

ABSTRACT

Title of Document: COMPARISON OF NF- κ B REGULATION IN NAÏVE AND ANERGIC PRIMARY CD8⁺ T LYMPHOCYTES

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Due to the cytotoxic potential of CD8⁺ T cells, maintenance of CD8⁺ peripheral tolerance is critical. A major mechanism of peripheral tolerance in T lymphocytes is the induction of anergy, a refractory state caused by T lymphocyte activation in the absence of costimulation. Hallmarks of anergy are decreased IL-2 secretion and decreased proliferation. Here we used a T cell receptor transgenic mouse model to determine whether there are defects in the NF- κ B signaling pathway in CD8⁺ T lymphocytes rendered anergic *in vivo*. In the anergic cell population, decreased NF- κ B-mediated gene transcription and NF- κ B p65 subunit DNA binding activity were observed. These changes were not due to inhibition of early NF- κ B activation events, including I κ B α degradation and NF- κ B p65 subunit nuclear translocation, which occurred normally in anergic T lymphocytes. Nor were they related to defective phosphorylation of p65 at Ser⁵³⁶ in the cytoplasm or Ser²⁷⁶ in the nucleus, as p65 was phosphorylated at these

residues in both naïve and anergic T lymphocytes with similar kinetics. However, the anergic CD8⁺ T lymphocytes failed both to phosphorylate the NF-κB p65 subunit at Ser³¹¹ an event implicated in the recruitment of histone acetyl-transferase molecules such as CBP and p300, and to acetylate p65 at Lys³¹⁰. Both of these posttranslational modifications have been shown to be critical for the positive regulation of NF-κB transcriptional activity. Thus, our results suggest that defects in key phosphorylation and acetylation events in p65 underlie defective NF-κB transactivation capacity and resultant lack of T cell function observed in anergic CD8⁺ T lymphocytes. Taken together these data provide a novel mechanistic explanation of how NF-κB p65 subunit is regulated in anergic CD8⁺ T lymphocytes leading to defective NF-κB transcriptional activity and suggest that recruitment of CBP/p300 and p65 DNA binding *in vivo* is abrogated in anergic T lymphocytes.

COMPARISON OF NF- κ B REGULATION IN NAÏVE AND ANERGIC PRIMARY
CD8⁺ T LYMPHOCYTES

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Dedication

To my wife Paola, for all her love, patience, support and help; for always being my strength during all this process. To my parents Rafael and María de Jesús for all the love and for all their teachings. To my sister María Belén and my brother Xavier for all their love and support. This goes for all of you.

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Abbreviations

ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen presenting cell
Bcl-10	B cell lymphoma-10
BSA	Bovine serum albumin
CARMA1	Caspase recruitment domain-containing membrane associated guanylate kinase protein-1
CBP	CREB binding protein
CD	Cluster of differentiation
cDNA	Complementary DNA
CK II	Casein kinase II
CPS	Counts per second
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
CR	Conserved region
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GRB2	Growth factor receptor-bound protein 2
HAT	Histone acetyl transferase
HDAC	Histone de-acetylase
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
I κ B	Inhibitor of kappa \square
IKK	Inhibitor of κ B kinase
IL-2	Interleukin 2
IP3	Inositol 1,4,5-trisphosphate
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker of activation of T lymphocytes
LZ	Leucine zipper
MALT-1	Mucosa associated lymphoid tissue lymphoma translocated protein-1
MHC	Major histocompatibility complex
MSK1	Mitogen- and stress-activated protein kinase
NFAT	Nuclear factor of activated T lymphocytes

NF- κ B	Nuclear factor- κ B
NLS	Nuclear localization sequence
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PDK1	Phosphoinositide-dependent kinase 1
PE	Phycoerythrin
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphatidylinositol 4,5 bisphosphate
PKA	Protein kinase A
PLC γ	Phospholipase C gamma
PMSF	Phenylmethylsulfonyl fluoride
RAG	Recombination activation gene
RHD	Rel Homology domain
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNase	Ribonuclease
RPS3	Ribosomal protein S3
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SLP-76	SH2 domain containing leukocyte protein of 76 KDa
SMAC	Supramolecular activation complex
TAD	Transactivation domain
TAK-1	Transforming growth factor- β activated kinase-1
TCR	T cell receptor
ZAP-70	Zeta chain-associated protein of 70 KDa.

Chapter 1: Introduction

The vertebrate immune system needs to respond to a tremendous variety of pathogens. Two types of immunity are involved in the protection of the organism against pathogens, innate and adaptive immunity. Innate immunity is the first line of defense and is mediated mainly by dendritic cells, macrophages, neutrophils, and natural killer cells. Adaptive immune response is mediated by B lymphocytes (humoral immune response) and T lymphocytes (cellular immune response). Lymphocytes are essential components of the adaptive immune response. T lymphocytes have the ability to recognize antigens presented by antigen presenting cells (APCs). APCs process antigens into peptides and present them in surface molecules called major histocompatibility complex (MHC). T lymphocyte activation requires interaction between MHC and the T cell receptor (TCR) and other surface molecules.

1.1 The T cell repertoire

T lymphocytes contribute to cell-mediated immunity in the context of the adaptive immune response. Processed antigens are recognized by T lymphocytes only when presented by the MHC on the surface of APCs. T lymphocytes can respond to extracellular and intracellular pathogens, as well as cells presenting abnormal cell surface molecules, such as cancer cells. T lymphocytes are classified according to which coreceptor they express at the cell surface as CD4⁺ or CD8⁺ T lymphocytes.

1.1.1 CD4⁺ T lymphocytes

CD4⁺ T cells are involved in activation of the immune response and recognize antigen peptides associated with MHC class II molecules. CD4⁺ T cells are required for the activation of other cells in the immune system such as macrophages, B cells, and CD8⁺ T cells and activate these cells via the production of cytokines and surface molecules such as CD40L.

To date, three types of CD4⁺ effector T cells have been characterized: Th1, Th2, and Th17. CD4⁺ Th1 effector cells recognize peptide antigens presented by APCs, activating macrophages and dendritic cells, and stimulating phagocytosis of the antigen [1]. Th1 cells control intracellular infections such as *Toxoplasma* and *Leishmania* by providing help to CD8⁺ T lymphocytes by IFN- γ and IL-2 [2]. These cells are characterized by the production of IL-2, IFN- γ , and TNF- α [3]. CD4⁺ Th2 effector cells can activate B cells to produce antibodies such as IgG1 and IgE. Th2 cells control extracellular pathogens. These cells express IL-4, IL-5, IL-6, IL-10, and IL-13 [3]. CD4⁺ Th17 cells are involved in autoimmune and inflammatory responses, as well as in the control of fungal infections [4]. These cells are characterized by the production of IL-17. CD4⁺ T cells are also involved in activation, proliferation, and survival of CD8⁺ T lymphocytes [5].

1.1.2 Regulatory T cells (Tregs)

Tregs are a subset of CD4⁺ cells generated in the thymus (natural Tregs) or in the periphery (induced Tregs) [6, 7]. These cells are characterized by the expression of CD25 and the transcription factor Forkhead Box P3 (FoxP3) (CD4⁺CD25⁺FoxP3⁺). 5% of CD4⁺

T cells have been reported to be CD25⁺ [8]. In both humans and mice, a lack of FoxP3 has been linked to an increase in autoimmune diseases [9] and Tregs have been suggested to regulate self-reactive T lymphocytes by production of high levels of IL-10 and TGF- β [10].

1.1.3 CD8⁺ T lymphocytes

CD8⁺ T cells are important for host defense response against viruses. They also help control intracellular pathogens such as bacteria (*Salmonella enterica*, *Listeria monocytogenes*), parasites (*Trypanosoma cruzi*, *Toxoplasma*), and are also involved in anti-tumor immune responses [11]. CD8⁺ T cells secrete cytokines including IL-2 and IFN- γ which are involved in proliferation, inflammation, and activation of macrophages. CD8⁺ T cells recognize peptides presented by MHC class I molecules, which present mainly endogenous peptides or peptides derived from proteins secreted into the cytoplasm by intracellular pathogens. After activation by interaction with APCs, CD8⁺ T cells differentiate into cytotoxic T cells (CTLs). CTLs destroy infected cells by the release of cytotoxic compounds such as perforin and granzyme, or by the Fas ligand (FasL) that interacts with Fas expressing cells. CD8⁺ T cells are critical for immune responses in cancer, as tumor cells express cell surface MHC class I molecules. Tumor cells recognized by CD8⁺ CTLs are lysed resulting in death. Since all nucleated cells in the body express MHC class I molecules, CD8⁺ T cells act as surveillance cells, recognizing infected cells and destroying them.

1.2 The T cell receptor (TCR) complex and associated molecules

The TCR complex is a membrane bound association of receptors, coreceptors, and associated molecules required for specific interaction with peptide-MHC (pMHC). The main molecules comprising the TCR complex are TCR, CD3, and ζ chain. The TCR is an immunoglobulin-like heterodimer composed of covalently linked α and β chains, each containing a constant ($C\alpha$ and $C\beta$) and a variable ($V\alpha$ and $V\beta$) region [12, 13]. Both TCR chains contain a short cytoplasmic domain (five residues) that is not involved in signaling [14]. The TCR interacts with the MHC in APCs and is non-covalently associated with the signaling molecules CD3 and ζ chain, which are responsible for initiating the signaling cascades required for the activation of T lymphocytes.

CD3 is a complex of γ , δ , and ϵ chains. CD3 and ζ chain both have long cytoplasmic domains that contains one and three immunoreceptor tyrosine-based associated motifs (ITAMs), respectively [14, 15]. ITAMs are conserved structural motifs for which the canonical model is: $YxxI/L_{(7-8 \text{ amino acids})}YxxI/L$, where Y is tyrosine, x is any amino acid, I is isoleucine, and L is leucine [16]. ITAMs are essential for the initiation of signal transduction.

Other coreceptors associated with the TCR complex are CD4 and CD8. CD4 is a monomer that interacts with the non-polymorphic region of MHC class II, whereas, CD8 is a heterodimer that interacts with the non-polymorphic region of MHC class I [17]. LFA-1 acts to stabilize the complex formed by the TCR and MHC. This molecule interacts with ICAM-1 on APCs [18].

The TCR complex cooperates with accessory receptors such as CD28, cytotoxic T lymphocyte antigen 4 (CTLA-4), inducible T cell stimulator (ICOS), programmed cell

death protein 1 (PD-1), and CD7 [19]. CD28, ICOS and CD7 act as costimulators, whereas CTLA-4 and PD-1 act as inhibitory receptors [19]. CD28 is a costimulatory molecule that is crucial for T cell activation [12, 20], whereas, CTLA-4 and PD-1 are considered inhibitors of T cell activation [20-22]. CD28 is constitutively expressed and contains a long cytoplasmic domain (~40 amino acids), containing four tyrosine residues necessary for protein-protein interaction [23]. In contrast, ICOS and CTLA-4 are expressed in activated and memory cells [19]. The CD28 and CTLA-4 homodimers interact with B7.1 (CD80) and B7.2 (CD86) in APCs, whereas ICOS interacts with B7.H2 and PD-1 interacts with B7.H1.DC [23].

1.2.1 Costimulation during T cell activation

T cell activation requires interaction between TCR and CD28 in the T cell and MHC and B7.1/B7.2 expressed in APCs, respectively. The interaction between CD28 and B7.1/B7.2 molecules (second signal/or costimulation) is essential for proper activation of the T cell, as TCR/CD3 stimulation alone is not sufficient for T cell activation [24]. Stimulation of T cells in the absence of costimulation results in decreased IL-2 production and cell proliferation [25]. Costimulation is involved in the activation of transcription factors such as NF- κ B and AP-1 [26, 27]. In addition, costimulation increases cell survival due to upregulation in the expression of anti-apoptotic molecules such as Bcl-2 and Bcl-x_L [28]. The Bcl-x_L promoter contains an NF- κ B responsive element, which is critical for transactivation [28] and clarifies why, in absence of costimulation, cells become apoptotic. Costimulation is also important for cell cycle entry and T cell metabolism [19, 29-31].

Other costimulatory molecules such as CTLA-4 and PD-1 act as inhibitors of T cell activation. Both are homologous to CD28, but they prevent T cell activation [21]. Moreover, it has been established that CD28 costimulation up-regulates CTLA-4, which blocks CD28 costimulation, generating a negative feedback loop [20, 21]. CTLA-4 binds to members of the B7 family with greater affinity than CD28 resulting in decreased IL-2 production and proliferation, as well as a block in cell cycle progression [32].

1.3 Signal transduction during TCR activation

In response to antigen presentation by APCs, the TCR complex initiates a series of signaling cascades involved in the activation of transcription factors essential for T cell activation, proliferation, and survival. These signaling cascades include the nuclear factor κ B (NF- κ B) pathway, the nuclear factor of activated T cells (NFAT) pathway, and the activator protein 1 (AP-1) pathway [12] (Fig. 1).

1.3.1 Proximal signaling events

Upon TCR complex (TCR/CD3/ ζ chain) ligation with a MHC molecule a series of rearrangements occur within the plasma membrane. The region where the TCR complex interacts with MHC molecules is called the immunological synapse (IS), also known as the supramolecular activation complex (SMAC) [33]. Rearrangements in the SMAC involve recruitment of receptors, coreceptors, and accessory molecules to an area characterized by the presence of cholesterol/sphingolipid rich domains called lipid rafts [34]. Two sub-regions can be observed within the SMAC, the central SMAC (cSMAC) and the peripheral SMAC (pSMAC). A model has been proposed for the recruitment of

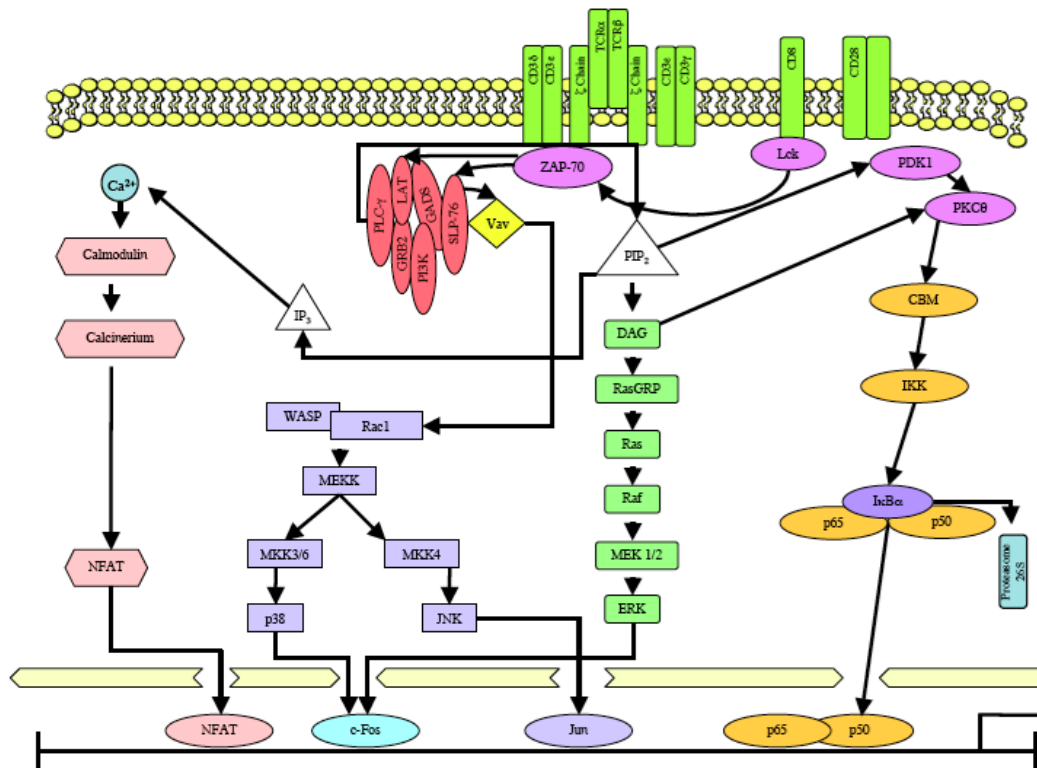


Figure 1. T lymphocyte signaling pathways. After TCR activation a series of signaling cascades are activated in T lymphocytes. Lck binds and phosphorylates ITAMs in CD3 and ζ chain. This event causes the recruitment of ZAP-70 to the ITAMS and its phosphorylation by Lck followed by its own phosphorylation. Activated ZAP-70 phosphorylates LAT and SLP-76. LAT phosphorylation causes the recruitment of Vav, PI3K, PLC γ , GRB2, and GADS. Phosphorylation of Vav causes the activation of WASP and Rac1 that are involved in activation and polymerization of actin. PLC γ hydrolyzes PIP2 into IP3 and DAG. IP3 is involved in the activation of the NFAT pathway, whereas DAG is involved in the activation of AP-1 and NF- κ B.

molecules to the SMAC. In this model, TCR complex, CD28, and CD4/CD8 are originally found in the pSMAC, while adhesion molecules such as LFA-1 are located in the cSMAC. Then, the TCR complex, CD28, and CD4/CD8 are recruited to the cSMAC and the adhesion molecules are recruited to the pSMAC to stabilize the interaction with the APC [35]. Recruitment of accessory molecules to lipid rafts in the cSMAC is essential for signal transduction across the plasma membrane [36].

