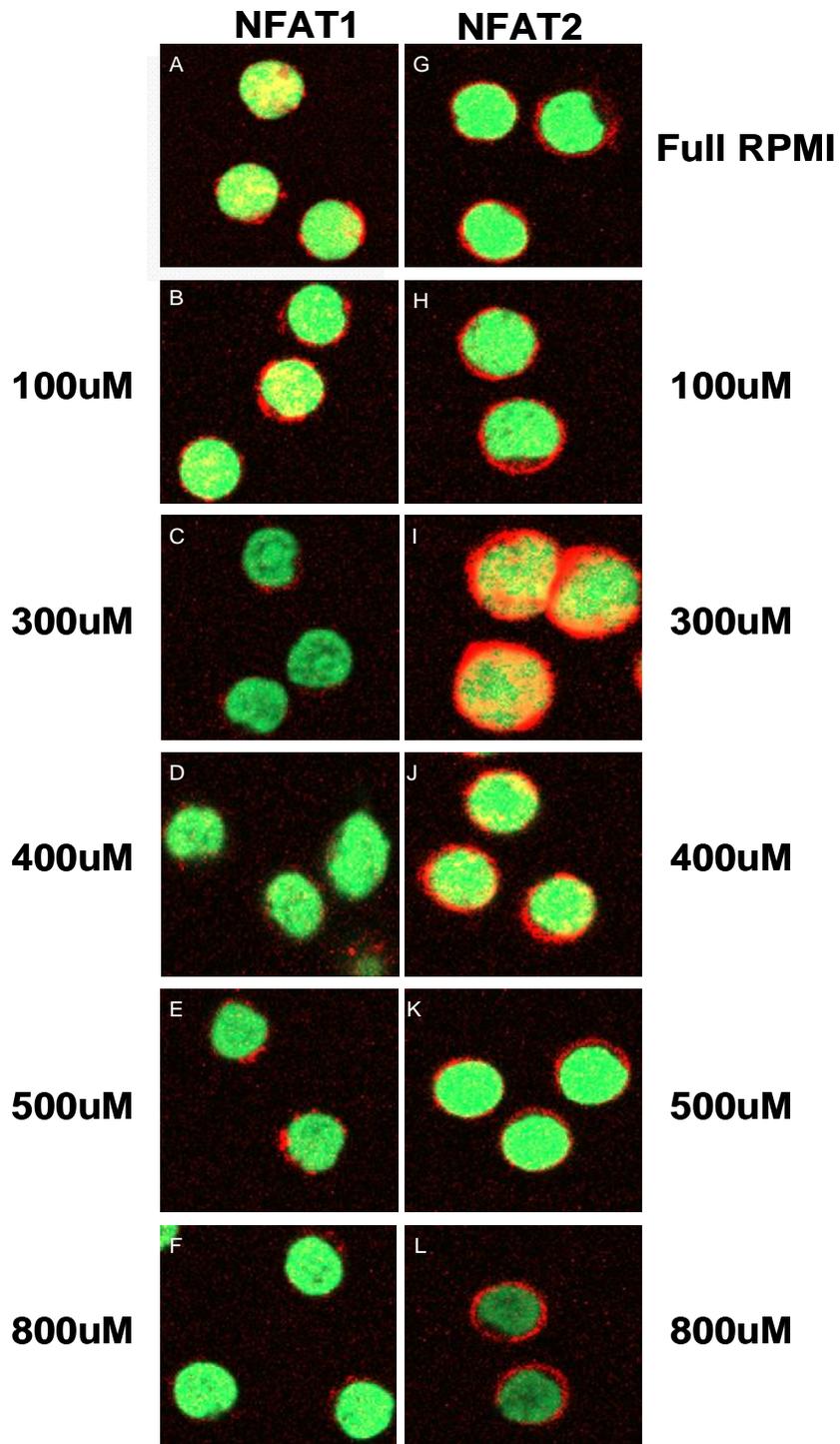
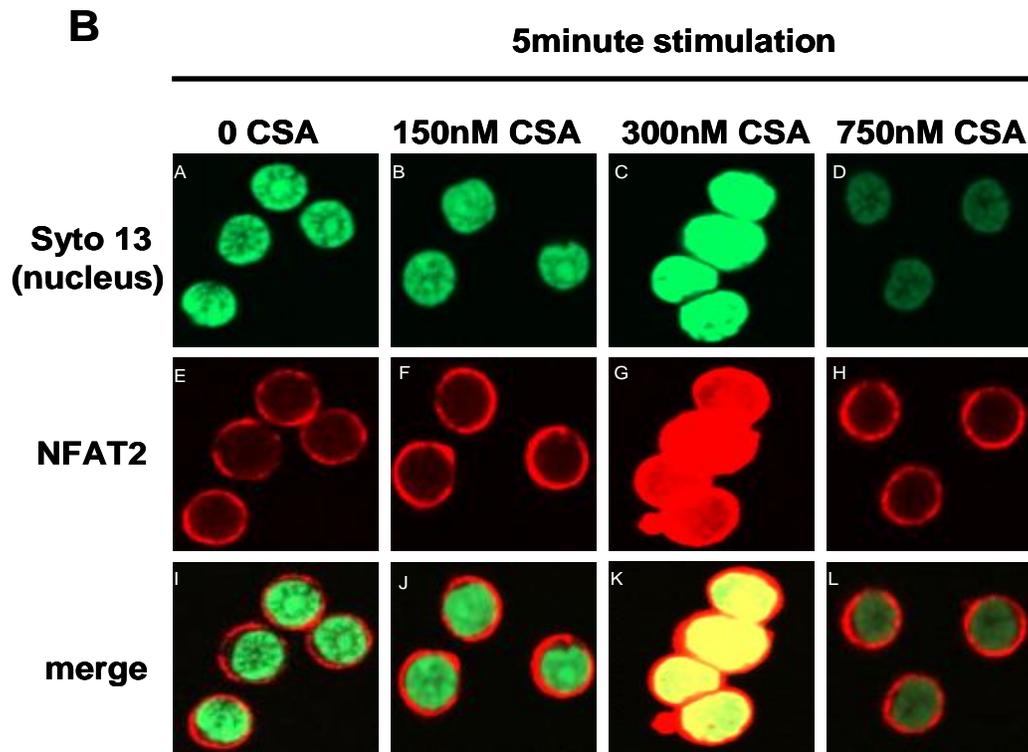
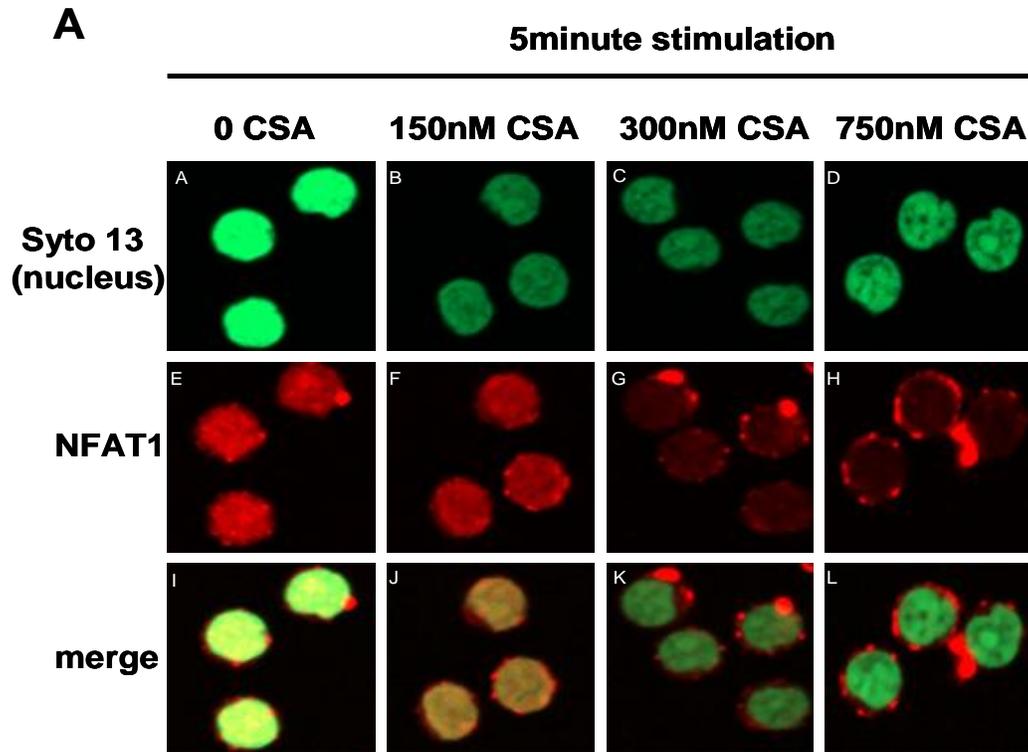


Figure 4.6: Differential NFAT1/2 translocation patterns are seen in responsive T cells stimulated in the presence of the calcium chelator BAPTA. A. NFAT1 and NFAT2 nuclear translocation patterns were determined in naïve (responsive) T cells stimulated with anti-CD3/anti-CD28 and crosslinking anti-hamster IgG antibodies for 5 minutes in the presence of 0, 100 μ M, 300 μ M, 400 μ M, 500 μ M and 800 μ M BAPTA by IF microscopy. Cells were incubated with anti-NFAT1/2 antibody followed by Alexa Fluor 594 anti-mouse IgG antibody (red). Nuclei were stained with SYTO13 (green). Shown are the merge images for NFAT1/2 and the nuclei, which depict colocalization (yellow). Shown is a representative image of two independent experiments.

these unwarranted effects. At calcium concentrations corresponding to the amount of calcium present in RPMI-1640 (500 μ M), NFAT1 and NFAT2 cellular distributions were similar to cells in complete medium. However, there was little if no consistency in the correlation between calcium concentrations and the translocation patterns during different experimental trials. It is possible that directly reducing calcium in the medium might have resulted in differences in the ionic strength that could affect calcium signaling and NFAT nuclear trafficking or had other effects on the cells.

4.2.4 NFAT2 localization in T cells is controlled at the level of nuclear import

The results described above led me to hypothesize that the altered pattern of NFAT1 and NFAT2 localization in anergic, and calcium-limited cells might be due to differential sensitivities of the two proteins to calcineurin. To confirm this hypothesis, we treated cells with cyclosporin A to probe the effects of partial inhibition of calcineurin on nuclear localization of the two isoforms. Cyclosporin treatment also allowed us to specifically target the activity of calcineurin. Thus, any other molecular modifications ensuing from limiting calcium to the cells were avoided. Naïve T cells were stimulated in the presence of titrated concentrations of cyclosporin A, and the cellular localization patterns were determined by immunofluorescence. As shown in Figure 4.7, partial inhibition of calcineurin with 300 nM cyclosporin A blocked NFAT1 translocation but induced NFAT2 translocation, similar to the effects of 600 μ M EGTA. Complete inhibition of calcineurin with 750 nM cyclosporin A caused cytosolic retention of both isoforms. Thus, a partial reduction in calcium signaling, by reducing either calcium influx or calcineurin activity, mimics the effects of T cell anergy on NFAT1 and NFAT2 localization patterns.