The first event after recruitment of accessory molecules to the cSMAC is the phosphorylation of tyrosine residues in ITAMs of CD3 and ζ chain by the Src family protein tyrosine kinase Lck (p56) [16]. This event causes the recruitment of the Syk family kinase ZAP-70 to the cSMAC where it associates with phosphorylated ITAMs through its two Src homology 2 (SH2) domains [37]. Once ZAP-70 is bound to ITAMs, it is activated by phosphorylation by Lck and by autophosphorylation [37]. Activated ZAP-70 then phosphorylates its transmembrane substrate LAT (linker for activation of T cells), as well as, the cytoplasmic substrate SLP-76 (SH2 containing leukocyte protein of 76 KDa) [38, 39]. LAT phosphorylation causes the recruitment of Vav, phosphatidylinositol-3-kinase (PI3K), phospholipase C γ (PLC γ), growth factor receptor-bound protein 2 (GRB2), and GRB2-related adaptor downstream of Shc (GADS) to the cSMAC [40, 41]. Phosphorylation of SLP-76 causes the recruitment of Vav and activation of WASP (Wiskott-Aldrich syndrome protein), and the activation of a Rho family GTPase (Rac1) which results in the recruitment and activation of WAVE2 [42]. WASP and WAVE2 are required for the activation of Arp2/3 which is necessary for actin activation and polymerization in the SMAC [43]. In the cSMAC, PLC γ is activated by phosphorylation at multiple tyrosine residues. Activated PLC γ hydrolyzes phosphatidyl

inositol 4,5-bisphosphate (PIP₂) on the inner side of the plasma membrane generating the second messengers inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) [42]. These molecules are essential for the activation of the NF- κ B, NFAT, and AP-1 signaling pathways.

1.3.2 Nuclear Factor of Activated T cells (NFAT)

IP₃ is involved in activation of NFAT. IP₃ generated by activated PLC γ binds to receptors on the surface of the endoplasmic reticulum (ER) [42]. This event causes the release of calcium from the ER into the cytoplasm and transport of extracellular calcium into the cell [42]. Increased cytoplasmic calcium levels activate calmodulin, which then activates the serine/threonine phosphatase calcineurin [44]. Activated calcineurin dephosphorylates NFAT. This event causes translocation of NFAT from the cytoplasm into the nucleus [45]. Once within the nucleus, NFAT is able to bind to promoters and impact gene transcription.

1.3.3 Activator Protein 1 (AP-1)

In T cells, the AP-1 transcriptional complex is comprised of c-Fos/c-Jun heterodimers and forms as a result of membrane associated DAG activation of the Ras-GTPase pathway. In the case of c-Fos, Ras-GTPase recruits Raf from the cytoplasm to the plasma membrane and activates it. Activated Raf phosphorylates and activates MEK, which phosphorylates and activates ERK (extracellular signal-regulated kinase) [46]. ERK phosphorylates and activates ELK, which binds to the *c-fos* promoter, resulting in the expression of c-Fos. In the case of c-Jun, Ras-GTPase recruits MEKK1 to the plasma

membrane and activates it. Activated MEKK1 phosphorylates and activates MKK4/SEK1, which phosphorylates and activates JNK. JNK phosphorylates and activates nuclear c-Jun [46]. Once c-Fos is expressed in the nucleus, it interacts with c-Jun to form the AP-1 transcription factor. AP-1 is required to regulate processes including T cell activation and cytokine production [47].

1.3.4 Nuclear Factor κ B (NF- κ B)

The NF- κ B family of transcription factors is composed of hetero- and homodimers of five NF- κ B subunits: p65 (RelA), c-Rel, p50, p52, and RelB. Dimers of the NF- κ B family of proteins are normally sequestered in the cytoplasm by I κ B proteins, however all members contain a nuclear localization sequence (NLS) and once freed from their inhibitory proteins, can enter the nucleus where they can impact gene transcription [48] (Fig. 2).

Signaling resulting in the activation of NF- κ B begins at the plasma membrane of the T cells with the PI3K products PIP2 and PIP3, which recruit and activate PDK1 (phosphoinositide-dependent kinase 1) from the cytoplasm to the cSMAC [49]. Activated PDK1 recruits the calcium independent serine/threonine kinase PKC θ to the cSMAC and phosphorylates it at Thr⁵³⁸ in its activation domain. At the same time, PDK1 recruits CARMA 1 (caspase recruitment domain (CARD) membrane associated guanylate kinase, MAGUK, protein1) to the cSMAC [49]. Once in the cSMAC, PKC θ interacts with and phosphorylates CARMA1 at Ser⁵⁵² [50]. Phosphorylation of CARMA1 results in conformational changes that trigger the recruitment of the B cell lymphoma 10 (Bcl-10)

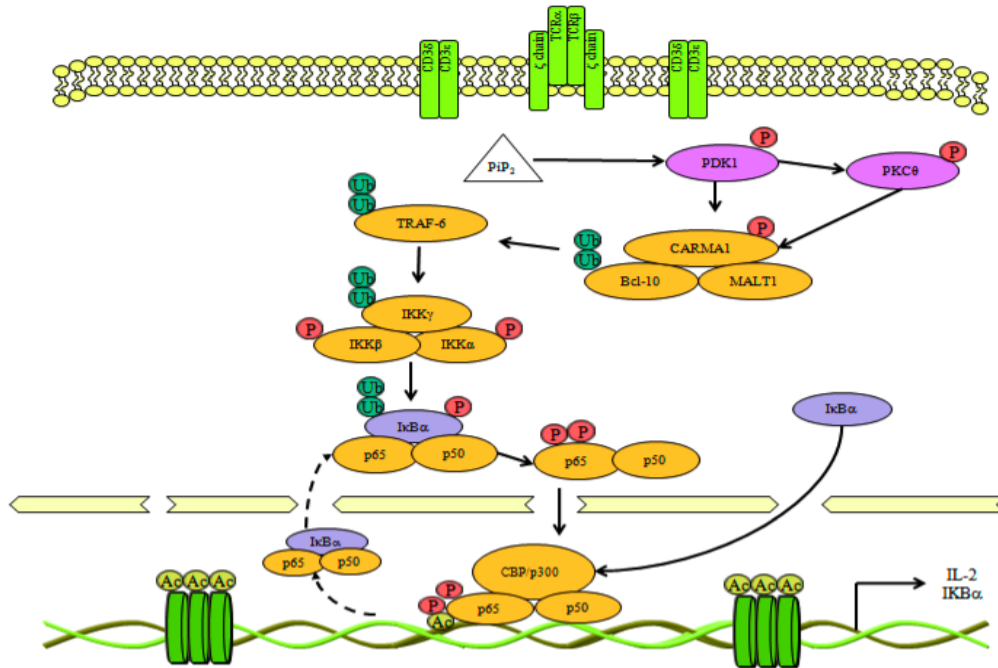


Figure 2. NF- κ B pathway in T lymphocytes. Activated DAG activates PDK1 that recruits and phosphorylates PKC θ . PDK1 also recruits CARMA1. PKC θ phosphorylates CARMA1 allowing for the recruitment of Bcl-10 and MALT1. MALT1 is involved in the ubiquitination of TRAF-6, which mediates the ubiquitination of NEMO (IKK γ). Ubiquitination of NEMO is required for the phosphorylation of IKK α and IKK β by TAK1. After phosphorylation, IKK β phosphorylates I κ B α , which is ubiquitinated and degraded by the proteasome 26S. Degradation of I κ B α frees NF- κ B, which is phosphorylated at Ser⁵³⁶ by IKK and Ser²⁷⁶ by cPKA in the cytoplasm. NF- κ B translocates into the nucleus where it is phosphorylated at Ser²⁷⁶ by MSK1, Ser³¹¹ by PKC ζ . These phosphorylation events cause the recruitment of CBP/p300 that cause the acetylation of p65 at Lys³¹⁰ and histones surrounding the NF- κ B complex. One of the targets of NF- κ B is the I κ B α promoter. Newly synthesized I κ B α translocates into the nucleus where it binds to p65 and re-shuttles it into the cytoplasm.

and mucosa associated lymphoid tissue lymphoma translocation protein 1 (MALT1) to the plasma membrane [50, 51]. MALT1 associates with Bcl-10 through its N-terminal domain and with CARMA1 through its C-terminal domain [52].

The CARMA1-Bcl-10-MALT1 (CBM) complex interacts via direct association with TRAF-6 through Bcl-10 and MALT1. TRAF-6 contains an N-terminal ring finger domain that is required to work as an E3 ubiquitin ligase [53, 54]. TRAF-6 induces polyubiquitination of its targets through recruitment of the UBC13/UEV1 complex [55]. MALT1 drives the TRAF-6 polyubiquitination of Bcl-10 [56]. The Bcl-10-MALT1-TRAF-6 complex induces activation of the I κ B kinase (IKK) complex [33].

The IKK complex is composed of three subunits, the catalytic subunits IKK α and IKK β , and the regulatory subunit IKK γ or NEMO [57, 58]. The Bcl-10-MALT1 complex activates TRAF-6, which mediates the polyubiquitination of NEMO at Lys³⁹⁹ (Lys³⁹² in mice) [59, 60]. Ubiquitination of NEMO does not involve degradation by the proteasome, however it is essential for IKK α phosphorylation at Ser¹⁷⁶ and Ser¹⁸⁰, and IKK β phosphorylation at Ser¹⁷⁷ and Ser¹⁸¹ by TAK-1 (transforming growth factor β (TGF- β) activated kinase 1), and efficient activation of the IKK complex [55, 59]. Both TAK-1 and the IKK complex are recruited to the pSMAC [59]. After Bcl-10-MALT1-TRAF-6 mediated ubiquitination of NEMO, the IKK complex can regulate the phosphorylation and degradation of the inhibitory κ B (I κ B) proteins.

To date, eight different mammalian I κ B molecules have been identified: I κ B α , I κ B β , I κ B ϵ , p105, p100, Bcl-3, I κ B ζ , and I κ B_{NS} [61, 62]. I κ B proteins have been divided in three groups, typical I κ Bs including I κ B α , I κ B β , and I κ B ϵ ; precursor I κ Bs, including p105 and p100; and atypical I κ Bs, including Bcl-3, I κ B ζ , and I κ B_{NS} [63]. Within their C-

terminus, I κ B molecules contain between five and seven regions of sequence homology of 30-33 amino acids known as ankyrin repeats [61, 64]. These ankyrin repeats are essential for protein-protein interactions and sequestration of NF- κ B dimers in the cytoplasm. I κ B α and I κ B β contain PEST (P-proline, E-glutamic acid, S-serine, and T-threonine) sequences in their C-terminus that are required for protein degradation [65, 66]. I κ B α contains a nuclear export signal (NES) that allows re-shuttling from the nucleus to the cytoplasm [67].

The eight I κ B molecules exhibit differential specificity. I κ B α binds mainly to p50-p65 and p65-c-Rel dimers, as well as p65 or c-Rel homodimers; I κ B β binds to p50-p65 and p65-c-Rel dimers [68-70]. I κ B ϵ is bound mainly to p65-c-Rel heterodimers in resting cells [71]. Bcl-3 is mainly localized in the nucleus where it binds to p50 and p52 homodimers [72]. I κ B ζ is mainly located in the nucleus and associates primarily with p50 homodimers [73]. I κ B_{NS} binds to p50 homodimers [74]. IKK α and IKK β rapidly phosphorylate I κ B α at Ser³² and Ser³⁶, and I κ B β at Ser¹⁹ and Ser²³ [75] and phosphorylated I κ B remains bound to NF- κ B dimers [76]. Phosphorylation targets I κ B α for ubiquitination at Lys²¹ and Lys²² and for degradation by the 26S proteasome (Fig. 1). I κ B α , β and ϵ are ubiquitinated by the SCF-bTrCP (Skp-1-Cull-F-box ligase containing the F box protein bTrCP) E3 ubiquitin ligase [77-79]. After phosphorylation, I κ B α is rapidly degraded, whereas I κ B β and I κ B ϵ are degraded at slower rates or not at all [71, 80]. Ubiquitinated I κ B α may remain bound to the NF- κ B dimer prior to proteasome degradation [77]. As with I κ B, p105 and p100 are phosphorylated, ubiquitinated and degraded by the 26S proteasome to give rise to the NF- κ B subunits p50 and p52

respectively [66]. p105 and p100 contain p50 or p52 at the N-terminus and ankyrin domains at the C-terminus [81, 82].

In resting cells, NF- κ B is maintained in the cytoplasm bound to I κ B molecules and protein kinase A (PKA) [83]. After stimulation, I κ B molecules are degraded, exposing the p65 NLS and activating the catalytic subunit of PKA (cPKA) [83]. Once cPKA is activated, it can phosphorylate p65 at Ser²⁷⁶ at a PKA consensus site [83] (Fig. 2). This phosphorylation event is necessary to reduce the interactions between p65 C-terminus and N-terminus, allowing interaction/binding with the acetyltransferase complex CBP/p300 and increasing NF- κ B transcriptional activity [84]. p65 is also rapidly phosphorylated in the cytoplasm by IKK α and IKK β at the conserved residue Ser⁵³⁶ in the C-terminal transactivation domain [27, 85]. It has been shown that this post-translational modification regulates p65 nuclear export [27]. Both phosphorylation events occur in the cytoplasm preceding p65 nuclear translocation.

An alternative mode for p65 phosphorylation at Ser²⁷⁶ has also been shown. After NF- κ B p65 translocates into the nucleus it is phosphorylated by the mitogen and stress activated protein kinase 1 (MSK1) at Ser²⁷⁶ [86, 87]. MSK1 is a kinase that contains a NLS and is known to be activated by ERK and p38 [87, 88]. As with the PKA pathway, this phosphorylation event allows p65 interaction/binding with the acetyltransferase complex CBP/p300, increasing NF- κ B transcriptional activity [86, 87, 89]. The p-p65-CBP/p300 interaction results in DNA removal of p50 homodimers bound to the histone deacetylase HDAC-1 [90]. p65 also associates with PKC ζ , leading to phosphorylation at Ser³¹¹ [91, 92]. Phosphorylation of p65 at Ser²⁷⁶ and Ser³¹¹ is required for CBP/p300 and RNA Pol II recruitment, p65 and histone acetylation, and enhanced p65 transcriptional

activity [92, 93]. It has been demonstrated that the CBP/p300 complex acetylates p65 at Lys³¹⁰ *in vivo* and *in vitro* and acetylates histone H4 at Lys⁸ and Lys¹⁰, causing relaxation of chromatin, facilitating transcription [89]. Acetylation of NF- κ B p65 subunit at Lys³¹⁰ and chromatin favors NF- κ B transcriptional activity but does not appear to regulate p65 DNA binding [94]. NF- κ B subunits can be deacetylated by HDAC3, allowing the binding of newly synthesized I κ B α [95].

The I κ B α promoter is known to contain NF- κ B binding sites [96]. In the nucleus of T lymphocytes, phosphorylated NF- κ B p65 subunit binds to the IL-2 promoter, the I κ B α promoter as well as several other promoters characterized by the consensus sequence 5'-GGGACTTTCC-3' [97]. Re-synthesized I κ B α translocates into the nucleus to re-shuttle deacetylated NF- κ B back to the cytoplasm [98]. (Fig.2)

1.3.4.1 The NF- κ B family domain structure

All NF- κ B subunits share the structural characteristics present in the v-Rel oncoproteins. The structure of NF- κ B subunits was revealed after p50 subunit cloning and structural analysis, and contains an N-terminal domain called the Rel homology domain (RHD), that is shared with all family members [99, 100]. The NF- κ B domain structure is represented in Figure 3. The N-terminal region contains the 300 amino acid RHD [99, 100]. The RHD is characterized by the presence of two immunoglobulin-like domains attached by a linker region that make contact with DNA molecules [101]. The RHD is necessary for DNA binding, protein dimerization, and cytosolic localization by interaction with I κ B molecules. The RHD also contains a NLS that is found in the C-

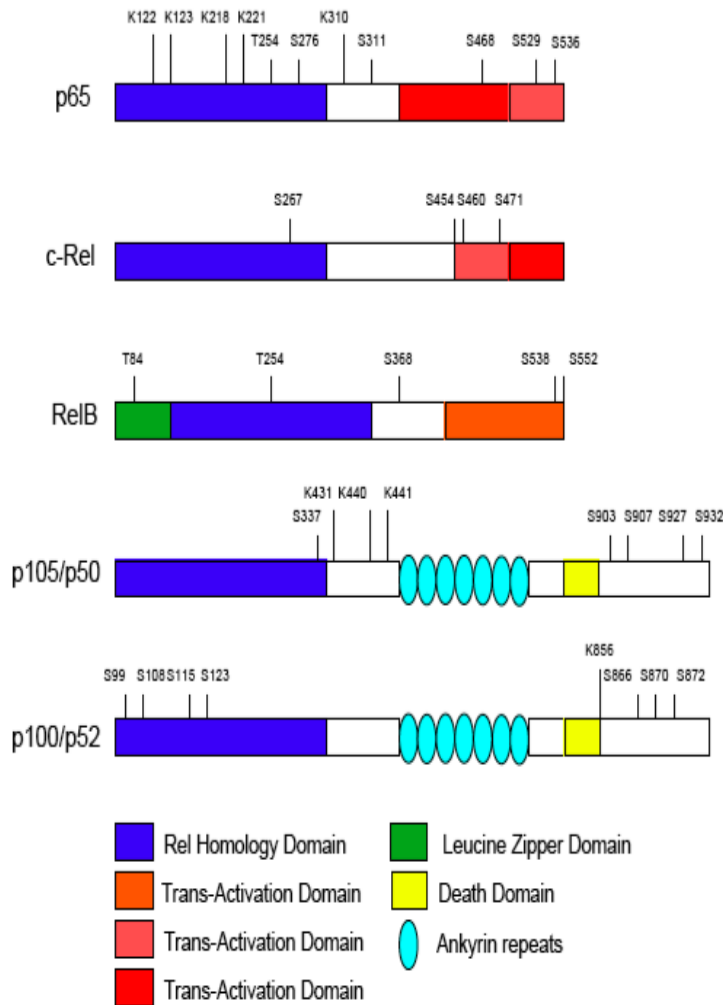


Figure 3: NF- κ B subunits structure and their post-translational modifications. NF- κ B is composed of five subunits, p65, c-Rel, RelB, p50, and p52. All subunits share a conserved region called Rel homology domain (RHD). p65, c-Rel, and RelB share between one or two transactivation domains (TADs). RelB structure also possesses a leucine zipper domain (LZ). p105/p50 and p100/p52 contain six ankyrin domains and one death domain (DD) each. All NF- κ B subunits are modified by different posttranslational modifications such as phosphorylation, acetylation, and ubiquitination. This figure shows some of these modifications affecting the NF- κ B subunits.

terminal side and is necessary for nuclear localization [48]. While all five NF- κ B subunits contain a NLS only p65, c-Rel, and RelB contain a C-terminal transactivation domain (TAD) and thus are the only subunits that can positively regulate transcription [102, 103]. This domain is also involved in stability during nuclear translocation and transactivation. It has been observed that p65 and c-Rel exhibit strong transactivation activity, while RelB exhibits moderate transactivation function [48, 103]. Transactivation domains are necessary for interaction with other proteins such as other I κ B family members, TATA-binding protein (TBP), and TFIIB [104]. Two independent transactivation domains have been identified, TAD1 and TAD2. In p65, TAD1 includes residues 521-551 and is involved in nuclear translocation and transactivation [105]. TAD2 includes residues 428-521 and has been divided into three conserved regions (CRs), CR1, CR2, and CR3 [105]. It has been observed that deletion of the N-terminus domain or C-terminus domain in p65 dramatically decreases p65 transactivation activity [106]. To be completely active, RelB requires a N-terminal LZ (leucine zipper) domain in addition to its RHD [48]. The N-terminal domain of the p50 and p52 subunits is required for DNA binding, whereas the C-terminal domain is required for dimerization and DNA binding [107]. Both, p50 and p52 lack transactivation domains. It has been suggested that both p50 and p52 act as negative regulators of transcription [62].

1.3.4.2 NF- κ B Post-translational modifications

Different post-translational modifications such as, phosphorylation, acetylation, and ubiquitination regulate NF- κ B activation (Fig. 3). In the RHD, p65 has five phosphorylation sites and four acetylation sites. Phosphorylation at Thr²⁵⁴ by an unknown

kinase in breast cancer samples stabilizes DNA binding and decreases NF- κ B-I κ B interaction [108]. Phosphorylation of p65 at Ser²⁷⁶ in response to LPS and TNF α by cPKA or MSK1 is required for recruitment of CBP/p300 and results in increased NF- κ B activation, DNA binding, and transcriptional activity [83, 86]. A lack of p65 phosphorylation at Ser²⁷⁶ causes the recruitment of HDACs to the NF- κ B complex [84, 109]. p65 is also phosphorylated at Ser²⁰⁵ and Ser²⁸¹ by unknown kinases in response to LPS, increasing NF- κ B transcriptional activity [110]. p65 is phosphorylated at one residue in the region between the RHD and TAD2, Ser³¹¹. Phosphorylation at Ser³¹¹ by PKC ζ in response to TNF α is necessary to recruit CBP/p300 and to increase NF- κ B transcriptional activity [92].

p65 is phosphorylated at five residues in the TAD. Phosphorylation at Thr⁴³⁵ by an unknown kinase decreases transcriptional activity [111]. Phosphorylation at Ser⁴⁶⁸ in response to IL-1 and TNF α by IKK β , IKK ϵ , and GSK3B decreases transcriptional activity [112, 113]. Phosphorylation at this residue recruits the SOCS1-E3 ligase and the COMMD1 ligase complex and results in ubiquitination of p65 [114]. Phosphorylation at Thr⁵⁰⁵ by checkpoint kinase (ChK1) and ATR increases HDAC recruitment and decreases transcriptional activity [115]. Phosphorylation at Ser⁵²⁹ in the TAD1 in response to IL-1 and TNF α by CKII enhances transcriptional activity, while phosphorylation at the conserved residue Ser⁵³⁶ in response to IL-1, TNF α , and LPS by IKK α , IKK β , IKK ϵ or RSK1 regulates nuclear localization, enhances DNA binding and transcriptional activity and regulates stability [27, 116, 117].

Acetylation also regulates p65 activity. p65 is acetylated at four lysine residues in the RHD, Lys¹²², Lys¹²³, Lys²¹⁸, and Lys²²¹, in response to TNF α stimulation.

Acetylation of p65 at Lys¹²² and Lys¹²³ by the HATs CBP/p300 and P-CAF decreases DNA binding and transcriptional activity, and increases IκB binding to p65 containing dimers [118]. p65 acetylation at Lys²¹⁸ and Lys²²¹ by CBP/p300 increases DNA binding and decreases IκB-NF-κB interactions [94]. Thus, the balance of these acetylations regulates p65 DNA binding. Acetylation of p65 between the RHD and TAD at Lys³¹⁰ by CBP/p300 increases transcriptional activity, but does not affect DNA binding [89, 94].

Phosphorylation is also critical to the activity of the other NF-κB subunits. c-Rel phosphorylation at Ser²⁶⁷ by PKA regulates nuclear localization and transcriptional activity [80]. Phosphorylation of c-Rel Ser⁴⁵⁴ and Ser⁴⁶⁰ by unknown kinases increases transcriptional activity [119], while phosphorylation at Ser⁴⁷¹ in response to TNFα by PKCζ enhances transcriptional activity [120, 121]. In contrast, c-Rel phosphorylation at Ser⁴⁵⁴ and Ser⁴⁶⁰ results in ubiquitination and protein degradation, [119]. RelB is phosphorylated at Thr⁸⁴, Thr²⁵⁴, Ser³⁶⁸, and Ser⁵⁵² by unknown kinases, leading to degradation of the protein by the proteasome [116].

p100/p52 phosphorylation at Ser⁹⁹, Ser¹⁰⁸, Ser¹¹⁵, Ser¹²³, Ser⁸⁶⁶, Ser⁸⁷⁰, and Ser⁸⁷² by IKKα increases ubiquitination and processing to generate p52 [122]. p105/p50 is phosphorylated at Ser³³⁷ by cPKA, enhancing its DNA binding activity [123]. It has been suggested that phosphorylation of p50 at this residue negatively regulates p65 binding to DNA [124]. p105 phosphorylation at Ser⁹⁰³ and Ser⁹⁰⁷ by GSK3β regulates protein stability [125], and phosphorylation at by IKKβ at Ser⁹²⁷ and Ser⁹³² regulates ubiquitination and processing to generating p50 [126]. p105/p50 is also acetylated at three residues, Lys⁴³¹, Lys⁴⁴⁰, and Lys⁴⁴¹ by p300, resulting in enhanced DNA binding

[127]. All these data show that NF- κ B is not only regulated by I κ B degradation or NF- κ B nuclear translocation, but it is also by multiple post-translational modifications.

1.3.4.3 NF- κ B mediated transcriptional regulation

NF- κ B has been extensively studied due to its central role in the immune system. It is activated by many different stimuli including: TCR and BCR stimulation, cytokines such as TNF α and IL-1; and bacterial and viral components such as LPS and dsRNA which work through Toll-like receptors (TLR). Genotoxic stress also activates the NF- κ B pathway. In this context, reactive oxygen species (ROS) and UV radiation activate NF- κ B. NF- κ B in turn regulates approximately 300 genes [128]. These genes include: immuno-regulatory proteins such as, the C3 complement subunit; pro-inflammatory cytokines and chemokines such as, IL-1, IL-2, TNF α , MIP-1 α , and MIP-2 γ ; adhesion molecules such as, ICAM and VCAM; growth factors such as, granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF); regulators of apoptosis, and cell survival such as, Bcl-x_L, Bcl-2, and Rel/I κ B proteins such as I κ B α and c-Rel [129]. These data shows that a wide range of functions in different types of cells is regulated by NF- κ B.

NF- κ B is mainly regulated via two mechanisms, localization and post-translational modifications. NF- κ B localization is predominantly regulated by I κ B degradation and resynthesis, while its transcriptional regulation is largely controlled by phosphorylation and acetylation events. As mentioned previously, p65 is phosphorylated at many residues, the majority of which regulate NF- κ B transcriptional activity and

CBP/p300 recruitment. CBP/p300 acetylates p65 and histones surrounding the NF- κ B complex, increasing transcriptional activity. It has been observed that in unstimulated cells, NF- κ B target gene promoters are bound by p50 homodimers associated with HDACs, resulting in transcriptional repression [90]. In activated cells, p65-HAT complexes displace p50-HDAC complexes to positively regulate NF- κ B transcriptional activity. On promoters bound by NF- κ B, histone H3 is phosphorylated and histones H3 and H4 are acetylated, promoting chromatin relaxation and gene transcription [130]. Deacetylation of promoter-bound p65 is necessary for NF- κ B-I κ B α interaction and re-shuttling of NF- κ B into the cytoplasm [131].

Among other genes, the *il-2* and the *nfkb1a* (*ikba*) genes are regulated by NF- κ B in T lymphocytes. The conserved 300 bp promoter of the *il-2* gene contains the following characterized consensus binding sites: NF- κ B, NF- κ B-like CD28 response element (CD28RE), two NFAT binding sites, two AP-1 binding sites (proximal and distal), and two Oct-1 binding sites (NFIL-2A and NFIL-2D) [132, 133]. T lymphocyte activation requires the coordinated interaction of all transcription factors recruited to the IL-2 promoter. In resting cells, the IL-2 promoter remains in a closed conformation [134, 135]. After activation, the promoter goes through a series of rearrangements at the chromatin level that allow the binding and accessibility of the transcriptional machinery [136]. Acetylation of histones is a key element in IL-2 transcription and causes the recruitment of the chromatin-remodeling complex, including RNA-Pol II, TFIID, and the TATA box binding protein (TBP) [137]. DNA methylation is associated with decreased transcriptional activity because it is involved in the generation of binding sites for

HDACs [137]. T cell stimulation causes a decrease in methylation in the IL-2 promoter [138] and a switch from promoter hypoacetylation to hyperacetylation [137].

Several proteins have been shown to regulate NF- κ B transcriptional activity. The ribosomal protein S3 (RPS3) positively regulates NF- κ B DNA binding affinity and transcriptional activity in specific genes such as I κ B α [139]. Other proteins such as Ikaros, Smad3, and FoxP3 negatively regulate NF- κ B transcriptional activity [137, 140]. It has been observed that NF- κ B binds to the I κ B α promoter rapidly upon entry into the nucleus [141]. In contrast to IL-2, rapidly induced NF- κ B target genes such as I κ B α do not require chromatin remodeling to be activated [141].

Transcriptional regulation of NF- κ B is essential to maintain the homeostasis of the immune response, since this transcription factor regulates many events during innate and adaptive immune responses. This is exemplified by the defective regulation of NF- κ B that is related to several disorders such as arthritis, asthma and cancer [142].

1.3.4.4 NF- κ B Mutants

Animal models and cell lines null for one or more of the NF- κ B subunits or containing mutated subunit genes have been generated to study the function of NF- κ B subunits. *p65*^{-/-} mice are embryonically lethal due to hepatic apoptosis. In conditional *p65*^{-/-} fibroblasts, I κ B α and I κ B β expression was abrogated and cell proliferation was decreased [143]. In another study it was shown that *p65*^{-/-} L929SA cells transfected with WT p65 rescued TNF α mediated IL-6 expression [86]. These data show the importance of p65 in the activation of cells.

c-rel^{-/-} animals develop normally but show proliferative defects in B and T lymphocytes [128]. Approximately 50% of *relb*^{-/-} animals die by three months due to inflammation in several organs, especially the spleen [128, 144]. Proliferation in B and T lymphocytes is decreased in these animals and, low numbers of dendritic cells are present in the thymus [144]. *relb*^{-/-} animals also showed defects in negative selection [68]. *nfkb1*^{-/-} (p105/p50) animals develop normally but exhibit defects in the humoral immune response characterized by decreased B cell maturation, proliferation, and antibody production [128]. *nfkb2*^{-/-} (p100/p52) animals are viable with defects in spleen and lymph node development, B cell proliferation, and magnitude of T cell antigen specific responses [145].