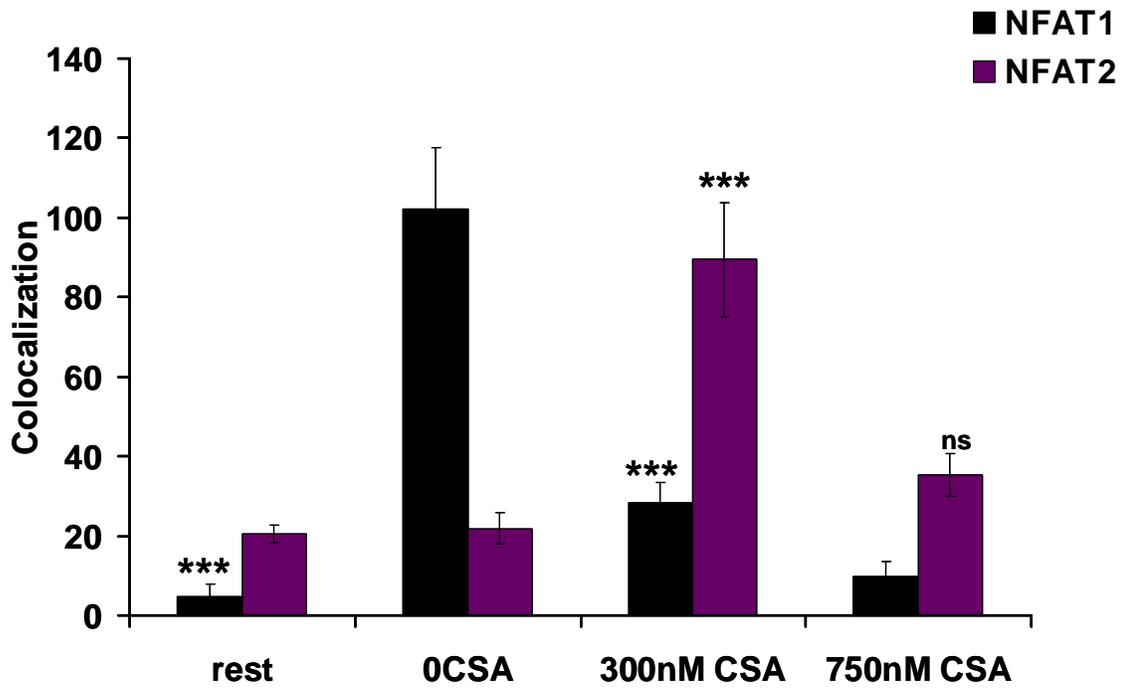
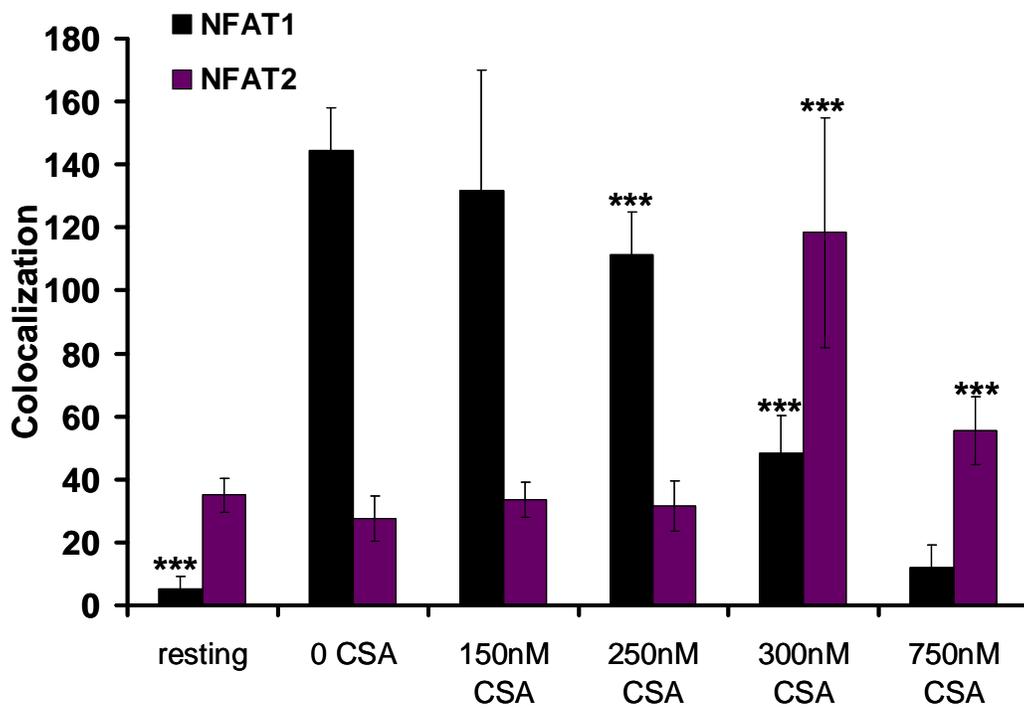
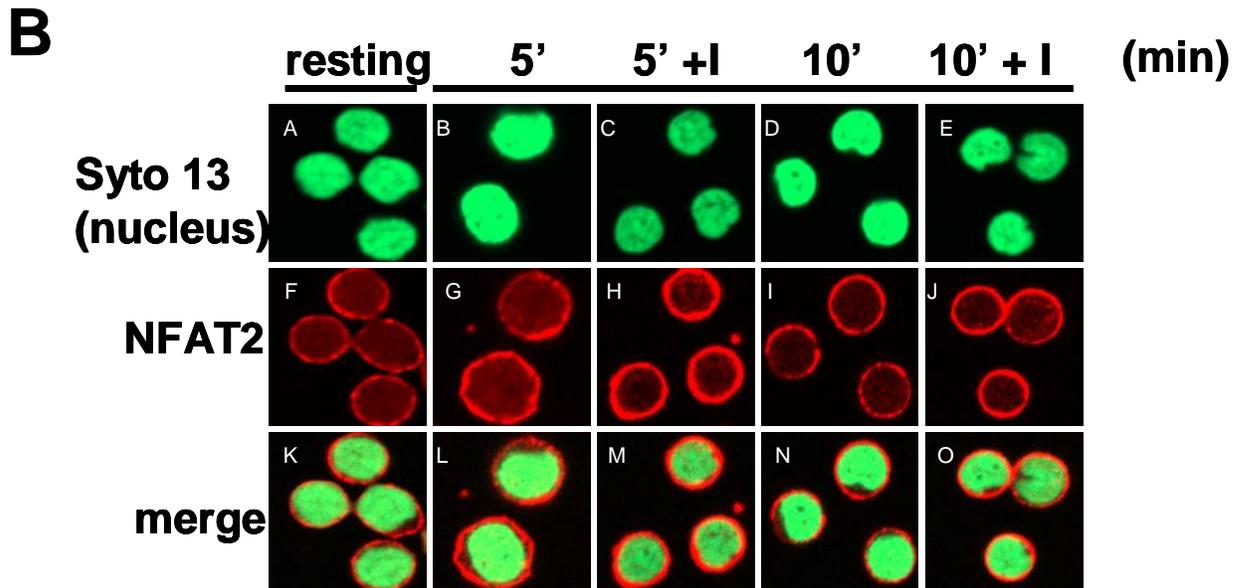
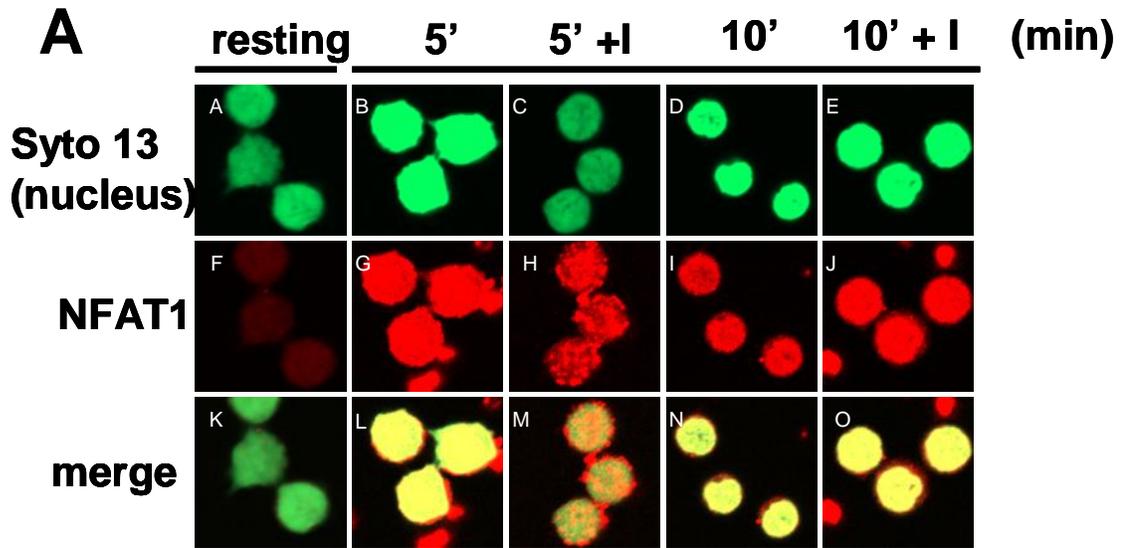
C**D**