Animal models null for multiple NF-κB subunits have also been generated. *p65*^{-/-} *nfkb1*^{-/-}, *p65*^{-/-} *c-rel*^{-/-}, *p65*^{-/-} *relb*^{-/-}, and *p65*^{-/-} *nfkb2*^{-/-} mice die embryonically due to hepatic apoptosis, similar to the effects of the individual p65 deficiency [128]. It has been observed that in *p65*^{-/-} *c-rel*^{-/-} cells only p50 homodimers are bound to DNA, whereas in *p65*^{-/-} *nfkb1*^{-/-} cells no other subunits are bound to DNA [143]. These data show that either p65 or p50 must be bound to DNA so that other NF-κB subunits can bind DNA. *c-rel*^{-/-} *relb*^{-/-}, *c-rel*^{-/-} *nfkb1*^{-/-}, and *c-rel*^{-/-} *nfkb2*^{-/-} animals develop normally but with decreased proliferation of T and B lymphocytes, and dendritic cells [143]. It has been observed that *c-rel*^{-/-} *nfkb1*^{-/-} mice exhibit irregular spleen architecture and humoral immune response [146]. Approximately 80% of *relb*^{-/-} *nfkb1*^{-/-} and 50% of *relb*^{-/-} *nfkb2*^{-/-} mice die 3 months due to inflammation of different organs [128, 147]. These animals also show defects in T and B lymphocyte proliferation. *nfkb1*^{-/-} *nfkb2*^{-/-} animals die post-weaning because the lack of teeth [128]. These mice also have defective T and B

lymphocyte proliferation and maturation [128]. In cells derived from these animals, only p65 homodimers bind to DNA [143]. Mice null for three NF- κ B subunits predominantly die embryonically due to inflammation of multiple organs. Only *nfkb1*^{-/-} *nfkb2*^{-/-} *c-rel*^{-/-} mice survive to birth, but die post-weaning due to a lack of teeth. Like the *nfkb1*^{-/-} *nfkb2*^{-/-} these animals also present deficiencies in T and B lymphocyte proliferation [128].

Mouse models null and mutant for kinases directly involved in phosphorylation of p65 have also been generated. *msk1*^{-/-} mice develop normally [86], but MSK1^{-/-} mouse embryonic fibroblasts (MEFs) stimulated with TNF α exhibit decreased p65 phosphorylation at Ser²⁷⁶ and decreased transcriptional activity [86]. PKC ζ ^{-/-} embryonic fibroblasts (EFs) stimulated with TNF α showed decreased phosphorylation of p65 at Ser³¹¹, and reduced recruitment of CBP/p300 and RNA Pol II [92]. These data show that these kinases are essential for the proper activation of the NF- κ B p65 subunit.

1.4 T cell tolerance

Recognition of antigen peptides presented by APCs requires a diverse repertoire of antigen receptors. The process of VJ somatic recombination on the TCR α chain and VDJ somatic recombination on the TCR β chain is essential to generate diversity for the recognition of foreign antigens [148, 149]. V(D)J somatic recombination can also give rise to autoreactive T cells with receptors specific for self-antigens. The responses generated against self-antigens are regulated by a group of mechanisms collectively referred as immunological tolerance. T cell tolerance can be divided in central tolerance and peripheral tolerance.

Following random rearrangement of the antigen receptor by somatic recombination, thymocytes express TCR molecules on the surface. Some of these TCRs can recognize self-antigens expressed in thymic stromal cells or presented by thymic APCs [150]. Antigen-TCR interactions with MHC class I or MHC class II molecules can be low or high affinity interactions. During fetal development, a low affinity interaction signal results in positive selection of lymphocytes and promotion of maturation into single positive (SP) lymphocytes. A MHC class I interaction positively selects CD8⁺ T lymphocytes whereas MHC class II interaction positively selects for CD4⁺ T lymphocytes [151]. Positive selection ensures that only T lymphocytes recognizing self-MHC molecules survive. High affinity interaction between lymphocytes and thymic stromal cells or hematopoietic cells results in central tolerance of lymphocytes, also known as negative selection [152]. Negative selection consists of the deletion of autoreactive lymphocytes by apoptosis [153]. Approximately 90-95% of the maturing thymocytes eventually die by apoptosis [154]. Negative selection helps to avoid further maturation of autoreactive T cells and autoimmune reactions.

Despite negative selection in the thymus, some autoreactive lymphocytes migrate to the periphery. The reason for this is that not all autoreactive antigens are expressed in thymic stromal cells or are presented by thymic APCs. Self-antigens such as proteins expressed during puberty, pregnancy, and in the mammary glands are presented to T lymphocytes after they migrate to secondary lymphoid organs and after they travel to the blood stream [155, 156]. Examples of self-antigens expressed late in life are spermatozoid-specific antigens that are not in contact with mature T lymphocytes, and thecal cell-antigens present in the Graafian follicles and corpus luteum in females [157,

158]. Peripheral tolerance is especially important for CD8⁺ T lymphocytes since they recognize peptides bound to MHC class I found in all nucleated cells in the organism. Nevertheless, autoreactive T cells in the periphery can be rendered unresponsive by suppression by regulatory T cells or by anergy. Peripheral tolerance can be attained by activation of Tregs that suppress self-reactive lymphocytes by production of inhibitory cytokines (IL-10 and TGF β), disruption of the calcium signaling and inhibition of the NF- κ B pathway in T lymphocytes [159, 160]. My research deals with a second mechanism of tolerance, anergy, which is discussed in detail in the next section.

1.4.1 Anergy

The interaction of T lymphocytes with APCs without costimulation causes a state of hyporesponsiveness called anergy [161] (Fig. 4). Anergy was first described in CD4⁺ T cell clones and is characterized by limitations in cell cycle progression, cell proliferation, and IL-2 production [162]. Initially it was thought that these cells were not anergic but that they were dying. However, when exogenous IL-2 was added to these cells, they proliferated [163]. Proliferation was possible because the IL-2 receptor is still functional in anergic cells [164]. Anergic T cells are arrested in G₀ or G₁ phase of the cell cycle or they fail to up-regulate proteins that regulate G₁ phase of the cell cycle [165, 166]. In this sense, anergy has been described as a protective mechanism for avoiding activation of peripheral autoreactive T cells [159, 165]. Expression of cell surface molecules such as TCR α/β chains, CD3, CD4, CD8, CD25, CD45, LFA-1, and IL-2R α is not affected in anergic cells, in fact, they are found in similar levels as in naïve cells [167, 168].

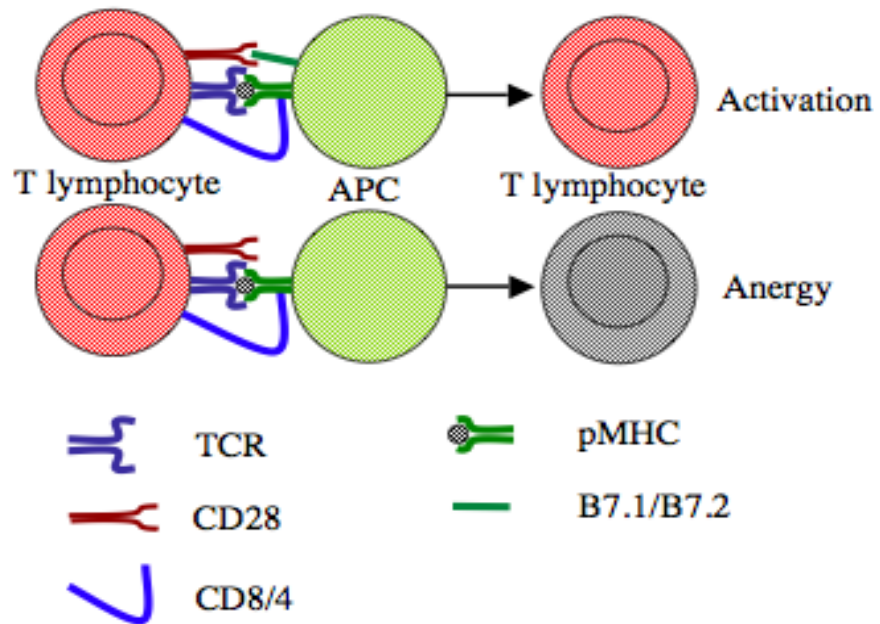


Figure 4. Anergy induction in T lymphocytes. Activation of T lymphocytes requires the association of two signals. Signal 1 involves the interaction of the TCR with peptide-MHC in APCs, and signal 2 (costimulation) involves the interaction of CD28 in the T cells and B7.1/B7.2 in the APC. Ligation of both signals induces activation of the T lymphocyte, while association of signal 1 alone, in the absence of costimulation causes anergy.

Anergy can be induced experimentally both *in vitro* and *in vivo*. *In vitro* induction of anergy can be achieved by several methods, including treatment with immobilized anti-CD3 in the absence of APCs or use of the calcium ionophore ionomycin [169, 170]. *In vivo* induction of anergy generally involves administration of antigen via intraperitoneal or intravenous injection, or via intranasal or oral dosage forms. Common stimuli used to induce *in vivo* anergy are partial agonist peptides (peptides created by single amino acid substitutions) [171] and superantigens, such as staphylococcal enterotoxin A or B (SEA/B), in the absence of other inflammatory signals [172]. Another means to inducing anergy is by induction of the negative signaling molecule CTLA-4. This molecule induces anergy by outcompeting the binding of B7.1/B7.2 on APCs [173, 174]. Another molecule that has been involved in anergy is PD-1, which is up-regulated in anergic cells [175].

T lymphocytes rendered anergic demonstrate alterations in the signaling pathways involved in IL-2 production. Anergic cells exhibit decreased phosphorylation of ZAP-70 and Lck [176], as well as reduced PLC γ phosphorylation, and ubiquitination and degradation of proteins involved in TCR signal transduction such as PLC γ and PKC θ [177, 178]. Studies have found that all three major transcription factor pathways are disrupted in anergic cells. Activation of ERK and JNK was shown to be defective in anergic murine T cell clones [179, 180]. The AP-1 and NF- κ B signaling pathways have also been shown to be disrupted in anergic CD4⁺ T cells [180, 181]. Other groups showed that defects in Ras activation are related to deficiencies in ERK and JNK activation [182, 183]. Recently, our lab showed that NFAT1 translocation into the nucleus was affected in anergic CD8⁺ T cells [184]. It has been suggested an anergy

mechanism involving Ikaros silences the *il-2* promoter until the proper CD28 stimulation is received [140, 185]. Ikaros is a ZF-binding protein that has been observed to induce histone deacetylation at the *il-2* promoter [140, 185]. ZEB1 has been associated with the recruitment of HDACs to the *il-2* promoter [140, 185]. *il-2* promoter methylation has also been implicated in the induction of anergy in CD4⁺ and CD8⁺ T lymphocytes [140, 185].

1.4.1.1 The role of NF-κB in T cell anergy

One of the defining characteristics of anergic cells is a decrease in IL-2 at the mRNA and protein levels. This suggests that there is a defect in the transcription factor signaling pathways involved in IL-2 regulation. It has previously been shown that NFAT, AP-1, and NF-κB are in some way affected in anergic T lymphocytes. Becker et al. showed that NF-κB activation is inhibited in T lymphocytes that were previously stimulated in the absence of costimulation [186]. It has also been found that the NF-κB heterodimer p65/p50 binding to the *il-2* promoter is inhibited in anergic cells [187]. Later, Grundstrom et al. showed that different NF-κB dimers are observed in nuclear fractions of naïve and anergic cells. They found that in stimulated naïve cells p65/p50 homodimers are the main components in nuclear extracts, whereas p50 homodimers are the main components of nuclear extracts in anergic cells [188]. Guerder et al. also showed the presence of different NF-κB dimers in naïve and anergic CD8⁺ T lymphocytes and showed that NF-κB transcriptional activity is decreased in anergic CD8⁺ T lymphocytes [180]. It has been also shown that in cells stimulated in the absence of costimulation, the NF-κB dimer c-Rel/p50 does not bind to a κB binding sequence [189]. The Sundstedt group showed only partial degradation and resynthesis of IκBα in

the cytoplasm of anergic CD4⁺ T lymphocytes, as well as inhibition of p65 nuclear translocation [188]. In the same way, another group showed a decrease in IκBα degradation in anergic CD4⁺ T lymphocytes. This group also showed impaired nuclear translocation in anergic cells [190]. These data show that all signaling pathways involved in IL-2 production are affected in other anergy systems, however data regarding post-translational regulation in anergic cells has not been presented yet.

1.5 Significance

The goal of this project is to delineate the role of NF-κB signaling during anergy in CD8⁺ T lymphocytes. Elucidating the role of the NF-κB signaling will help us to understand the mechanisms involved in the generation of anergy. Data suggesting a role for NF-κB have already been obtained in other T cell lines, such as tumor cells and CD4⁺ T cell clones, however these cell lines present a different cellular context relative to naïve primary CD8⁺ T lymphocytes. Tumor cells are constitutively activated and established cell lines are subject to genetic changes as a result of prolonged culture. In addition, data regarding regulation of NF-κB by posttranslational modifications in anergic T cells have not yet been presented yet, giving this project a major relevance and significance.

Understanding the process of anergy has potential implications for the treatment of cancer, graft rejection in transplantation, and for treatment of autoimmune diseases. It has been observed that T cells specific for tumor antigens become unresponsive, and reversing this state could help to improve T cell anti-tumor therapies. It has also been observed that induction of anergy could be important to reduce graft vs. host disease in

transplantation [191]. Our studies in NF- κ B regulation in CD8⁺ T lymphocytes might help to provide new targets to allow reversal of the anergy state in lymphocytes associated with cancer cells or in pathogenesis, or promote anergy during transplantation or to prevent graft vs. host disease.

In chapter 3, I will address the hypothesis that there is a defect in the transcriptional activity of NF- κ B in anergic CD8⁺ T lymphocytes. In my studies, I show that NF- κ B transcriptional activity is decreased in anergic cells. In this chapter I also address whether two of the central events in the cytoplasm are associated with activation of the NF- κ B pathway in naïve and anergic cells. Here, I analyze the kinetics of I κ B degradation/resynthesis at the protein and mRNA level, and the nuclear translocation pattern of the NF- κ B subunit p65. I show that I κ B α is degraded in naïve and anergic cells, but it is resynthesized in naïve cells only. I κ B β is degraded with faster kinetics in anergic cells after stimulation compared to naïve cells. Finally, I show that p65 translocates normally to the nucleus in anergic cells.

In chapter 4, I will address the post-translational events affecting the NF- κ B p65 subunit in naïve and anergic CD8⁺ T lymphocytes. I show that p65 is phosphorylated at Ser⁵³⁶ and Ser²⁷⁶ with the same kinetics in naïve and anergic lymphocytes. However, the phosphorylation of p65 at Ser³¹¹ and acetylation at Lys³¹⁰ are inhibited in anergic CD8⁺ T lymphocytes. The observations presented in this work could explain the NF- κ B defect seen in our T cell anergy model.

Chapter 2: Materials and Methods

2.1 Mice

2C TCR-Transgenic/RAG^{-/-} mice and 2C TCR-Transgenic/RAG^{+/+} mice expressing the 2C $\alpha\beta$ T cell receptor alloreactive for the L^d MHC class I receptor have been described previously [165, 192], and were maintained on a C57BL/6 background. NF- κ B-Luciferase-Transgenic (NF- κ B-luc) mice [193] were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed with 2C TCR-Transgenic/RAG^{+/+} mice. 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) at the University of Maryland animal facility (College Park, MD). All protocols were approved by the University of Maryland Institutional Animal Care and Use Committee. Animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (Bethesda, MD). All of the mice were euthanized by carbon dioxide inhalation, as recommended by the American Veterinary Medical Association Panel on Euthanasia.

2.2 Antibodies and Reagents

The H-2K^b restricted 2C TCR reactive peptide SIYRYYGL was purchased from NeoMPS (San Diego, CA). Anti-CD3 (mAb 145-2C11), anti-CD28 (mAb 37.51), Armenian hamster IgG, Syrian hamster IgG, PE conjugated anti-V β 8, PE conjugated

anti-Thy 1.2, and FITC conjugated anti-CD8 α were purchased from eBioscience (San Diego, CA). Goat anti-hamster IgG was purchased from Pierce (Rockford, IL). Anti-I κ B α , anti-actin, anti-NF- κ B-p65, anti-phospho-p65 (Ser311), and anti-lamin A/C antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phospho-p65 (Ser536), and anti-phospho-p65 (Ser276) antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Anti- α -tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti-Ac-p65 (Lys310) antibody was from Abcam. HRP conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Bio-Rad (Richmond, CA).

2.3 Preparation of Antibody-conjugated Magnetic Beads

DynaBeads M-450 (Invitrogen, Carlsbad, CA) were resuspended and 4×10^8 beads were aliquoted into a sterile tube and placed in a MPC-1 magnet (Invitrogen) for one minute. Supernatant was removed, and beads were washed twice in buffer B (0.1M boric acid, pH 9.5). Supernatant was removed, and the beads were resuspended in 1 mL buffer B + antibodies. For control beads, 75 μ g/mL each of Armenian hamster IgG and Syrian hamster IgG were used. For stimulation beads, 75 μ g/mL each of anti-CD3 Ab and anti-CD28 Ab were used. The suspension containing the beads was incubated for 24 hours at 37 $^{\circ}$ C with constant rotation. After the incubation, the beads were placed in the MPC-1 magnet and antibody solution was removed. Beads were washed with constant rotation twice for 5 minutes at 4 $^{\circ}$ C in buffer D (1X PBS, and 0.1% BSA), once in buffer E (0.2M Tris, pH 8.5, and 0.1% BSA) for 24 hours at room temperature, and once with

buffer D for 5 minutes at 4°C. Supernatant was removed and beads were resuspended in buffer D + 0.02% NaN₃ to a final concentration of 4 x 10⁷ beads/ml.

2.4 Cell Culture

All cells were maintained in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 2mM L-glutamine (Mediatech, Manassas, VA), 10% FBS (Hyclone, Logan, UT), 10mM HEPES buffer (Hyclone), penicillin (100U/ml)/streptomycin (100µg/ml) (Mediatech), and 55µM β-mercaptoethanol (Gibco, Grand Island, NY). Cells were cultured at 37°C in an incubator with 5% CO₂

2.5 T Cell Purification

T lymphocytes and CD8⁺ T lymphocytes were purified using the EasySep mouse T cell enrichment negative selection kit and the EasySep mouse CD8⁺ T cell enrichment negative selection kit respectively (STEMCELL technologies, Vancouver, British Columbia, Canada). Spleens collected from mice were macerated in PBS supplemented with 2% (v/v) FBS. Splenic extracts were sterile filtered through a nylon membrane and centrifuged at 500 x g for 5 minutes at room temperature. Cell pellets were resuspended at a concentration of 1 x 10⁸ cells/ml in PBS supplemented with 2% (v/v) FBS containing 5% (v/v) normal rat serum. Mouse T cell enrichment cocktail was added to the suspension at a concentration of 50µl/ml and then incubated at 4°C for 15 minutes. Cells were then incubated with biotin selection cocktail (100µl/ml) at 4°C for 15 minutes. Magnetic nanoparticles (50µl/ml) were added and the mixture was incubated at 4°C for

15 minutes. Finally, cells were collected using an EasySep magnet for 5 minutes at room temperature. For all experiments, two rounds of magnetic separation were performed to achieve greater purity.

2.6 In Vivo Anergy Induction

To induce anergy in vivo, 2C TCR transgenic mice (6-8 weeks old) were injected intraperitoneally (i.p.) with 25-50 nmol of 2C peptide dissolved in 200 μ L sterile 1X PBS or with 200 μ L of 1X PBS alone. Seven days after peptide injection the mice were sacrificed and their spleens were removed. T lymphocytes were purified from spleens as described in 2.5 and restimulated in vitro.

2.7 Estimation of T Cell Purity by Flow Cytometry

100 μ L of the purified T lymphocytes were stained with 20ng of PE anti-Thy1.2 or PE conjugated Rat IgG. Cells were incubated for 15 minutes at room temperature and then 300 μ L of FACS buffer (1X PBS containing 1% (v/v) FBS) was added to each sample for analysis. Samples were analyzed for PE fluorescence using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The purity of the isolated T lymphocytes generally ranged between 90-95%.

2.8 T Lymphocyte Stimulation

Purified primary T lymphocytes were stimulated using soluble anti-mouse CD3 and anti-mouse CD28 antibodies. To begin, the cells were incubated with anti-CD3 and

anti-CD28 (10 μ g/ml each) antibodies on ice for 30 minutes, while unstimulated cells to be used as a control were incubated with Armenian hamster IgG and Syrian Hamster IgG antibodies (10 μ g/ml each). Following incubation, 5ml of ice-cold 1X PBS was added to each sample and the samples were centrifuged at 500 x g for 5 minutes. Cell pellets were resuspended with complete RPMI 1640 medium containing 10 μ g/ml goat-anti Syrian hamster IgG as secondary cross-linking antibody and incubated at 37°C for appropriate time points. Reactions were stopped by adding 5ml ice-cold 1X PBS, followed by centrifugation at 500 x g for 5 minutes at 4°C.

For luciferase assays, purified T lymphocytes were stimulated using magnetic beads conjugated to anti-CD3 and anti-CD28 antibodies. For stimulation, beads were resuspended and washed in 1X PBS. Purified T lymphocytes and beads were plated at a bead:cell ratio of 3:1 in complete RPMI 1640 medium and incubated for the appropriate time points at 37°C in a 5% CO₂ atmosphere. As a control, unstimulated cells were incubated with hamster IgG-conjugated beads.

2.9 Proliferation and Cytokine Assays using APC Stimulation

For proliferation assays, splenocytes obtained from C57BL/6 mice were used as APCs to stimulate purified T lymphocytes. 2 x 10⁵ splenocytes were cultured with 5 x 10⁴ T cells in each well and titrated doses of 2C peptide. The cells were incubated with 2C peptide at 37°C in a 5% CO₂ atmosphere for 3 days.

Proliferation was then analyzed by [³H] thymidine incorporation into DNA. 1 μ Ci of [methyl-³H] thymidine (MP Biomedicals, Solon, OH) was added to each well and cells

were incubated for 8 hours at 37°C. Cells were harvested onto glass fiber filters using a 96 well cell harvester (Tomtec, Hamden, CT) and filters were dried by placing in a microwave on “high” for 45s. Dried filters were sealed in plastic bags with 3mL of CytoScint scintillation fluid (MP Biomedicals), and radioactivity was read using a 1450 Microbeta Trilux scintillation counter (Wallac, Turku, Finland). Data were plotted as the average counts minute (cpm) of triplicate samples \pm standard deviation.

For cytokine assays, splenocytes obtained from C57BL/6 mice were used as APCs to stimulate purified T lymphocytes. Plates were incubated at 37°C in a 5% CO₂ atmosphere for 48 hours at which time supernatants were collected for analysis of IL-2 and IFN- γ levels by ELISA.

2.10 Enzyme Linked Immunosorbent Assay (ELISA)

IL-2 and IFN- γ levels in cell supernatants were determined by sandwich ELISA. For this assay, 96 well plates were coated with purified anti-mouse IL-2 antibody (1 μ g/ml) (eBioscience, San Diego, CA) or anti-mouse IFN- γ antibody (2 μ g/mL) (eBioscience) diluted in coating buffer (0.1M NaHCO₃, pH 8.2) overnight at 4°C. Plates were washed with 1X PBS + 0.05% Tween-20 (PBS/0.05% Tween) and blocked with 1X PBS containing 3% (w/v) BSA for one hour at room temperature. Culture supernatants were added to blocked plate in triplicate (50 μ L/well) and incubated for one hour at room temperature. The plates were washed with PBS/0.05% Tween and then incubated for 45 minutes at room temperature with biotin-conjugated anti-mouse IL-2 or biotin-conjugated anti-mouse IFN- γ (eBioscience) diluted to 1 μ g/ml in PBS/0.05% Tween. The plates were

washed with PBS/0.05% Tween and then incubated for 30 minutes with alkaline phosphatase-conjugated streptavidin (1:3000 in PBS/0.05% Tween) (Jackson ImmunoResearch Laboratories, West Grove, PA). Plates were washed with PBS/0.05% Tween, and the reaction was detected using colorimetric alkaline phosphatase substrate (Sigma-Aldrich, St. Louis, MO) diluted to 1mg/mL in substrate buffer (10% diethanolamine, 1M MgCl₂, and 0.02% NaN₃). Plates were analyzed at 405nm using Versamax spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were analyzed using the Softmax Pro software (Molecular Devices), and cytokine levels were calculated based on a standard curve of recombinant mouse IL-2 or IFN- γ (eBioscience). Data points are presented as the mean of triplicate wells \pm standard deviation.

2.11 Luciferase Assay

Purified T lymphocytes (3×10^6 /sample) were stimulated at 37°C for 48 hours with anti-CD3/anti-CD28-conjugated magnetic beads at a bead:cell ratio of 3:1. Samples were washed twice with PBS, and cell pellets were resuspended in supplemented RPMI 1640 medium without phenol red at a density of 1×10^7 cells/ml. Luciferase activity was assessed by adding an equal volume of Bright-Glo Luciferase Assay System Reagent (Promega, Madison, WI) to each sample and incubating samples at 23°C for 15 minutes. Samples were loaded in triplicate in Optiplate 96 well plates (PerkinElmer, Shelton, CT). Luciferase activity was recorded using a 1450 Microbeta Trilux scintillation counter (Wallac) in luminometer mode and reported as counts per second (cps). Data points are presented as the mean of triplicate wells \pm standard deviation.

2.12 Western Blot

Cells were stimulated in vitro for the appropriate time points and then lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10mM NaF, 1mM Na₃VO₄ in 1X PBS) at a concentration of 5 x 10⁷ cells/ml. Proteins were resolved by SDS-PAGE on a 12% gel and electrotransferred at 200V for 1 hour onto nitrocellulose membranes. The membranes were blocked overnight with 5% non-fat dried milk in PBS-0.1% Tween-20. The membranes were then probed with primary antibody diluted in PBS-Tween-20 and then incubated with the appropriate peroxidase conjugated secondary antibodies. Specific bands were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL).

2.13 Total RNA Isolation

Purified T cells were stimulated for 0, 5, 10, 15, 30, 45 and 60 minutes with anti-CD3 and anti-CD28 antibodies, or for 36 hours with anti-CD3 and anti-CD28 antibodies conjugated to beads. After stimulation, cells were centrifuged at 500 x g for 5 minutes. Total RNA was isolated using the Total RNA Isolation NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA) as per the manufacturer's instructions with slight modifications.

Briefly, cell pellets were resuspended in 350µl buffer RA1 and 3.5µl β-mercaptoethanol. This mixture was passed through a 1ml syringe with a 21G1 needle to disrupt the pellet and transferred to a Nucleospin Filter column followed by centrifugation at 11,000 x g for 1 minute. The resulting supernatant was mixed with 350µL 70% ethanol, loaded on a Nucleospin RNA II Column and centrifuged at 11,000 x

g for 30 seconds. 350µl of membrane desalting buffer were added to each column and columns were centrifuged at 11,000 x g for 1 minute. 95µl of DNase reaction mixture were then added and columns were incubated at room temperature for 45 minutes. Columns were washed once with buffer RA2 and twice with RA3 buffer and RNA was eluted with 60µl RNase free water by centrifugation at 11,000 x g for 1 minute.

2.14 cDNA Synthesis

RNA was quantified using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Newark, DE) and an iSCRIPT cDNA synthesis kit (Biorad, Hercules, CA) was used to produce cDNA from total RNA. In a 0.2ml microtube, 4µl of 5x iScript reaction mix were mixed with 25ng of purified RNA, 1µl of iScript reverse transcriptase, and nuclease free water to final volume of 20µl. The reverse transcription reaction was performed using an iCycler Thermal Cycler (Biorad) programmed as follows: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. cDNA was stored at 4°C.

2.15 Polymerase Chain Reaction (PCR)

Following a hot start at 95°C for 2 minutes, cDNA was amplified for 35 cycles as follows: 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute using an iCycler Thermal Cycler (Biorad). PCR primers used in the reactions are listed below:

IL-2 forward primer: 5'-TGCTCCTTGTC AACAGCG-3'

IL-2 reverse primers: 5'-TCATCATCGAATTGGCACTC-3'

IκBα forward primer: 5'-GCTCTAGAGCAATCATCCACGAAGAGAA-3'

I κ B α reverse primers: 5'-CGGAATTCGCCCCACATTTCAACAAGAG-3'

I κ B β forward primers: 5'-ACACAGCCCTGCACTTGGCTG-3'

I κ B β reverse primers: 5'-GGTATCTGAGGCATCTCTTGGG-3'

18sRNA forward primers: 5'-ATGCGGCGGCGTTATTCC-3',

18sRNA reverse primers: 5'-GCTATCAATCTGTCAATCCTGTCC-3'

PCR products were resolved on 1% agarose gels and visualized using an EpiChemi³ Darkroom (UVP BioImaging systems, Upland, CA).

2.16 Quantitative Real-time PCR (qPCR)

Primer sets for qPCR were the same as described in section 2.15. qPCR was performed using the iCycler iQ system (Biorad, Hercules, CA) with iQ SYBR Green Supermix reagents (Biorad, Hercules, CA) or SensiMix SYBR & Fluorescein kit (Bioline, Taunton, MA). Data was analyzed using MyiQ software (Biorad, Hercules, CA). The presence of a single PCR product was confirmed by melt curve analysis. After a hot start at 95°C for 3 minutes, the PCR cycling protocol used was as follows: 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C-60°C for 30 seconds, and extension at 72°C for 30 seconds. Fold induction was obtained using the $\Delta\Delta C_t$ method, and 18S rRNA was used to normalize loading differences. Data points are presented as the mean of triplicate wells \pm standard deviation.

2.17 Cellular Fractionation

A cellular fractionation protocol was modified from Park et al. [194]. Briefly, stimulated cells were centrifuged at 500 x g for 5 minutes at 4°C and cell pellets were lysed on ice for 15 minutes at a concentration of 5×10^7 /mL using Hypotonic Lysis buffer (10mM HEPES, pH7.9, 10mM KCl, 0.1mM EDTA, protease inhibitors, PMSF, 10mM NaF, 1mM Na₃VO₄, and 200nM Trichostatin A). Triton X-100 was added to each tube to a final concentration of 1% and cells were vortexed briefly followed by incubation on ice for 10 minutes and centrifugation at 16,000 x g for 1 minute at 4°C. Supernatant containing the cytosolic fraction was collected and stored at -80°C. Pellets were washed one time with Hypotonic Lysis buffer for 5 minutes and then centrifuged at 16,000 x g for 1 minute at 4°C. The remaining pellet was resuspended at a concentration of 1×10^8 /mL using Nuclear Extraction buffer (20mM HEPES, pH7.9, 0.4M NaCl, 1mM EDTA, protease inhibitors, PMSF, 10mM NaF, 1mM Na₃VO₄, and 200nM Trichostatin A) and cells were incubated at 4°C for 30 minutes with constant agitation. Samples were centrifuged at 16,000 x g for 5 minutes at 4°C and supernatant containing the nuclear fraction was collected and stored at -80°C.