Figure 4.7: NFAT1 and NFAT2 reciprocal nuclear translocation is regulated by calcineurin activity. Purified naïve T cells were left untreated or treated with 150nM, 300nM and 750nM of cyclosporin A and stimulated for 5 minutes as described above. The nuclei were stained with SYTO 13 (green). NFAT1 (**A**) and NFAT2 (**B**) nuclear localization (panels I, J, K and L) was visualized by immunofluorescence using Alexa Fluor 594 anti-mouse IgG antibodies to detect anti-NFAT1 or anti-NFAT2 (red), and quantified using Image J, as in Figure 4.3 (**C,D**). **C.** Displayed are combined averages (\pm s.d.) Statistical significance was determined between colocalization in the presence of EGTA and other concentrations of EGTA or unstimulated cells for both NFAT1 and NFAT2. 25 cells per sample were quantified in each individual experiment. Statistical significance was determined between colocalization in the presence of various concentrations of CSA versus colocalization in the absence of CSA, or colocalization in unstimulated cells versus colocalization in cells stimulated in the absence of CSA. Significance values were generated for comparisons between colocalization of NFAT1 in the various samples. NFAT2 colocalization analysis was done independently, using the same comparisons. ***, $p < 0.001$. **, $p < 0.01$. **D.** Quantification of unstimulated cells (resting) and cells stimulated in the presence of 0 CSA, 150nM CSA, 250 nM CSA, 300nM CSA and 750nM CSA. Shown are averages (\pm s.d.) from 25 cells per sample from one experiment. Statistical significance was determined between colocalization in the presence of various concentrations of CSA versus colocalization in the absence of CSA, or colocalization in unstimulated cells versus colocalization in cells stimulated in the absence of CSA. Significance values were generated for comparisons between

colocalization of NFAT1 in the various samples. NFAT2 colocalization analysis was done independently, using the same comparisons. ***, $p < 0.001$

The nuclear localization of NFAT1/2 is determined by a balance in the rates of dephosphorylation by calcineurin and rephosphorylation of the same serine residues by NFAT kinases, which promote nuclear export. Some of these kinases, such as GSK3 [153] and CK1 [139], act on both isoforms. Other kinases show specificity for one isoform, with p38 MAPK acting on NFAT1 [144] and JNK acting on NFAT2 [146]. To discern the effect of these proteins on the nuclear residence of NFAT1 and NFAT2, I stimulated T cells in the presence of pharmacological inhibitors of p38 and JNK and determined translocation patterns. The p38 inhibitor SB203580 had no effect on NFAT2 (Figure 4.8). This is consistent with a role for p38 being a NFAT1 specific kinase. However, inhibition of JNK did not lead to increased nuclear NFAT2 out to 10 minutes of stimulation (Figure 4.9 A-C). The working concentration of the p38 inhibitor SB203580 was obtained from previously published studies while the concentrations used for the JNK inhibitor SP600125 was obtained by measuring inhibition of phosphorylation of c-Jun, a substrate of JNK activity (Figure 4.9 D). From these results, it was inferred that regulation of NFAT2 in naïve T cells is upstream of nuclear entry.

To confirm that the cytosolic localization of NFAT2 in stimulated naïve T cells was not actually due to rapid export, I stimulated T cells in the presence of the nuclear export inhibitor leptomycin B, and examined the pattern of NFAT1 and NFAT2 localization. As shown in Figure 4.10, treatment with 200nM leptomycin retained NFAT1 in the nucleus out to 30 minutes after stimulation. However, NFAT2 remained cytosolic even in the presence of leptomycin. The enhanced nuclear localization of NFAT1 in cells stimulated for 30 minutes, confirms that leptomycin B functions as a nuclear export inhibitor.



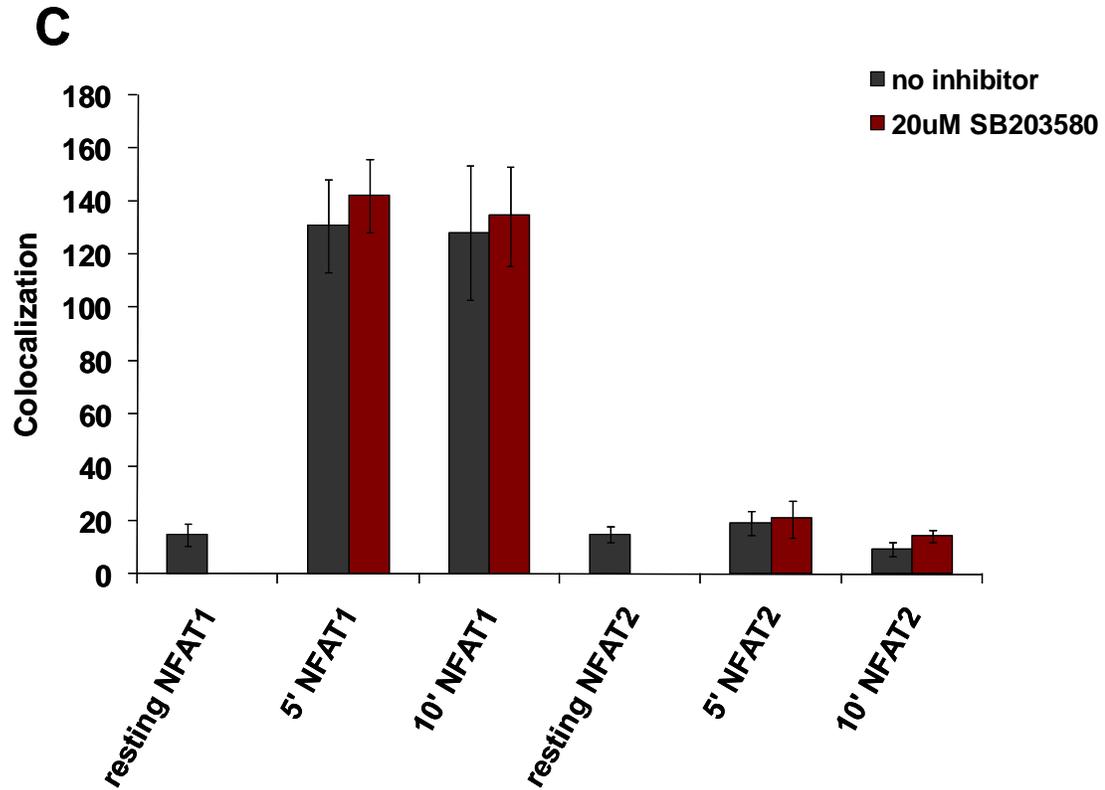
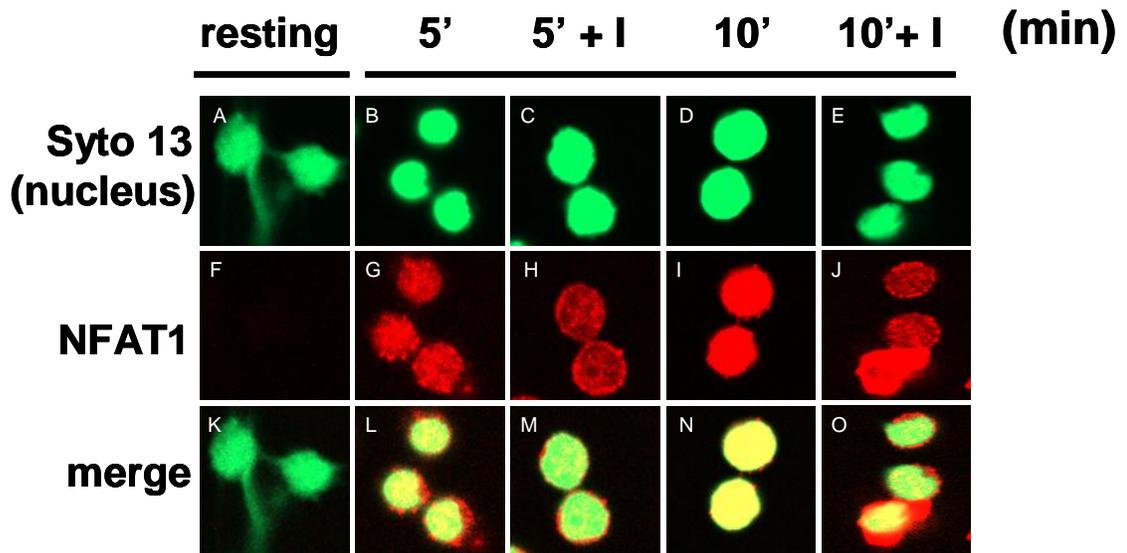
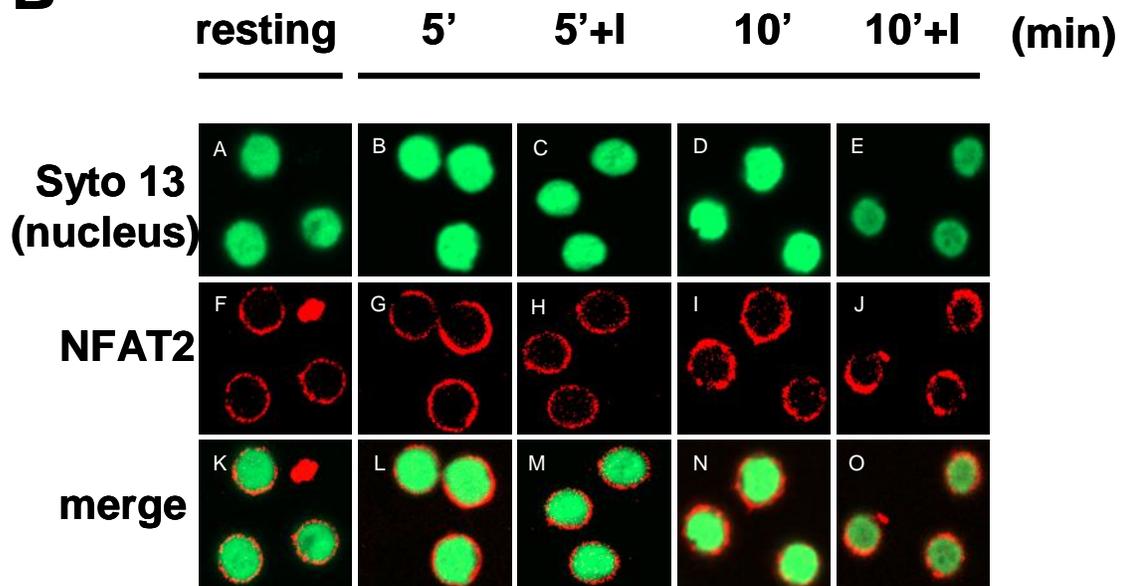


Figure 4.8: p38 inhibition does not affect NFAT2 cellular localization in responsive T cells. NFAT1 and NFAT2 nuclear translocation patterns were determined for naïve T cells treated with the p38 inhibitor SP230580. T cells were stimulated with anti-CD3/anti-CD28 and secondary crosslinking anti-Hamster IgG antibodies for 5 and 10 minutes in the presence or absence of 20 μ M SB230580 or left unstimulated. NFAT1 (A) and 2 (B) nuclear translocation patterns were determined by immunofluorescence, and represented as colocalization (yellow, panels K-O) between NFAT1/2 (red) and Syto 13 (green). C. Colocalization was quantified using ImageJ with data from 25 cells per sample from one experiment. Displayed are averages (\pm s.d.).