2.18 Immunofluorescence

Primary T lymphocytes were stimulated with anti-CD3 and anti-CD28 antibodies for the appropriate time points. After stimulation, cells were centrifuged at 500 x g for 5 minutes at 4°C and pellets resuspended in 100µl RPMI/BSA (RPMI 1640, 10mM HEPES, pen/strep, and 0.6% BSA) and mounted on poly-L-lysine microscope slides

(Polysciences, Inc, Warrington, PA). Samples were incubated on ice for 40 minutes, the media were aspirated, and the cells were fixed for 15 minutes at 4°C with ice-cold methanol (Thermo Scientific, Fair Lawn, NJ). Samples were then washed twice with ice-cold 1X PBS. Cells were permeabilized and blocked by incubation on ice for 20 minutes with PB buffer (RPMI 1640, 10% FBS, 10% saponin, 1M glycine, and 1M HEPES) followed by one hour incubation on ice with anti-p65 antibody at a concentration of 4µg/ml. After incubation, cells were washed three times with PB buffer and incubated on ice for a further hour with 2µg/ml ALEXA Fluor 594 linked anti-IgG (Molecular Probes, Eugene, OR) as a secondary antibody. After washing the cells three times with PB buffer, the samples were incubated at room temperature for 20 minutes with SYTO-13 (Molecular Probes) in the dark, washed twice with PB buffer and post fixed at room temperature for 10 minutes with 4% paraformaldehyde (Electron Microscopy Sciences, Washington, PA). Cells were washed with 1X PBS and coverslips were fixed onto the slide using Aqueous Mounting Media (Biomed, Foster City, CA). Cells were analyzed on a LSM 510 confocal microscope (Carl Zeiss Microimaging, Thronwood, NY) or a Leica SP5 X confocal microscope (Leica Microsystems Inc, Bannockburn, IL). Confocal data was analyzed using Zeiss LSM image browser and the degree of nuclear co-localization was ascertained using the co-localization tool in the Leica Application Suite AF software (Leica Microsystems Inc). To quantify co-localization, 15-20 cells were analyzed individually and data was presented as the mean of the intensity \pm standard deviation.

2.19 Transcription Factor ELISA

Transcription Factor ELISA assays were performed as per the manufacturer's instructions using the TransAM NF- κ B p65 Transcription Factor Assay kit (Active Motif, Carlsbad, California). Briefly, complete binding media was added to each well followed by nuclear extracts (0.05 μ g) diluted in complete lysis buffer. Nuclear extracts analyzed were derived from fractionated naïve and anergic T lymphocytes, and Jurkat nuclear extract diluted in complete lysis buffer was used as a positive control. Serially diluted recombinant p65 (10ng/well-0.0ng/well) was included in the assay for purposes of quantification. Samples were covered and incubated for 60 minutes at room temperature with agitation followed by three washes in 1X washing buffer. Then NF- κ B p65 antibody diluted in 1X antibody binding buffer (1:1000) was added to each well. The plate was covered and then incubated for a further 60 minutes at room temperature followed by three washes in 1X washing buffer. Next, HRP-conjugated antibody diluted in 1X antibody binding buffer (1:1000) was added and the covered plate incubated for 60 minutes at room temperature followed by four washes in 1X washing buffer. Developing solution was added to each well for four minutes at room temperature avoiding exposure to direct light and reaction was stopped with stopping solution. Signal was detected on a Versamax spectrophotometer (Molecular Devices) at 450nm with a reference wavelength of 655nm and data analyzed using Softmax Pro software (Molecular Devices). Standard curves were generated to determine p65 concentration. Data points are presented as the mean of duplicate wells \pm standard error. Competitive binding experiments were performed using excess wild type and mutated NF- κ B oligonucleotides.

2.20 Chromatin Immunoprecipitation (ChIP)

Enzymatic chromatin shearing was performed as per the manufacturer's instructions using the ChIP-IT kit (Active Motif). Briefly, 4.5×10^7 EL-4 cells were fixed for 10 minutes at room temperature with 0.75% formaldehyde and then washed with ice-cold PBS. Cells were centrifuged at $500 \times g$ for 10 minutes at 4°C and pellets resuspended in ice-cold lysis buffer. Samples were incubated on ice for 30 minutes followed by homogenization with an ice-cold Dounce homogenizer. Samples were transferred to microtubes and centrifuged at $2,400 \times g$ for 10 minutes at 4°C . Samples were incubated for 5 minutes at 37°C with enzymatic shearing cocktail and then incubated on ice for 10 extra minutes with ice-cold EDTA. Samples were centrifuged at $10,000 \times g$ for 10 minutes at 4°C and supernatant containing the sheared chromatin was stored at -80°C .

Samples were pre-cleared by incubation with protein G agarose beads ($40\mu\text{l}$ of a 50% bead slurry) (Sigma-Aldrich) overnight at 4°C with rotation. Tubes were then centrifuged at $10,000 \times g$ for 10 minutes at 4°C . 5% of the supernatant was removed and stored (input sample). Samples were then incubated with rotation for two hours at 4°C with $2\mu\text{g}$ of anti-p65 antibody. After two hours, protein G agarose beads ($50\mu\text{l}$ of a 50% bead slurry) were added to each tube and samples were incubated for a further hour at 4°C with rotation. Tubes were centrifuged at $10,000 \times g$ for 5 minutes and the agarose beads washed for 3 minutes on a rotating platform in the following order: once with TSE-150 buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl), once with TSE-500 buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl), once with LiCl washing buffer (0.25M LiCl, 1% NP-40,

1% DOC, 1mM EDTA, 10mM Tris-HCl pH 8.1), and twice with TE buffer (10mM Tris pH 8.1, 1mM EDTA). Samples were centrifuged at 10,000 x g for 5 minutes and then resuspended in elution buffer for 15 minutes at room temperature. Samples were centrifuged at 10,000 x g for 5 minutes and the supernatants collected. Pellets were resuspended for an extra 15 minutes at room temperature in elution buffer and supernatants collected and combined with previous eluate. Samples were incubated for 4 hours at 65°C with 5M NaCl and then for one hour at 45°C with proteinase K.

DNA purification was performed as per the manufacturer's instructions using the ChIP DNA clean & concentrator kit (Zymo Research, Irvine, California). Briefly, ChIP DNA binding buffer was added to the immunoprecipitated samples and transferred to a Zymo-spin column in a collection tube. Samples were centrifuged at 10,000 x g for 30 seconds. Supernatant was discarded and the columns were washed twice with wash buffer. Samples were centrifuged 10,000 x g for 30 seconds and then eluted with elution buffer. Samples were centrifuged 10,000 x g for 30 seconds and eluted DNA was stored at -80°C. Samples were analyzed by PCR as described in 2.15

2.20 Statistics

Statistical analysis was performed using the Prism software, version 5 (GraphPad Software Inc, San Diego, CA). The minimal level of confidence at which experimental results were considered significant was $p < 0.05$. An unpaired two-tailed T test was used to determine statistical significance between PBS and 2C samples in cytokine ELISA assays, and between PBS and 2C samples in luciferase assays. A two-way ANOVA test followed by Bonferroni post-test was used to determine statistical significance between

treatments (PBS vs. 2C) and stimulation times in quantitative real time PCR, transcription factor ELISA, and immunofluorescence (nuclear colocalization)

Chapter 3: Analysis of NF- κ B activation in anergic CD8⁺ T lymphocytes

The NF- κ B family of transcription factors is composed of hetero- and homodimers of the five NF- κ B subunits: p65 (RelA), c-Rel, p50, p52, and RelB. Dimers of the NF- κ B family of proteins are normally sequestered in the cytoplasm by I κ B proteins, and once freed from their inhibitory proteins, can enter the nucleus where they can impact gene transcription [195]. Because NF- κ B is important for expression of many immunologically important genes, including IL-2, we were interested in whether regulation of NF- κ B was affected in anergic CD8⁺ T lymphocytes.

An in vivo model system was previously described [165] for the induction of anergy in TCR transgenic mice in a RAG2^{-/-} background. In RAG-sufficient mice, the transgenic 2C TCR is expressed in 20-95% of peripheral T lymphocytes, where most of the T lymphocytes are CD4⁻ CD8⁺ [192]. By maintaining the TCR transgene on the RAG-deficient background, the fraction of T cells expressing the 2C TCR is increased to 100%, and essentially all T cells are CD4⁻ CD8⁺ (K.A. Frauwirth, unpublished observations). The use of this system confers several advantages, such as a uniform T lymphocyte population and TCR transgenic cells that can be monitored by the use of antibodies [196]. In our model, we induce anergy by intraperitoneal injection of the 2C antigenic peptide in the absence of costimulation. The peptide binds to MHC molecules on the surface of antigen presenting cells in the mouse (although the identity of the relevant cell population is unknown) and interacts with the TCR in the surface of T lymphocytes. A high fraction of T lymphocytes recovered from peptide-injected mice

have been shown to be antigen experienced since CD44 expression levels are upregulated (K.A. Frauwirth, unpublished observations).

3.1 RESULTS

3.1.1 Anergic CD8⁺ T lymphocytes exhibit decreased IL-2 production.

In order to understand the signaling alterations in anergic T lymphocytes, the 2C TCR/RAG2^{-/-} *in vivo* mouse system [165] was utilized, as describe above. A single injection of 2C peptide into 2C TCR transgenic mice generates anergic cells leading to decreased IL-2 production and cell proliferation [165]. IL-2 and IFN- γ secretion by naïve and anergic T lymphocytes were assessed by ELISA in order to confirm that the injection of 2C peptide into 2C TCR transgenic mice induced anergy of lymphocytes. Analysis of IL-2 levels after 24 hours incubation at 37°C with APCs demonstrated a ~90% reduction in IL-2 secretion by T lymphocytes derived from 2C peptide injected mice relative to control (Fig. 5A). IL-2 mRNA is not expressed in resting cells, but is induced in naïve T lymphocytes by 24 hours of stimulation (Fig. 5B). In contrast, increased IL-2 transcription is not observed in anergic cells after stimulation for 24 hours (Fig. 5B). Previously it was reported that IFN- γ levels are affected to a lesser degree in anergic cells [184, 197], thus we determined the levels of IFN- γ after 24 hours incubation. Consistent with these reports, secreted IFN- γ levels are comparable between anergic and naïve cells (Fig. 5C). Previously our group demonstrated that [³H] thymidine incorporation was reduced by 90% in T cells derived from 2C peptide injected mice relative to control [184]. Taken together, our results confirm that injection with 2C peptide results in the

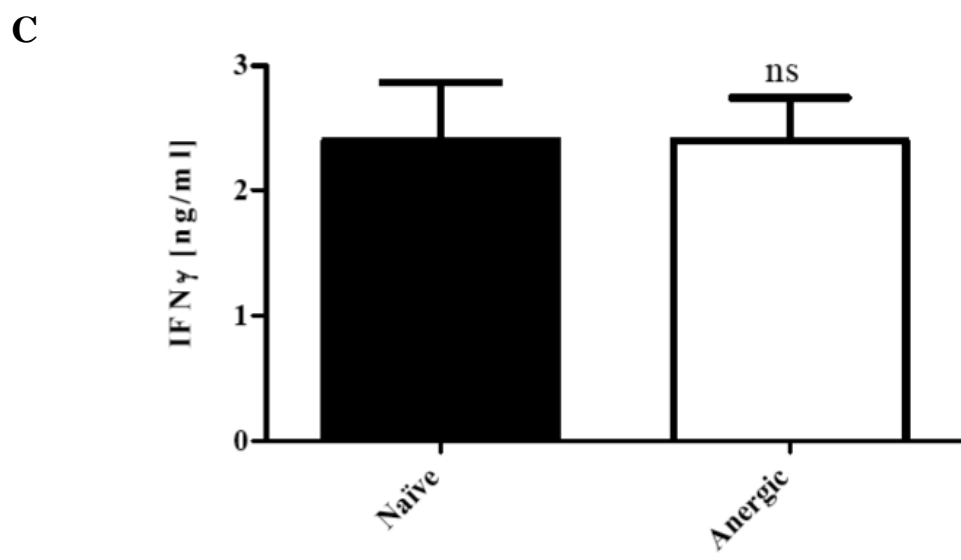
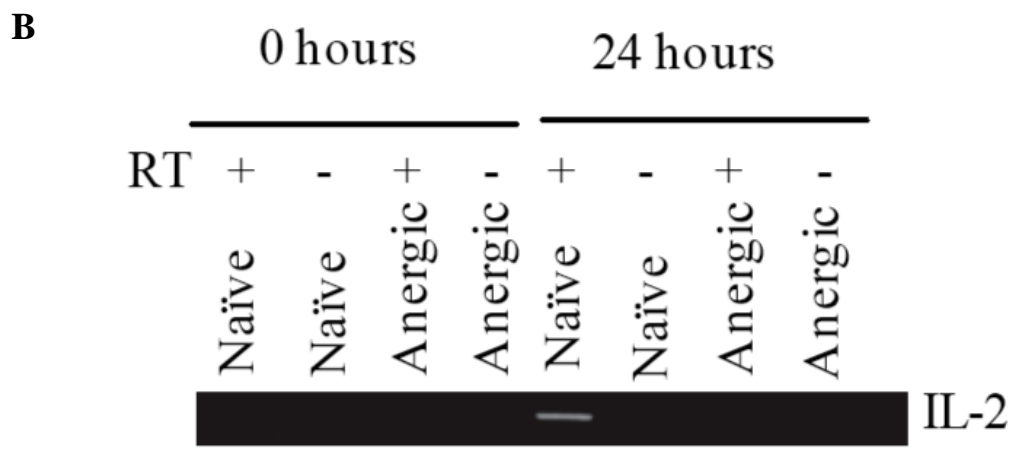
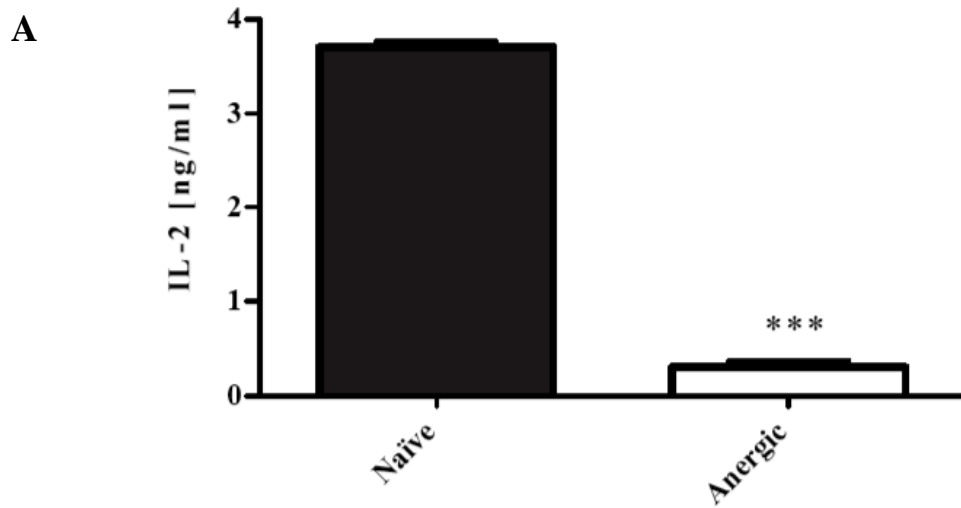


Figure 5. Injection of peptide antigen induces anergy in 2C TCR transgenic T lymphocytes. A. IL-2 secretion was quantified by sandwich ELISA. 1×10^6 purified T lymphocytes were incubated with 4×10^6 splenocytes as APCs and peptide, and then supernatants were analyzed. $***p < 0.0001$, different from Naïve. Data are representative of three independent experiments. B. IL-2 mRNA levels were quantified by PCR. 1×10^6 purified T lymphocytes were stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours or left unstimulated and RNA was purified and reverse transcribed. cDNA was amplified using IL-2 primers and analyzed by agarose gel. C. IFN- γ secretion was quantified as for IL-2. ns, not significantly different from Naïve. Data are representative of three independent experiments.

induction of T cell anergy as ascertained by a selective reduction in IL-2 cytokine production at the protein and mRNA levels.

3.1.2 NF- κ B activation is defective in anergic CD8⁺ T Lymphocytes.

In T lymphocytes, the regulation of IL-2 production and cell proliferation is regulated by three inducible transcription factors, NFAT, AP-1 and NF- κ B, all of which bind to the *il-2* promoter. Previously our lab showed that the NFAT pathway is affected in anergic cells [184], while others have found that the NF- κ B pathway is defective in a variety of anergy systems [180, 187, 190]. To address whether the NF- κ B pathway is defective in the 2C TCR anergy model we analyzed NF- κ B activation in untreated and anergized T lymphocytes.