A**B**

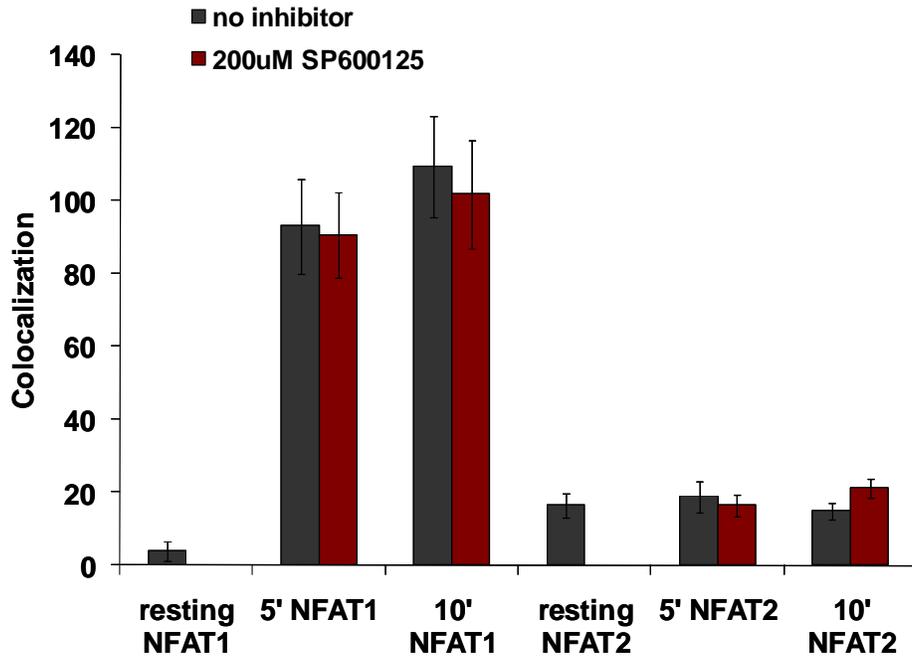
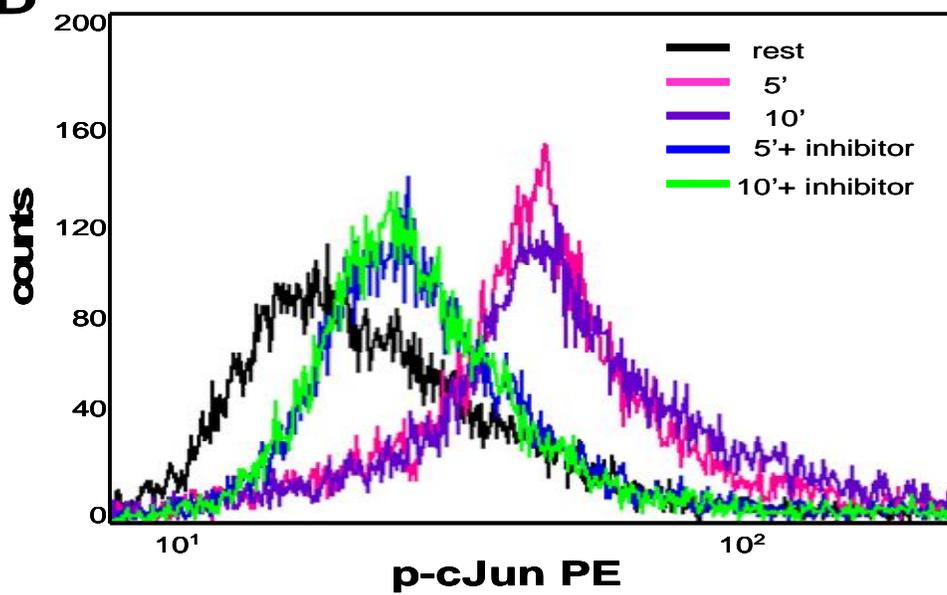
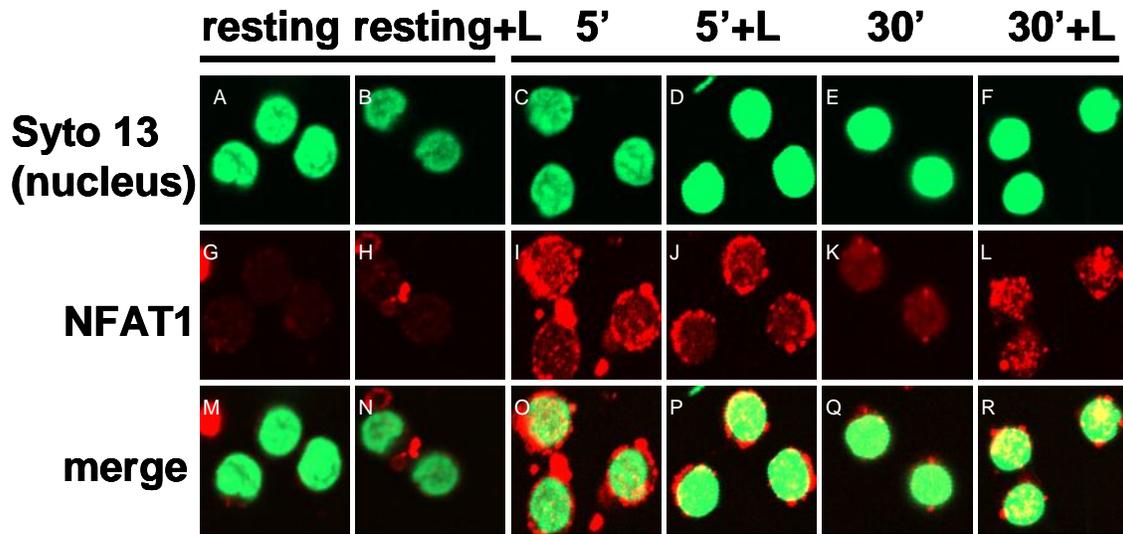
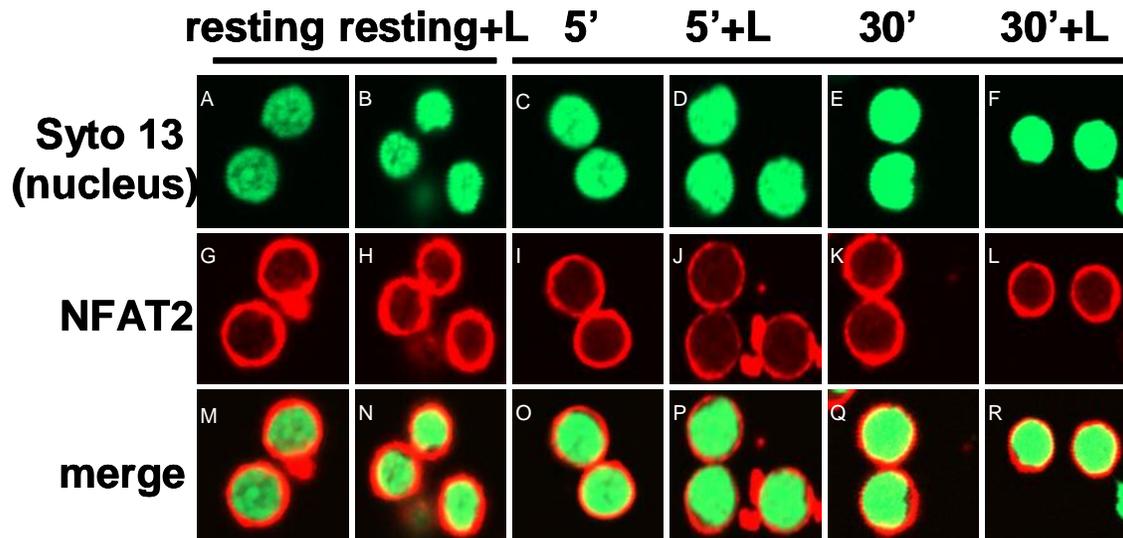
C**D**

Figure 4.9: Inhibiting Jnk activity does not affect translocation patterns of NFAT2 in responsive T cells. NFAT1 and NFAT2 cellular localization was determined in naïve T cells, stimulated in the presence of the JNK inhibitor SP600125. T cells were stimulated as described for 5 and 10 minutes in the presence or absence of 200 μ M SP600125 or left unstimulated. NFAT1 (**A**) and 2 (**B**) nuclear translocation patterns were determined by immunofluorescence and represented as colocalization (yellow, panels K-O) between NFAT1/2 (red) and SYTO 13 (green). **C.** Colocalization was quantified using ImageJ with data from 25 cells per sample for each individual experiment. Shown are averages (\pm s.d.) from two independent experiments. **D.** Inhibition of JNK activity by SP600125 was determined by phosphorylation of c-Jun. Naïve T cells were stimulated for 5 and 10 minutes, in the presence or absence of 200 μ M SP600125, and phospho c-Jun values were determined by intracellular staining with PE- tagged antibodies by flow cytometry. Shown is a representative image of three independent experiments.

A



B



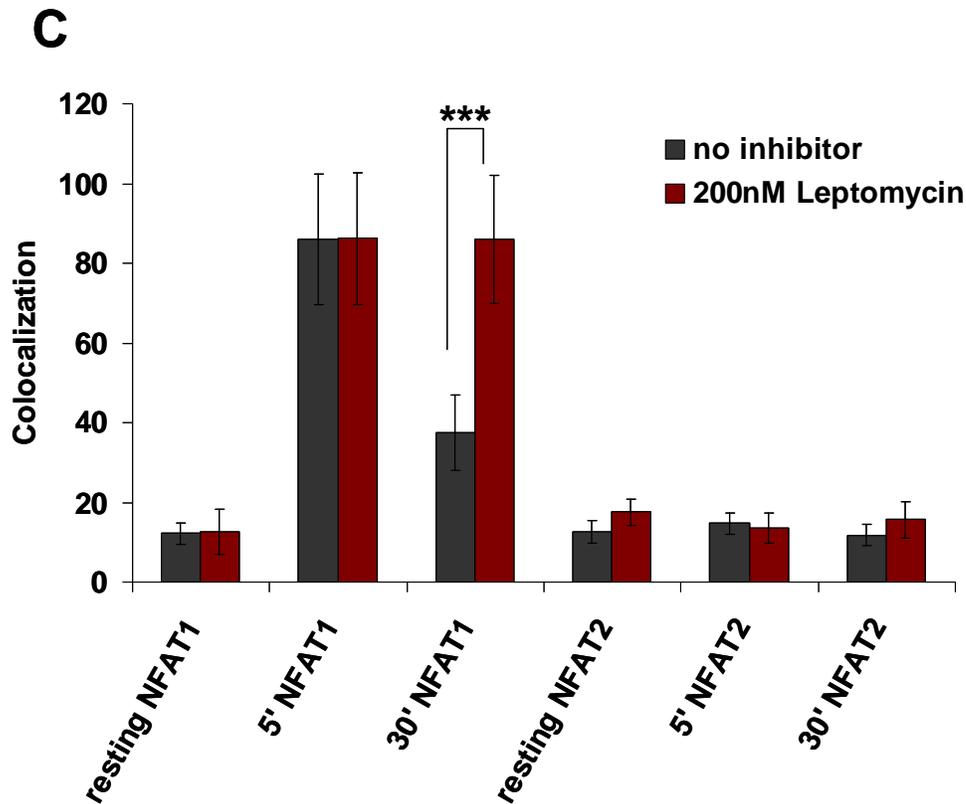


Figure 4.10: NFAT2 localization is controlled at the level of nuclear import. NFAT1 (A) and NFAT2 (B) nuclear translocation in the presence of the nuclear export inhibitor Leptomycin B was determined by immunofluorescence. Responsive T cells were stimulated for 5 minutes and 30 minutes in the presence of 200 μ M Leptomycin B or left untreated. Cells were stimulated as described above, and nuclear localization was determined as the colocalization between NFAT1/2 (red) and the nuclei, stained with Syto 13 (green), represented in panels M-R. C. Colocalization was quantified using ImageJ with data from 25 cells per sample from one experiment. Shown are averages (\pm s.d.). ***, $p < 0.001$

Thus, the cellular localization NFAT2 is controlled at the level of nuclear import. Together, these results indicate that the altered regulation of NFAT translocation in anergic T cells is due to the reduced calcium flux and resultant reduction in calcineurin activity.

4.2.5 Conditions that promote NFAT2 nuclear localization in naïve T cells inhibit T cell proliferation.

NFAT1 and NFAT2 have been shown to bind the IL-2 promoter ARRE2 site in vitro [154]. In vivo, a cooperative interaction with AP-1 at the IL-2 promoter sites results in transcriptional activation. This suggests that under normal conditions of activation, when AP-1 formation is not affected, NFAT1 or NFAT2 nuclear residence and subsequent activation should result in IL-2 production and proliferation. To determine the proliferative ability of naïve T cells under conditions of NFAT2 nuclear entry, whole spleen cells were stimulated for 72 hours using titrated concentrations of anti-CD3 antibody in the presence of 600 μ M EGTA or 300nM CSA. Whole spleen cells were used to provide the antigen presenting cells that provide costimulatory signals. The T cells treated with EGTA or CSA, displayed minimal to negligible proliferation and cell expansion (Figure 4.11 A). IL-2 production was also minimal in the cells that were stimulated in the presence of 600 μ M EGTA or 300nM CSA (Figure 4.11 B). To ascertain that AP-1 formation is not affected by EGTA or CSA treatment, I looked at the phosphorylation of c-Jun and total c-fos levels in naïve T cells that were stimulated under conditions that promote NFAT2 nuclear entry. Phosphorylation of c-Jun was comparable in T cells treated with EGTA or CSA and cells left untreated up to 30 minutes after stimulation. Similarly, there were no differences in total c-fos levels during early

activation of T cells between EGTA or CSA treated cells, and untreated cells (Figure 4.12). This suggests that the differences in IL-2 cytokine production and proliferative ability are not due to defective assembly of the AP-1 protein. T cells treated with EGTA and CSA differ from their untreated counterparts only in the NFAT2 versus NFAT1 nuclear localization, upon stimulation. The inability to produce IL-2 is therefore an inherent characteristic of the NFAT2 that is found in the nucleus in these cells. Thus, though NFAT2 has been shown to bind the IL-2 promoter in vitro, it does not initiate IL-2 transcription in naïve T cells. This suggests that NFAT2 has distinct function from NFAT1, at least in the transcriptional activation of IL-2. Stimulation of naïve T cells under conditions that promote NFAT2 nuclear localization, results in an unresponsive state, similar to anergy.

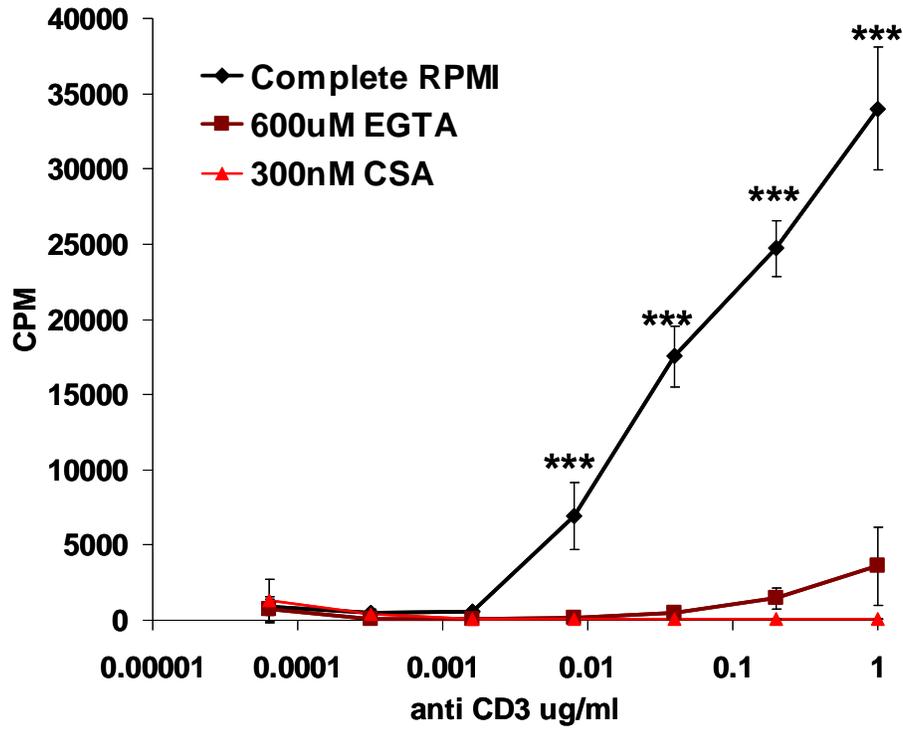
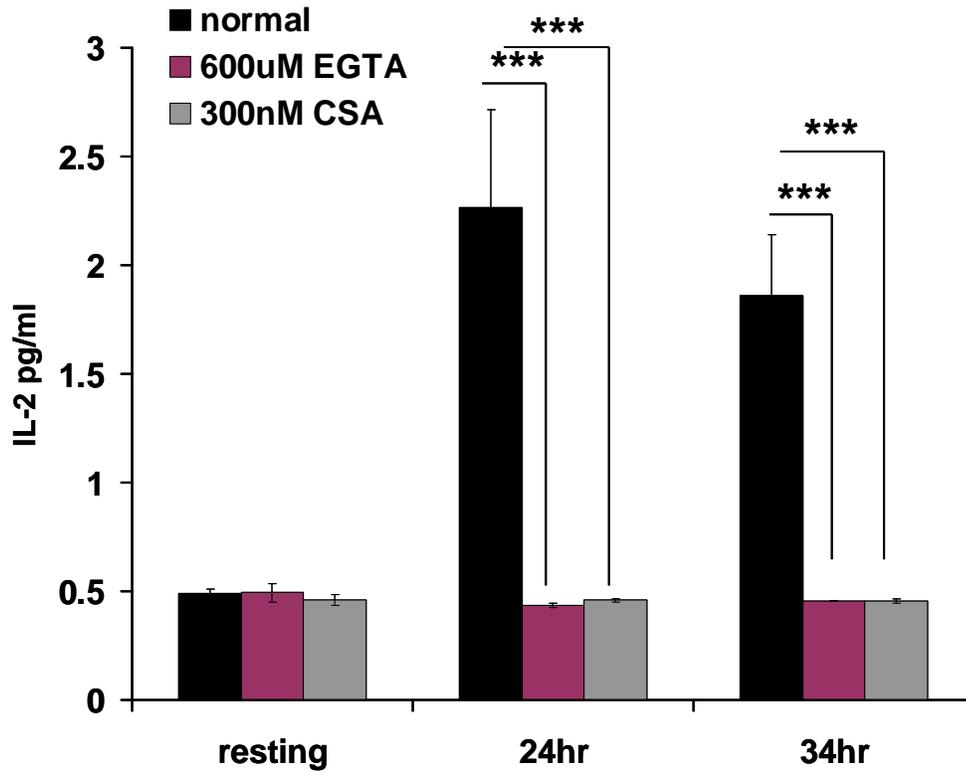
A**B**

Figure 4.11: T cell proliferation and IL-2 production is inhibited in conditions that promote NFAT2 nuclear localization. **A.** Proliferation measurements for naïve T cells stimulated with anti-CD3 antibodies and splenic APCs in the presence of 600 μ M EGTA or 300nM CSA. Splenic cells were stimulated in the presence of graded concentrations of anti-CD3 antibodies and EGTA/CSA for 72 hours at 37°C. [3 H] Thymidine was added to the cells during the last 8 hours of stimulation. Proliferation was assayed by [3 H] thymidine uptake in the cells. Shown is a representative of three independent experiments. Statistical significance was determined between proliferation using complete RPMI and both treatments. ***, $p < 0.001$ **B.** IL-2 production in naïve T cells was determined by ELISA. 1×10^6 purified T cells were stimulated in the presence of EGTA or CSA, with anti-CD3/anti-CD28 coated beads for 24 and 34 hours, and the supernatants were assayed for IL-2 production by sandwich ELISA. ***, $p < 0.001$