To determine if NF- κ B transcriptional activation is impacted by anergy of CD8⁺ T lymphocytes, we crossbred 2C TCR transgenic mice with NF- κ B-luc mice. NF- κ B-luc mice harbor a NF- κ B luciferase reporter gene controlled by two NF- κ B responsive elements from the κ B light chain enhancer upstream of a minimal Fos promoter [193]. This allows measurement of NF- κ B activity by quantifying luciferase activity. Anergy was induced in 2C/NF- κ B-luc mice by peptide injection, and T cells were stimulated with anti-CD3 and anti-CD28 antibodies. Upon measuring luciferase activity we found that NF- κ B dependant luciferase activity is significantly reduced in anergic cells relative to naïve cells (Fig. 6), indicating that components functionally important in the NF- κ B pathway are downregulated or inactivated in anergic cells.

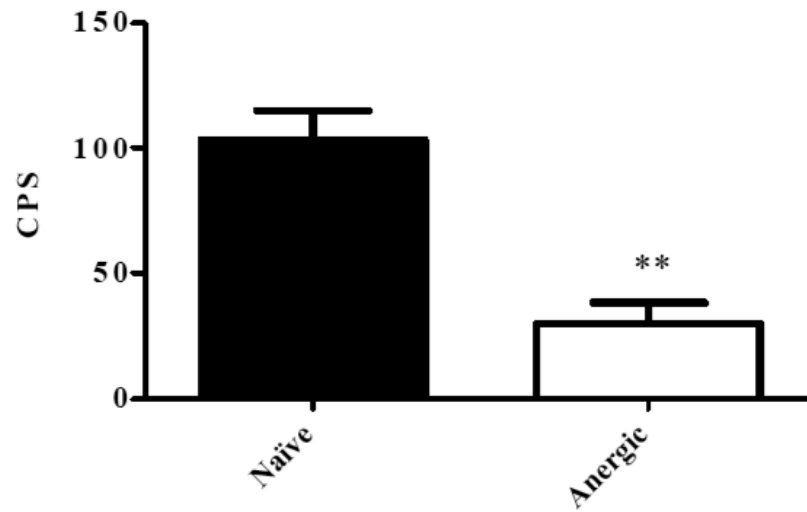


Figure 6. Anergic T lymphocytes display deficient NF- κ B dependent transcription. 1×10^6 purified naive and anergic 2C TCR/NF- κ B-Luc transgenic CD8⁺ T lymphocytes were stimulated for 48 hours with anti-CD3 and anti-CD28 conjugated beads and luciferase activity was analyzed. ** $p < 0.001$, different from Naïve. Data are representative of three independent experiments

3.1.3 I κ B α is degraded in both naïve and anergic T lymphocytes, but is resynthesized only in naïve T lymphocytes.

A critical event in the regulation of the NF- κ B pathway is the degradation of the inhibitory proteins, the I κ Bs [64]. The I κ Bs are phosphorylated by the I κ B kinase (IKK) complex, resulting in ubiquitination and degradation by the 26S proteasome, and release of NF- κ B. In unstimulated cells, NF- κ B is bound by I κ B, inhibiting translocation to the nucleus. In stimulated cells, I κ B is degraded, unmasking the NF- κ B nuclear localization sequence and allowing for nuclear translocation. I κ B is resynthesized in a NF- κ B dependent manner, generating a negative feedback loop [106, 198]. Therefore, we asked if the degradation of two members of the I κ B family, I κ B α and I κ B β is impacted in anergic T lymphocytes.

To address this question, we stimulated naïve and anergic CD8⁺ T lymphocytes *in vitro* with anti-CD3 and anti-CD28 antibodies and visualized I κ B α expression by western blot. Consistent with previous observations [198], we showed a pattern of degradation followed by resynthesis of I κ B α in naïve T cells (Fig. 7A). In the naïve cell population I κ B α levels decrease within 5 of stimulation, and increase again by 30 minutes post stimulation (Fig. 7A, left panel). The strong induction of I κ B α levels at 30 minutes is suggestive of I κ B α resynthesis due to enhanced nuclear NF- κ B levels. In anergic cells, we observed that I κ B α is degraded with similar kinetics to naïve cells, but in contrast, resynthesis of I κ B α does not occur in the anergic T cell population (Fig. 7A, right panel). Since I κ B α is a known NF- κ B target gene [199], we examined whether the defect in I κ B α resynthesis in anergic cells is due to a defect in gene transcription. To determine whether I κ B α transcription is impacted in anergic CD8⁺ T lymphocytes, we evaluated

I κ B α mRNA levels by standard PCR (Fig. 7B) and by quantitative real time PCR (qPCR) (Fig. 7C) following CD8⁺ T lymphocyte stimulation. In naive CD8 T lymphocytes, I κ B α mRNA expression increased between 15 and 30 minutes post-stimulation. (Fig. 7B and 7C). In contrast, a similar increase was not observed in I κ B α mRNA expression in the anergic CD8⁺ T cell population, indicating that I κ B α transcription is inhibited in anergic CD8⁺ T lymphocytes. These data indicate that NF- κ B transcriptional activity in anergic T lymphocytes is defective despite normal I κ B α degradation.

In addition to I κ B α , I κ B β is a NF- κ B inhibitor that is phosphorylated and degraded allowing for NF- κ B nuclear translocation [200]. To analyze the kinetics of I κ B β degradation/resynthesis in naïve and anergic CD8⁺ T lymphocytes, we purified and stimulated naïve and anergic lymphocytes with anti-CD3 and anti-CD28 antibodies and visualized I κ B β expression by western blot. This demonstrated that I κ B β is degraded in naïve cells after 45 and 60 minutes of stimulation (Fig. 8A left panel). I κ B β is also degraded after stimulation in anergic T lymphocytes, and appears to follow more rapid kinetics, showing degradation after 5 minutes of stimulation (Fig. 8A right panel). Next, we wanted to analyze whether I κ B β transcription is also impacted in anergic cells. As with I κ B α , I κ B β is regulated by NF- κ B activation, generating a negative feedback loop [201]. Real time PCR data demonstrated that I κ B β is resynthesized starting at 30 minutes of stimulation in both naïve and anergic T cells. In naïve cells, I κ B β mRNA levels remain static between 30-60 minutes of stimulation; while in anergic cells I κ B β mRNA expression show a slight increase (Fig. 8B). Here we show that unlike I κ B α , there is no obvious defect in I κ B β re-expression

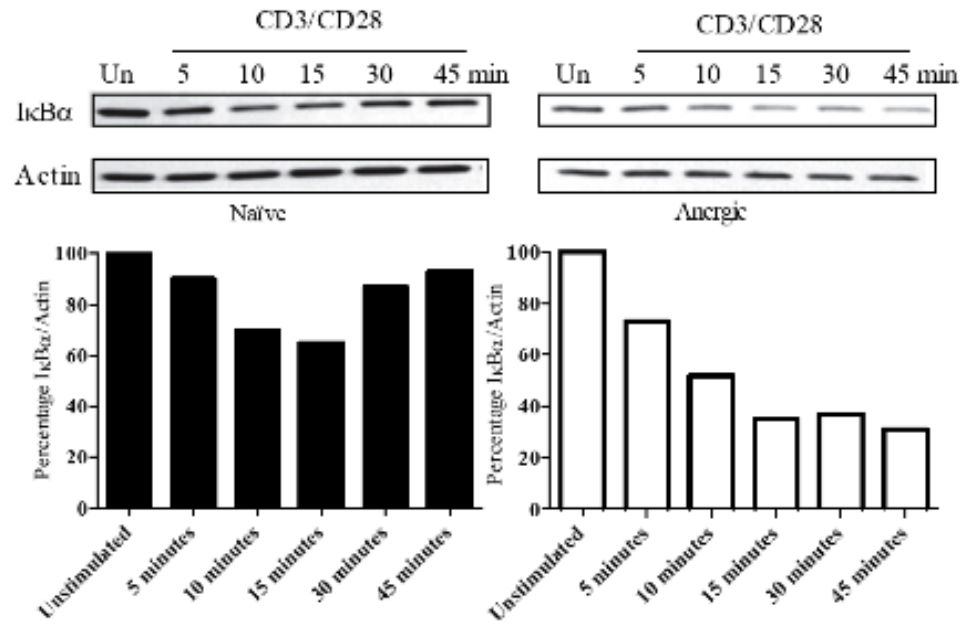
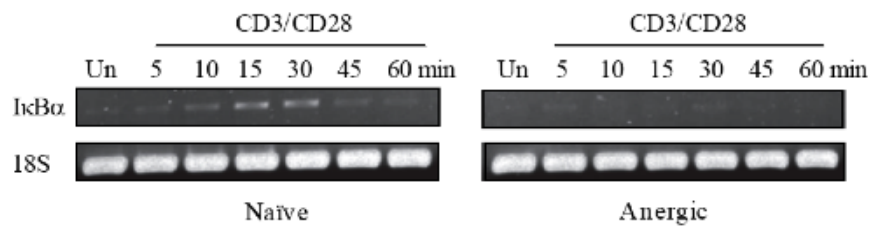
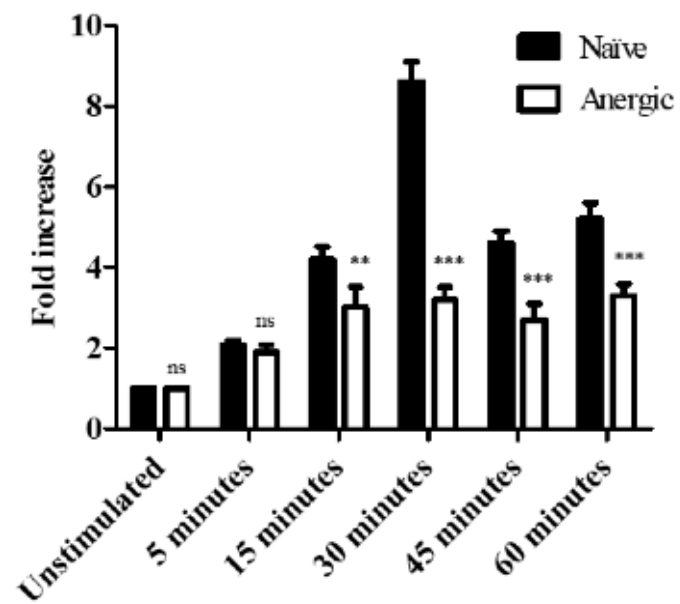
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Figure 7. Anergic T cells show normal degradation, but impaired re-expression, of I κ B α . A. Purified naïve and anergic 2C TCR transgenic T cells were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated times and I κ B α levels were determined by western blot. The relative intensity of the bands is defined as the ratio of intensity of I κ B α to actin, with unstimulated cells set to 100%. B. Purified naïve and anergic cells were stimulated as in (A) for the indicated times and I κ B α mRNA levels were compared by RT-PCR. C. Purified naïve and anergic cells were stimulated as in (A) for the indicated times and I κ B α mRNA levels were compared by RT-qPCR. ** p <0.001, different from Naïve; *** p <0.0001, different from Naïve; ns, not significantly different from Naïve. Data are representative of three independent experiments.

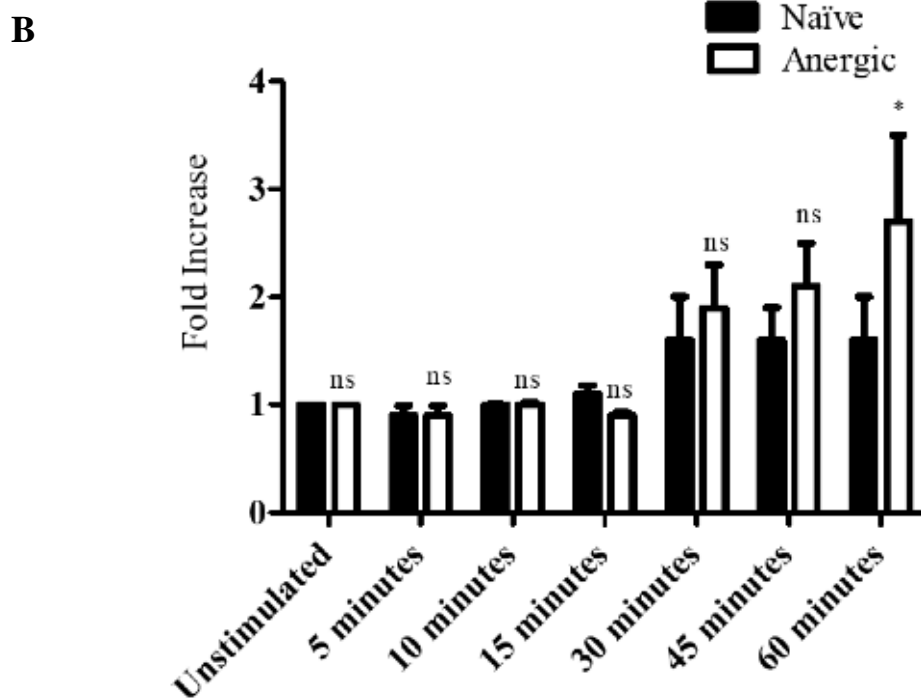
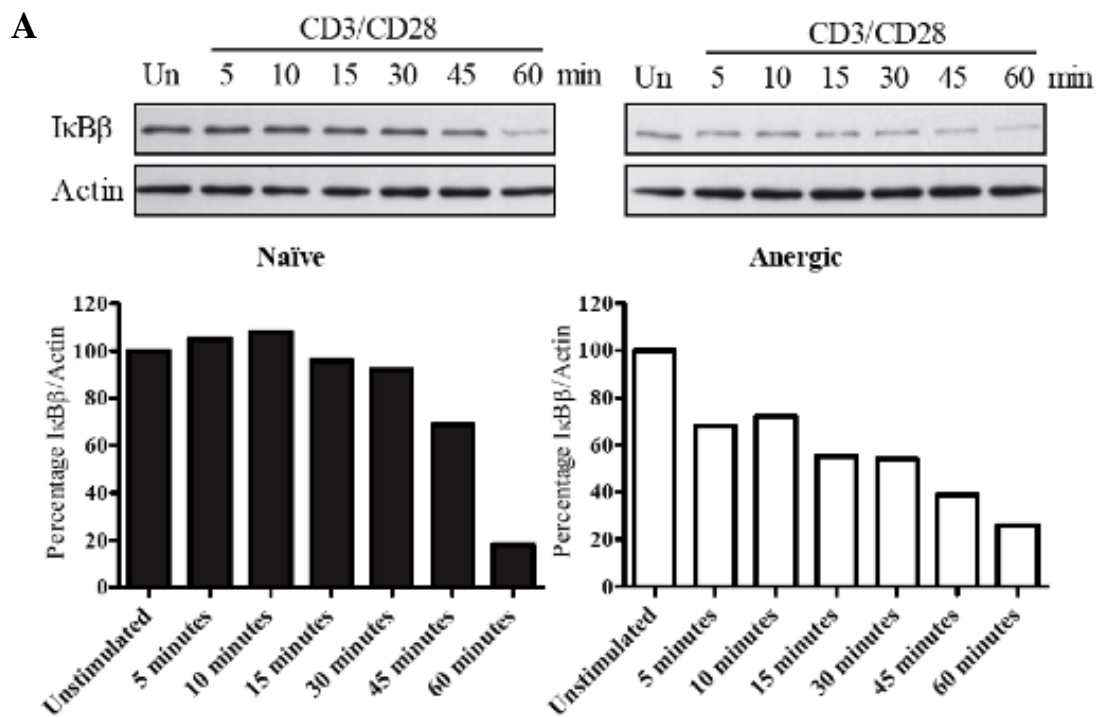
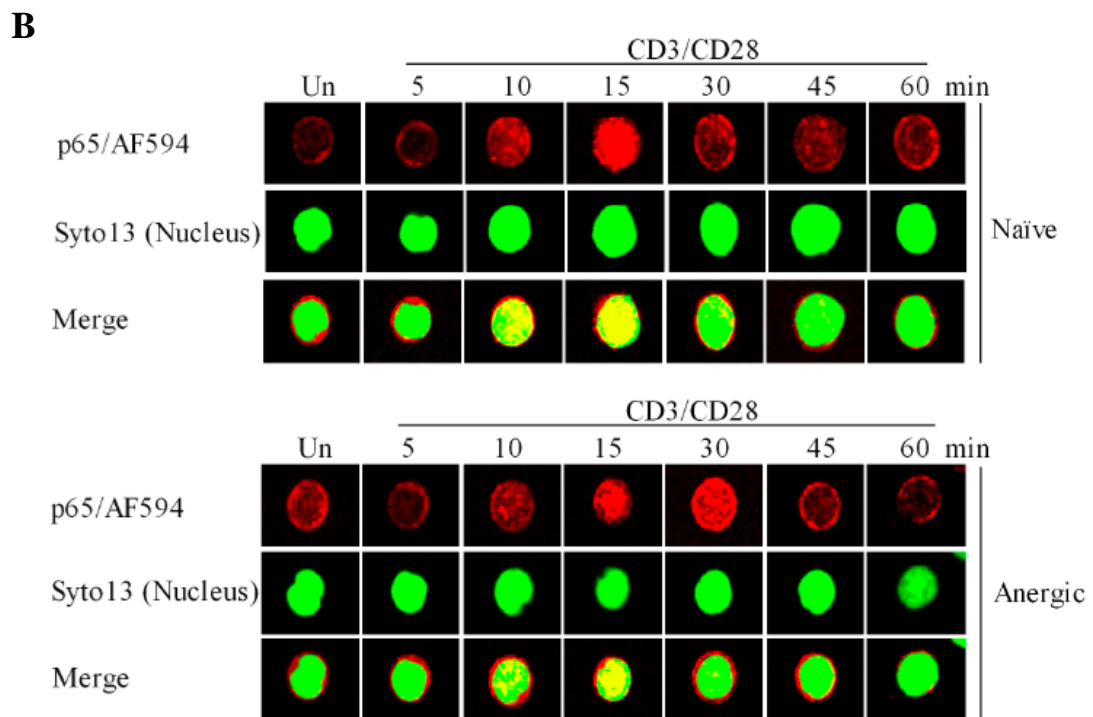
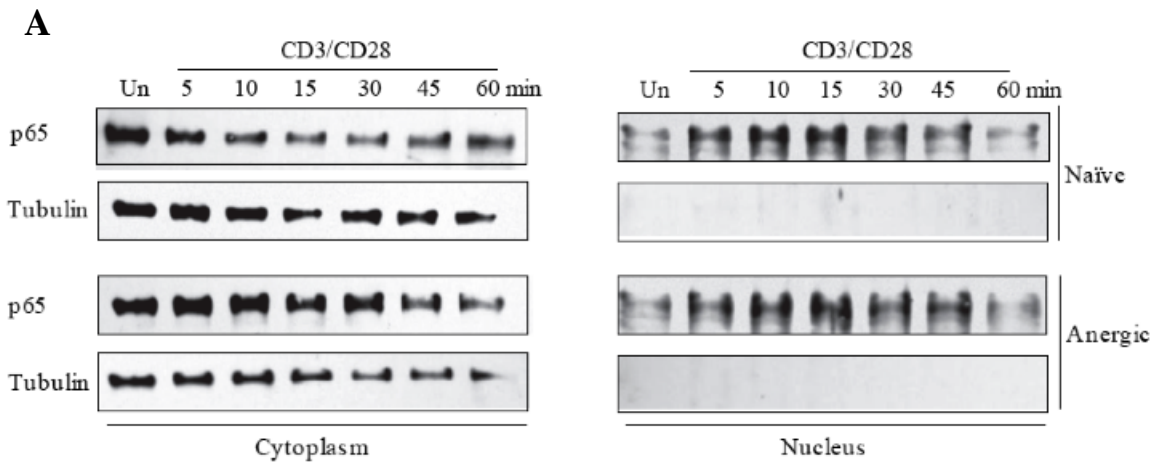