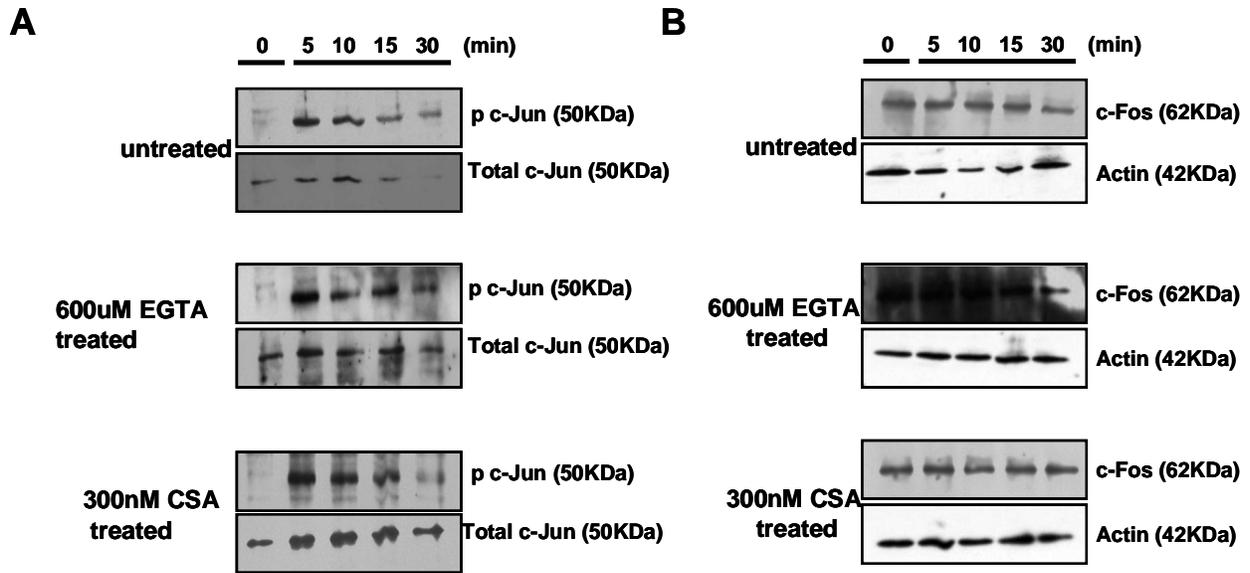


Figure 4.12: AP-1 components are unaltered by conditions that promote NFAT2

nuclear localization. Phospho c-Jun levels and c-fos levels were assayed by western blot analysis. Responsive T cells were stimulated in the presence of 600 μ M EGTA or 300nM CSA with anti-CD3/anti-CD28 coated beads for 5, 10, 15 and 30 minutes, or left unstimulated. Control cells were stimulated in the absence of EGTA or CSA. Cells were then lysed with RIPA buffer and assayed by western blot for (A) phospho c-Jun using antibodies specific for phosphorylation at Ser63 and (B) c-fos. Total c-Jun and actin levels were also assayed for as loading controls for A and B respectively. Shown are representative images of two experiments.

4.3 Discussion

Intracellular mobilization of calcium is an important target of modulation in anergic T cells. NFAT regulation is one of the important downstream effects of calcium signaling in T cells. I therefore focused on the NFAT transcription factor family members NFAT1 and NFAT2 and their cellular distribution, as these isoforms are expressed in peripheral T cells and nuclear translocation of these isoforms requires the activation of the calcium/calmodulin-dependent phosphatase calcineurin [69, 131].

NFAT1 distribution was nuclear within five minutes of stimulation in naïve T cells, but NFAT1 failed to translocate in anergic T cells. The failure of NFAT1 to accumulate in the nuclei of anergic T cells could be explained by a decrease in nuclear import (due to reduced calcineurin activity) or an increase in nuclear export (via activation of nuclear NFAT kinases). The pattern of NFAT1 localization in anergic T cells treated with the nuclear export inhibitor leptomycin was examined, to distinguish between these options. Treated cells showed an increase in nuclear NFAT1, but required prolonged TCR ligation to approach the level seen in stimulated naïve T cells. Thus, although there may be some increase in NFAT kinase activity in anergic cells, this is unlikely to account fully for the blockade in nuclear NFAT1 accumulation. I hypothesize that, in concert with any increased NFAT kinase activity, the reduced calcium flux lowers calcineurin activity enough to shift the balance from import to export.

Interestingly, NFAT1 was undetectable in resting cells, but was detected in the cytosol after two minutes of stimulation in both naïve and anergic T cells. As I could detect

NFAT1 in Western blots of lysates from resting cells, this result suggests that nuclear localization of NFAT1 occurs in at least two distinct steps. First, TCR ligation causes a conformational shift in NFAT1, or induces release of a binding partner, which reveals the antibody epitope. This event appears to be independent of calcium flux, or has a relatively low threshold, as it occurs in both naïve and anergic T cells. The second step is the dephosphorylation and translocation of NFAT1, which is sensitive to calcium flux and is impaired in anergic cells. Although we hypothesize that these two steps are independently regulated by multiple pathways, they might both be mediated by calcineurin. Calcineurin dephosphorylates up to 20 serine residues on the serine rich region (SRR) and the serine proline xx-repeat motif (SP)₁ domains of NFAT1 in a specific order [73]. Dephosphorylation of the first residues may have a lower calcium requirement, whereas the final steps in uncovering the nuclear localization signal (NLS) require higher calcium concentrations. Further characterization of this process may reveal novel drug targets for the regulation of T cell activation.

I also determined the cellular distribution of NFAT2 in anergic T cells. Although NFAT1 and NFAT2 show a high degree of homology in their DNA-binding Rel similarity domain (>70%) [62, 131], their translocation patterns after stimulation differed in naïve and anergic T cells. To my knowledge, this observation had not previously been reported. Unlike NFAT1, there was no nuclear translocation of NFAT2 for at least 30 minutes after stimulation of naïve T cells. Further, stimulation of anergic cells did lead to nuclear localization of NFAT2 within five minutes. Thus NFAT1 and NFAT2 seem to be reciprocally regulated in naïve and anergic T cells.

By limiting calcium in the culture medium, I was also able to demonstrate that the reduction in calcium influx in anergic T cells is sufficient to account for the switch from NFAT1 to NFAT2 as the predominant translocating NFAT family member. Analysis of the nuclear translocation pattern of NFAT1 and 2 using the pharmacological inhibitors cyclosporin (inhibits calcineurin) and leptomycin (inhibits nuclear export) led to the conclusion that the reciprocal regulation takes place at the level of dephosphorylation prior to nuclear import. The involvement of nuclear NFAT kinases, which rephosphorylate the serine residues targeted by calcineurin, in controlling NFAT2 localization in T cells seems to be limited, as stimulating naïve T cells in the presence of a JNK inhibitor during stimulation did not retain NFAT2 in the nucleus. Together, these results support a model in which localization of NFAT2 is controlled mainly by calcium-dependent calcineurin activity, rather than rephosphorylation and nuclear export by JNK and other NFAT kinases.

The reciprocal regulation of cellular distribution of NFAT1 and NFAT2 is not just a unique characteristic of CD8⁺ anergic T cells, but can be initiated in a mixed population of naïve T cells by modulating calcineurin activity. This phenomenon of differential regulation has also been reported in other cell types. Developing skeletal muscles have been reported to show NFAT2 nuclear localization in conjunction with cytosolic NFAT1 [155]. The differences have been attributed to the activity of NFAT kinases that mediate export. This is the first instance where differential regulation upstream of nuclear entry has been reported. Increased knowledge about the various proteins that interact with

NFAT1 and NFAT2 in the cytosol and function as scaffolding proteins will aid in understanding the mechanism of NFAT1/2-specific regulation. Park et al, have identified a second calcineurin binding site for NFAT2 and NFAT4, toward the C terminal end of the regulatory domain [70]. This region is variable from the conserved PxIxIT arrangement and has been found to be necessary for calcineurin interaction with NFAT2 and 4 in vivo. Interestingly, the peptide stretch corresponding to the second calcineurin binding region in NFAT1 did not mediate calcineurin binding in vitro. Calcineurin binding to this second site is therefore NFAT2-specific and could have important implications in NFAT1/2 dephosphorylation and cellular localization. For instance, the presence of two binding sites might decrease the concentration of calcineurin required to effect dephosphorylation and movement into the nucleus. Thus, the decreased calcium influx could stimulate sub-optimal calcineurin activity enough for NFAT2 regulation, but lower than the threshold required for NFAT1 binding and subsequent dephosphorylation.

Proteins that actively compete with calcineurin for NFAT binding have been identified for the PxIxIT binding site, common in all the members. The protein Homer, for instance, has been shown to bind the calcineurin binding site that is closest to the N terminus of the regulatory domain [147]. Mutational analysis has also shown that Homer does not bind to residues that have been implicated in the second calcineurin binding site of NFAT2 [147]. The cytosolic organization of the NFAT-scaffolding protein complex might play an important role in the accessibility of calcineurin or the facilitation of translocating into the nucleus. Many molecules such as CABIN1 [77] and the DYRK kinases [143] have been identified to bind NFAT in the cytosol. A thorough analysis of the specific NFAT

proteins these molecules bind needs to be undertaken, to obtain a more comprehensive picture of NFAT cytosolic regulation upstream of nuclear entry. Differential regulation of NFAT1 and NFAT2 can be achieved by the individual activity or by concerted action of a number of NFAT interacting proteins, or by modulating upstream signaling events that feed into NFAT regulation.

It is interesting to note that altering the NFAT1/NFAT2 nuclear entry patterns in naïve T cells is sufficient to inhibit proliferation and IL-2 production. This suggests that NFAT1 and 2 have inherently different functions in the transcriptional activation of IL-2. It is tempting to speculate that NFAT2 might repress IL-2 transcription while NFAT1 might be the positive regulator of transcription, although detailed DNA binding and transcriptional analysis is required to confirm or disprove this hypothesis. Most studies regarding NFAT1 or 2 binding to the IL-2 promoter have been done using in vitro gel shift assays, which do not provide any information about positive or negative regulation of the gene involved. Analysis of expression patterns of genes such as TNF α [156, 157] and IL-4 [158] in T cells, and IL-13 [159] in mast cells have shown differences in DNA binding patterns and transcriptional activity of NFAT1 and NFAT2. Thus NFAT1 and NFAT2, in spite of their similarities in DNA binding in vitro, might have a differential contribution to the physiological gene expression patterns of genes that have NFAT binding consensus sequences in their promoter/enhancer sites.

This difference in NFAT utilization between responsive and anergic cells could be significant, in the establishment and maintenance of the anergic phenotype. One

hypothesis is that NFAT1 is responsible for the regulation of activation induced genes (e.g. IL-2), whereas NFAT2 is important for the expression of anergy related genes. Identification of NFAT2 controlled genes may therefore shed light on the factors responsible for maintaining the hyporesponsiveness of anergic T cells. It is important to distinguish this from the events involved in establishing the anergic state, which occur during the initial exposure of T cells to antigen and are likely to involve NFAT1-regulated genes [83].

Despite the high degree of homology between NFAT1 and NFAT2, they have previously been shown to be non-redundant in T cells [69, 139, 144, 146]. Notably, NFAT1 and NFAT2 knockouts mice have different immunological phenotypes. NFAT1 deficient T cells are skewed toward a Th2 response, while NFAT2 knockout cells have a Th1 bias [65, 67, 68, 160]. Although it is not clear how this may relate to T cell anergy, particularly in CD8⁺ T cells, our observations suggest that regulation of calcium flux may also play a previously unsuspected role in T cell differentiation. Strong calcium signaling during stimulation may bias T cells toward a Th1 program by activating NFAT1, while sub optimal calcium signals would preferentially activate NFAT2, directing cells toward a Th2 bias. Investigation of the factors involved in the fine control of cellular calcium levels may therefore uncover novel targets for immunomodulation.

5. Conclusion

Anergy as a mechanism of tolerance has been extensively studied using various models of induction. T cells that have been stimulated in anergizing conditions, all primarily display hyporesponsiveness and decreased clonal expansion, but there is no consensus on the molecular modulations that effect these functional changes. Presently, all these experimental *in vitro* and *in vivo* models of induction have been included in the definition of anergy, but may represent distinct sub optimally activated states of T cells. The comprehensive characterization of the signaling and transcriptional modifications corresponding to the different anergy models is necessary, for the further use of T cell anergy as a means of controlling T cell activation and function, primary goals in studies of autoimmunity, transplantation and tumor immunotherapy.

The primary goal of my thesis is the characterization of the anergic phenotype of CD8⁺ T cells that interact with their cognate antigen *in vivo*, in a tolerogenic setting. Given the underlying biochemical differences and similarities in the various *in vitro* and *in vivo* models, I have attempted to characterize the important signaling manifestations that are exhibited during the secondary re-stimulation phase of anergy. Two major signaling pathways frequently found to be altered in anergic T cells are the ras/MAPK cascade and calcium signaling [92]. Although I could not detect consistent defects in MAPK activation in our model of *in vivo* anergy induction, I found that calcium responses in anergic cells were blunted relative to naïve cells. This is consistent with multiple other *in vivo* anergy models, spanning different induction methods and both CD4⁺ and CD8⁺ T

cells [96, 118, 126], suggesting that the calcium limitation is a common feature of *in vivo* T cell anergy.

The decrease in the intracellular mobilization of calcium can be attributed to modulations in the proximal signaling events after TCR engagement. The activation of PLC γ that leads to the generation of IP₃ appears to be the significant cause of lower calcium influx rates. Other systems have also reported alterations in the phosphorylation patterns of other molecules such as LAT and ZAP-70, along with a general decrease in the level of tyrosine phosphorylation. I have not examined these patterns, but reduced phosphorylation of PLC γ , which occurs downstream of phosphorylation of ZAP-70 and LAT, suggests that the 2C Tg anergic T cells might also display these defects.

Nevertheless, these specific signaling events need to be characterized in our system of anergy, as the network of proximal signaling immediately after TCR engagement contributes to multiple downstream effects. A detailed analysis of events that precede PLC γ phosphorylation might aid in mapping out the key regulatory events that control the hyporesponsive phenotype. Mediating complete release of ER calcium in anergic T cells generates calcium responses similar to naïve T cells. This suggests that changes in the pre-ER signals dominate the calcium phenotype in anergic T cells. There are certain caveats to this interpretation, as discussed previously. However, the determination of downstream events mediated by calcium, such as NFAT cellular translocation in anergic T cells treated with thapsigargin or cell permeable IP₃, will aid in determining if the calcium signaling defect in anergic T cells can be overcome by increasing the efficiency of ER release.

Another important consideration is the contribution of the second messengers cADPR and NAADP to calcium release from the intracellular stores. cADPR has been shown to bind the ryanodine receptors on the ER membrane [161], resulting the calcium release from the ER, in much the same way as IP₃-IP₃R interactions [35]. Administration of NAADP into T cells has also been shown to result in calcium influx through the plasma membrane channels [41]. The signals downstream of TCR engagement, that these second messengers recognize have not yet been characterized, but a potential role for these molecules in determining the calcium influx in anergic T cells cannot be overlooked. The effect of administration of cell permeable cADPR or NAADP on calcium signaling in anergic T cells would be informative in defining the role of these molecules in anergy regulation.

The demonstration of defective calcium signaling not only helps in understanding the biochemical changes occurring in anergic T cells, but also provides a system to study the underlying mechanisms of calcium regulation in T cells. In an important study, human peripheral T cells isolated from some patients afflicted with severe combined immune deficiency showed pronounced calcium mobilization defects [56]. Further genetic analyses of these T cells and cells from the kin of the patients, contributed to the identification of Orai1, a protein that serves as the functional pore forming subunit of the plasma membrane CRAC channels [54], whose molecular identity had eluded scientists for many years [26]. The T cells isolated from these patients displayed impaired cytokine production and unresponsiveness to activating stimuli [55], but were distinct from anergic

T cells in that the defect in calcium signaling was post ER in the formation of the plasma membrane calcium channels, and therefore could not be restored by enabling complete ER release of calcium. Nevertheless, these studies demonstrate that the defective calcium signaling phenotype can be utilized as a model to identify key proteins that are involved in calcium regulation in T cells.

The signaling alterations in anergic T cells cause an attenuation of intracellular calcium mobilization rather than a complete abrogation. This has been seen in my experiments with 2C TCR transgenic CD8⁺ T cells, as well as other CD4⁺ and CD8⁺ T cell anergy systems that have reported deviations in calcium signaling [118, 126]. The significance of this sub-optimal but detectable calcium influx has not been addressed before. My observations on NFAT1 and NFAT2 cellular localization properties might therefore, be important in defining the functional relevance of anergic T cells. NFAT2 has been consistently detected in the nucleus of restimulated anergic T cells, and the nuclear localization of this protein is predominantly dependent on a low intensity of calcium influx. These results, therefore, suggest that the nuclear presence of NFAT2 in anergic T cells has transcriptional significance.

Anergy is a reversible process. The proliferative ability of these cells can be restored by addition of IL-2 during restimulation [162-164]. Although the functional responsiveness of these anergy reversed T cells have been analyzed, the signaling mechanisms that underlie the restoration of T cell responsiveness has not been addressed. Studies on rapamycin induced anergy have shown that anergy reversal is inhibited in the presence

of rapamycin, implicating mTOR as a possible signaling regulator in anergy reversal [100]. IL-2/IL-2 receptor signaling is mainly effected through the downstream Jak-STAT pathways, resulting in the upregulation of anti-apoptotic genes such as Bcl-2 [165], and in CD8⁺ T cells, genes that are important for cytolytic effector functions, such as perforin and granzyme [166]. Among the other important signaling pathways that are activated by IL-2 /IL-2 receptor binding are the MAPK pathway, leading to upregulation of c-Fos and the PI-3K pathway, which mediates proliferation through multiple mechanisms [167]. It is necessary, therefore, to study the specific signaling and transcriptional effects of IL-2 on anergic T cells, to determine the signaling components important for anergy reversal.

The role of calcium signaling and the downstream NFAT activation cascade, in restoring functional responsiveness has not been characterized yet. Possible interactions between IL-2 receptor mediated signaling and the calcium-NFAT cascade might explain the potential role for NFAT in anergy reversal. Since anergy is not a terminal differentiation process, the complete abolishment of critical signaling components by ubiquitination might be counter productive to the cells. By partially impairing the important signaling cascades that mediate full activation, resulting in NFAT2 nuclear localization, anergy, in T cells might be an intermediate state that might be reverted in the presence of proliferating signals such as IL-2. It is therefore important to determine the gene expression patterns of anergy reverted T cells, in comparison to activated or anergic cells, and to also determine if there is a repression in the transcription of the anergy related genes, such as the E3 ubiquitin ligases or DGK α . An intriguing possibility then would be that NFAT2 nuclear accumulation in anergic T cells is to facilitate a transcriptional

repression of these anergy genes, when synergized with IL-2/IL-2 receptor signaling effects. This hypothesis, therefore suggests the possibility of NFAT2 functioning as a transcriptional repressor, as opposed to being a positive regulator, of the anergy genes, as was hypothesized before. Thus, rather than maintaining the anergic phenotype, NFAT2 might be the enabler of anergy reversal (Figure 5.1). Determination of cellular localization patterns of both NFAT1 and NFAT2 during anergy reversal might provide more information in proving or disproving this hypothesis. Also specific gene array analysis from anergic and anergy reverted cells might also provide information about the transcriptional regulation of the anergy related genes.

Yet another mechanism of tolerance is active suppression. Studies have shown that the state of anergy can lead to the generation of regulatory T cells [168]. In fact in a number of CD4⁺ studies, the terminology of anergy is also used to describe regulatory T cells, due to the functional unresponsiveness also seen in the suppressor cells. Regulatory T cells are characterized by the upregulation in expression of the transcription factor Foxp3 [169]. Foxp3 expression is controlled by NFAT-Smad3 cooperation [86]. This study was done looking at NFAT1-Smad3 interactions, so it is not known if NFAT2 also elicits Foxp3 transcription. Interestingly, NFAT/Foxp3/DNA interaction has also been reported [85, 132, 170, 171]. NFAT/Foxp3/DNA interactions that are formed are analogous to the NFAT/AP-1/DNA interactions, but act as functional repressors of IL-2, while promoting transcription of genes involved in the regulatory phenomenon, such as CD25 and CTLA-4 [85]. It is therefore a pertinent question to ask if NFAT2 nuclear movement is a developmental move for the anergic cell to progress into a distinct differentiation stage of

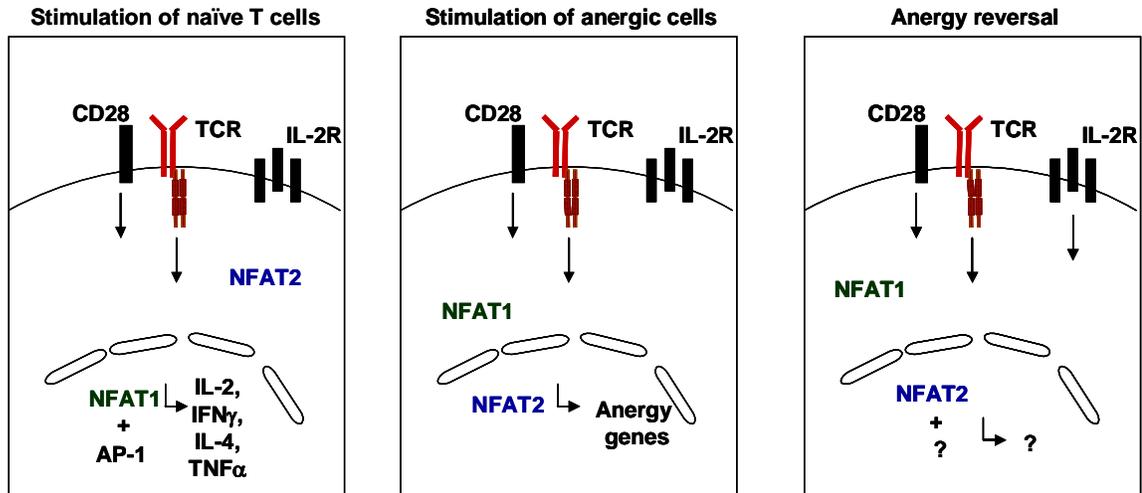


Figure 5.1: Possible roles of NFAT2 during anergy maintenance and anergy reversal.

Nuclear localization of NFAT1 under conditions of complete T cell activation results in the upregulation of genes such as IL-2 and IFN γ that dictate the productive immune response. NFAT2 is localized in the nucleus during anergy and might contribute to the activation of genes that are responsible for the unresponsive phenotype. NFAT2 could also be involved in the regulation of genes during anergy reversal, in cooperation with signals generated downstream of the IL-2/ IL-2R signaling cascades. Thus NFAT2 could potentially regulate gene expression of both negative or positive regulatory genes in anergic T cells.

T cells. CD8⁺ T cells have traditionally not been described as suppressor cells, but there is increasing evidence that a pool of CD8⁺ regulatory cells can exist [172].

My studies therefore have shown that anergy in CD8⁺ T cells results in the differential regulation of cellular accumulation of NFAT1 and NFAT2. This is a previously unreported finding that highlights the possible functional differences in proteins that share a high degree of sequence similarity and a broadly similar mechanism of activation in stimulated T cells. I have shown that the 2C TCR Tg CD8⁺ anergic cells display defective intracellular mobilization of calcium, and that this defect is the predominant causal feature of the differential NFAT mobilization that is seen. This differential regulation, which can be emulated in naïve T cells by regulating calcium availability or calcineurin activity, thus provides a novel means of controlling the activation of specific transcriptional factors and expression of the relevant genes involved, based on the strength of TCR stimulation.

It is important to measure transcriptional activity of NFAT1 and NFAT2 before assigning physiological significance to the nuclear accumulation of NFAT2 in anergic T cells. I acknowledge that an important caveat to all the hypotheses presented above is that NFAT2 nuclear accumulation in anergic T cells might not have any transcriptional significance. Many more experiments need to be conducted to characterize the functional activity of nuclear NFAT2, both in anergic T cells and in cells that have been stimulated in conditions promoting NFAT2 nuclear entry, looking at expression patterns of genes

involved in both activation and anergy. The results from those studies will definitely enrich conclusions from my studies on differential regulation of NFAT in T cells.

Over the past few years, NFAT has emerged as a master regulator of T cell differentiation, owing to the vast number of genes, whose expression is regulated by NFAT activity. NFAT, either alone, or in concert with various other binding partners is involved in regulating gene expression during Th1 differentiation, during Th2 differentiation and during the establishment of the Th17 phenotype. Thus productive immune response under any antigenic context is dependent on NFAT activity. NFAT has also been implicated in anergy induction and the establishment of the T regulatory phenotype. Thus NFAT also controls of inhibition of T cell responses. The specificity of the isoform involved in these gene expression events has not been comprehensively identified. My results, which show that the strength of calcium signaling activates one or the other isoform of NFAT, reiterates the importance of studying the differential usage of NFAT1 and NFAT2. Thus, along with the particular binding partner involved, the specificity of the NFAT isoform might be an important determinant in driving naïve T cells down distinct programs of differentiation.

5.1 Future studies

Future studies will focus on determining differential transcriptional activity of NFAT1 and NFAT2 in anergic T cells, and also in anergic T cells that have been treated with IL-2. Comprehensive gene array experiments comparing expression patterns of genes involved in activation, anergy, and anergy reversal will help in identifying the underlying

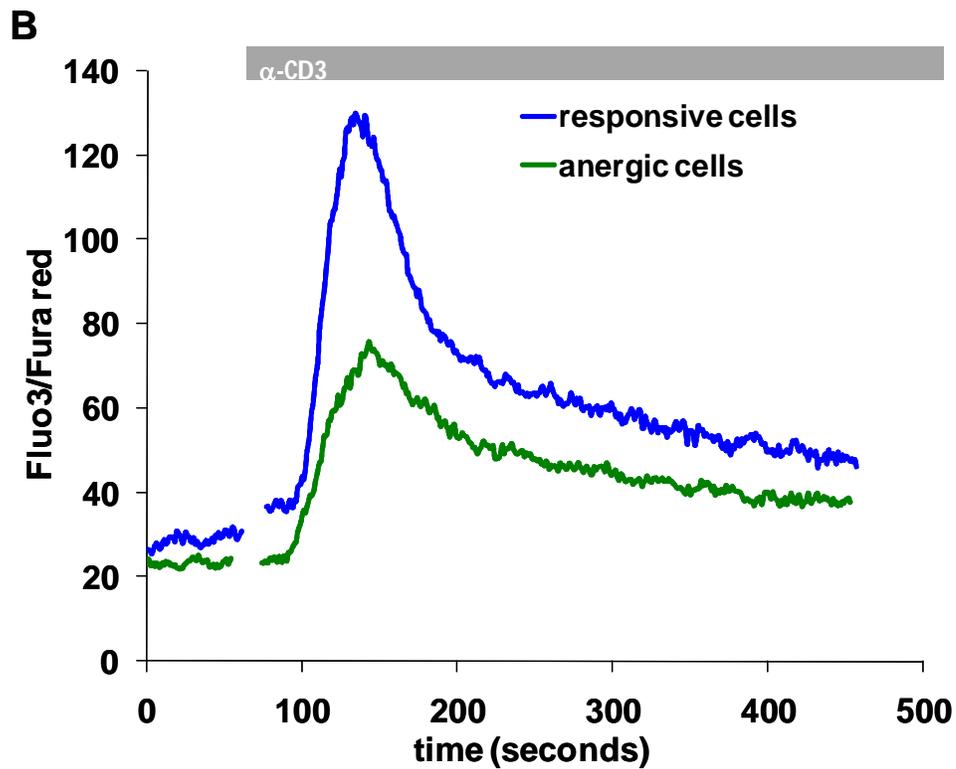
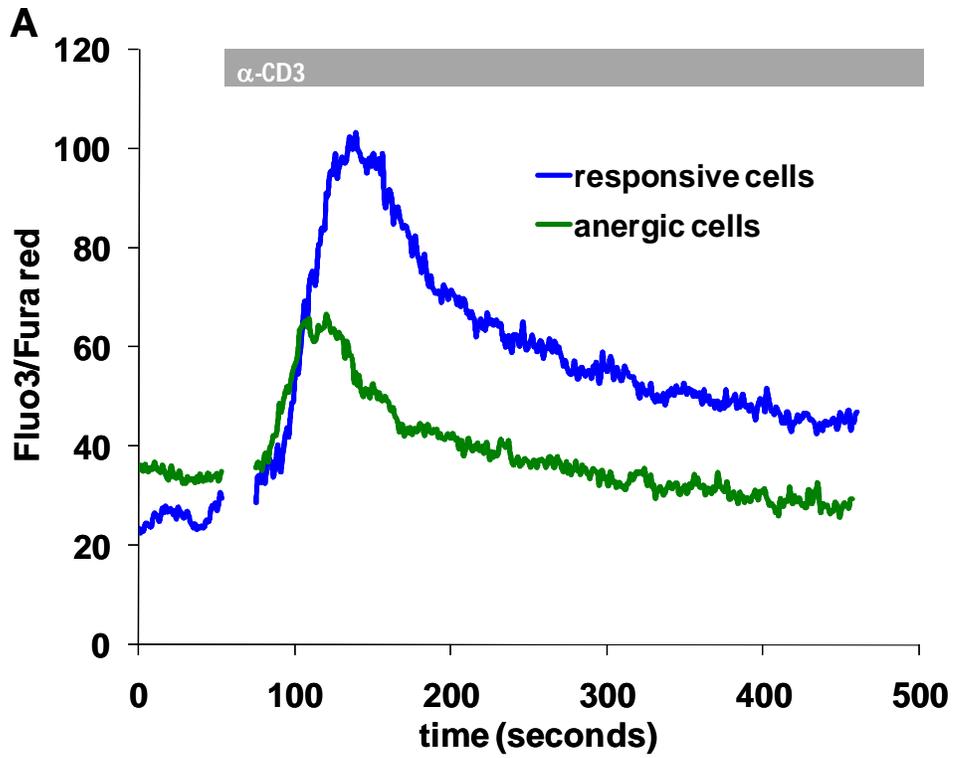
molecular mechanisms that regulate these phenotypes. Chromatin immunoprecipitation (ChIP) assays for NFAT1 and NFAT2 will aid in identifying specific genes as being regulated by NFAT1 or 2. CHIP-on-ChIP assays are based on gene array studies on DNA that is generated from chromatin-IP experiments, and can therefore be important tools to identify all the genes that are specifically regulated by each NFAT family member. Gene expression patterns generated from these experiments will provide conclusive evidence regarding the transcriptional role of NFAT2. These experiments will also aid in identifying new genes involved in calcium regulation, and establishing their expression patterns in anergic T cells.

Knockout mice have been previously used to elucidate the roles NFAT1 and NFAT2 play in T cell development and anergy induction. Mutant mice cannot be used in the studies discussed above, due to the dual role of NFAT in anergy induction and anergy maintenance. Therefore siRNA knockdown of NFAT1 or NFAT2 can be used to determine the phenotype of T cells that undergo stimulation in activating or anergizing conditions, in the absence of NFAT1 or NFAT2. Analysis of expression of specific genes in these siRNA targeted cells is yet another way of determining the transcriptional activity of NFAT2. Anergic T cells are highly refractory to genetic manipulation, due to their quiescent state, and to circumvent this limitation, the Gajewski lab have generated CAR (coxsackie/adenovirus receptor) Tg mice, which allows for efficient transduction of resting, non proliferating cells. CAR Tg T cells can be transduced with recombinant adenovirus carrying the gene or construct of interest [173]. By genetically engineering 2C TCR Tg cells that also express this receptor, these technical difficulties can be

circumvented. Conducting these experiments in T cell clones derived from cell lines is another option for elucidating transcriptional activity specific for NFAT1 or NFAT2.

The determination of NFAT1 and NFAT2 transcriptional activity by the above mentioned strategies will be important in establishing the individual role, these transcription factors play in regulating anergy and reversal of the hyporesponsive phenotype.

Appendix



Appendix: Anergic CD8+ T cells display a calcium signaling defect. Anergic and naïve T cells were loaded with Fluo3 and Fura Red, and intracellular calcium responses to anti-CD3 and secondary crosslinking antibody stimulation were determined by flow cytometry. The basal fluorescence levels of Fluo3 and Fura red in the cells were measured for the first 60 seconds, before addition of the stimulatory crosslinking anti-hamster IgG antibody. **A,B.** Intracellular calcium responses to anti-CD3 stimulation were depicted as a ratio of Fluo3/ Fura Red fluorescence. Shown is data from 2 additional independent experiments.

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