Figure 8. Anergic T cells show normal degradation of I κ B β . A. Purified naïve and anergic 2C TCR transgenic T cells were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated times and I κ B β levels were determined by western blot. The relative intensity of the bands is defined as the ratio of intensity of I κ B β to actin, with unstimulated cells set to 100%. Data is representative of three independent experiments. B. Purified naïve and anergic cells were stimulated as in (A) for the indicated times and I κ B β mRNA levels were compared by RT-qPCR. * p <0.05, different from Naïve; ns, not significantly different from Naïve. Experiment was performed only once.

3.1.4 p65 translocation is normal in anergic CD8⁺ T Lymphocytes.

As a result of TCR engagement, I κ B α is phosphorylated and degraded, releasing sequestered NF- κ B to the cytoplasm and allowing it to translocate into the nucleus where it can bind to the promoters of multiple genes. It is known that I κ B α preferentially binds and inhibits p50-p65 heterodimers [202]. Although I κ B α and I κ B β are degraded after stimulation of anergic T lymphocytes, it would be possible that NF- κ B remained sequestered in the cytoplasm, perhaps due to binding by other inhibitory factors. Therefore, we wanted to determine whether differences in NF- κ B function are due to altered nuclear translocation in the anergic CD8⁺ T lymphocyte population. To address this, we analyzed translocation of p65 during stimulation using cellular fractionation. Naïve and anergic cells were purified, fractionated into cytoplasmic and nuclear fractions and proteins of interest were visualized using anti-p65 antibodies. Western blot analysis revealed that in resting cells, p65 is predominantly located in the cytoplasm. Between 5 and 15 minutes of stimulation, p65 increases in the nuclear fraction of both naïve and anergic T cells (Fig. 9A). These results suggest that the NF- κ B defect in anergic T lymphocytes is not due to a failure in translocation. In order to confirm the results from the cellular fractionations, a second approach was also used. p65 localization was determined in intact cells using immunofluorescence microscopy. Consistent with the fractionation results, p65 was seen to translocate to the nucleus between 10 and 15 minutes of stimulation in both naïve and anergic T lymphocytes (Fig. 9B). Colocalization analysis with the nuclear marker SYTO13 confirmed the nuclear nature of p65 localization following stimulation of either naïve or anergic T lymphocytes (Fig. 9C).



C

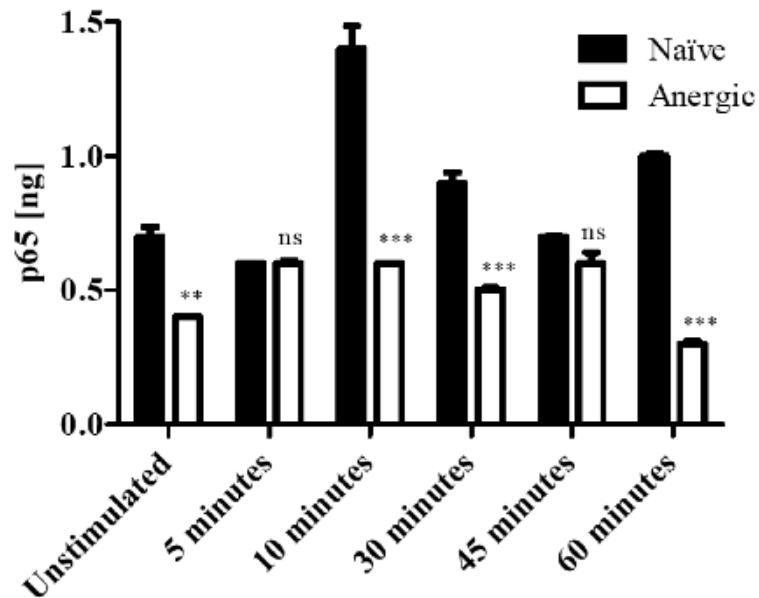


Figure 9. NF- κ B p65 translocates to the nucleus normally in anergic cells. Naïve and anergic T lymphocytes were purified and stimulated with anti-CD3 and anti-CD28 for the indicated time points. A. Cytoplasmic and nuclear fractions were collected and resolved by SDS-PAGE. Proteins were analyzed by western blot using anti-p65 antibody. Blots were re-probed with anti-tubulin antibody as a loading control for cytosolic fractions and to confirm the purity of nuclear fractions. B. Naïve (upper panels) and anergic (lower panels) T lymphocytes were stimulated for the indicated time points and NF- κ B p65 localization was determined by immunofluorescence microscopy. Nuclei were stained with the nuclear dye SYTO-13. C. Quantification of p65 nuclear localization shown in B. * $p < 0.05$, different from Naïve; ns, not significantly different from Naïve. Data show mean percentage of p65/SYTO-13 colocalization of 15 individual cells per individual experiment \pm S.D., and are representative of three independent experiments.

Thus, although we observed a defect in NF- κ B transcriptional activity, both I κ B α and I κ B β degradation and p65 nuclear translocation are intact in anergic T lymphocytes.

3.2 Discussion

Previously our laboratory demonstrated that T cell anergy can be induced in TCR transgenic mice by administration of antigenic peptide in the absence of adjuvants [165, 184]. Defective proliferation observed in anergic cells is related to the absence of IL-2 expression, as IL-2 is a known growth factor required for T lymphocyte proliferation [29].

The IL-2 promoter contains binding sites for various transcription factors, including NFAT, NF- κ B, and AP-1. Our group has previously demonstrated that the regulation of NFAT family members is altered in anergic T lymphocytes [184], although it remains unclear how this affects NFAT mediated gene expression. Given the likelihood that a combined action between multiple transcription factors is involved in the induction of IL-2 production and in promoting cell proliferation, both of which are repressed in anergic CD8⁺ T lymphocytes, we decided to study another transcription factor that is essential in IL-2 production, NF- κ B. NF- κ B is a pleiotropic transcription factor that regulates multiple genes, including IL-2. We studied NF- κ B activation using a NF- κ B responsive luciferase reporter gene and showed that in anergic CD8⁺ T lymphocytes, the level of NF- κ B activity is decreased in comparison to naïve cells. These data are suggestive of inefficient NF- κ B activation of anergic T lymphocytes. Consistent with our data, others have demonstrated that NF- κ B transcriptional activity is negatively impacted in anergic cells. Stimulation with peripheral self-antigens resulted in reduction in NF- κ B

transcriptional activity in CD8⁺ T lymphocytes [180], and in CD4⁺ T lymphocytes NF-κB transcriptional activity is dramatically reduced in tolerant cells [188]. Our data are thus in agreement with the data obtained by others showing a significant reduction in NF-κB transcriptional activity in anergic CD8⁺ T lymphocytes.

Previously it was shown that the activation of NF-κB as well as other transcription factors is defective in anergic cells. In anergic CD4⁺ T lymphocytes activated with three SEA injections, NF-κB p50-p50 homodimers predominate over p50-p65 heterodimers [187] suggesting that p50-p50 homodimers possess inhibitory characteristics due to the lack of transactivation domain. In autoreactive CD8⁺ T lymphocytes the transcriptional activity of NF-κB and AP-1 was reduced [180]. Defective NF-κB activation has also been shown in T lymphocytes stimulated with anti-CD3 alone [189], showing that costimulation is required for proper T lymphocyte activation. In anergic CD4⁺ T lymphocytes stimulated with three SEA injections, partial degradation of IκBα caused a defect in nuclear translocation of p65 [188]. Our laboratory has shown that the NFAT pathway is altered in anergic cells. Srinivasan et al. showed that NFAT1 translocation to the nucleus was decreased in anergic cells while, NFAT2 translocates to the nucleus only in anergic cells [184].

A key event in the NF-κB pathway is the degradation of IκB. We hypothesized that the NF-κB activation defect could be the result of defective IκB degradation in anergic T lymphocytes. Therefore, we examined whether expression levels of IκBα are impacted by T cell engagement. We found that IκBα is normally degraded in naïve and anergic T lymphocytes, but that the resynthesis of this protein is abrogated in anergic cells. These results differ from previously reported data in CD4⁺ T lymphocytes

stimulated with SEA or cytochrome c peptide, which showed that I κ B α is only partially degraded, and is resynthesized in anergic T lymphocytes, leading to reduced NF- κ B nuclear localization [188, 190]. It is possible that the decreased degradation of I κ B α in anergic cells reported by these two groups may be a feature of CD4⁺ T lymphocytes or that the differences in stimuli may cause different effects on the T lymphocytes. Understanding this difference will require a more direct comparison of the various anergy models. In our anergy model, the absence of I κ B α resynthesis may be due to a lack of NF- κ B binding on the I κ B α promoter or to the binding of NF- κ B to the I κ B α promoter in absence of the necessary machinery to start transcription.

We also analyzed the degradation of another member of the I κ B family in anergic T lymphocytes. We showed that I κ B β is degraded in naïve T lymphocytes at 45 minutes of stimulation. Our results in naïve cells are consistent with previous reports showing that I κ B β degradation kinetics are delayed relative to I κ B α degradation or may not occur at all [203]. In both naïve and anergic T lymphocytes we observed a minor increase in the levels of I κ B β mRNA at 30 minutes of stimulation. However, this experiment was performed only once and, it would be important to replicate it to confirm our data on I κ B β resynthesis. To this end, it has been reported that I κ B β can regulate p65 and c-Rel [69] and that I κ B β may prevent I κ B α association with DNA-bound NF- κ B, causing a prolonged activation of NF- κ B [204]. I κ B β has also been suggested to be a target for NF- κ B, thus we hypothesize that we should observe resynthesis of I κ B β in naïve cells but at later time points. More studies are needed to observe the kinetics of I κ B β resynthesis in anergic cells.

Degradation of I κ B α generally correlates with translocation of NF- κ B from the cytoplasm to the nucleus. However, we hypothesized that nuclear translocation could be impacted in anergic cells even though we observed I κ B degradation. In the cytoplasm NF- κ B translocation is regulated by different members of the I κ B family [198] so, there is the possibility that other I κ B proteins might be blocking the NLS of p65 causing the retention of NF- κ B molecules in the cytoplasm of anergic cells. However, using two complementary techniques, we observed that p65 translocates into the nucleus with similar kinetics after stimulation of naïve and anergic CD8⁺ T lymphocytes. In contrast, others have shown or suggested that p65 nuclear translocation is reduced or limited in anergic CD4⁺ T lymphocytes [188, 190]. The reasons for these differences remain unclear, but may reflect important differences in the regulation of NF- κ B signaling between CD4⁺ T lymphocytes and CD8⁺ T lymphocytes. Thus, the early events of NF- κ B activation, I κ B α degradation and p65 translocation, are intact in anergic CD8⁺ T lymphocytes, leading us to hypothesize that the defect in the NF- κ B pathway in anergic cells occurs in the nucleus. This hypothesis will be examined further in chapter 4.

Chapter 4: Comparison of post-translational modifications associated with activation of NF- κ B in naïve and anergic CD8⁺ T lymphocytes.

As described in chapter 3, we showed that NF- κ B transcriptional activity is defective in anergic T lymphocytes. To find out the reason of this defect we analyzed the degradation of I κ B at different stimulation times, and found that I κ B is degraded with similar kinetics in naïve and anergic T lymphocytes. We also studied the NF- κ B p65 subunit nuclear translocation patterns in naïve and anergic T lymphocytes. We showed that p65 translocates into the nucleus comparably in both naïve and anergic T lymphocytes. These findings led us to hypothesize that the decrease in NF- κ B activity is related to a defect in one or more of the post-translational modifications affecting NF- κ B activity. In this chapter, we analyze the phosphorylation and acetylation of p65 at different residues.

4.1 RESULTS

4.1.1 NF- κ B p65 phosphorylation at Ser⁵³⁶ is not affected in anergic cells

NF- κ B function is regulated not only by I κ B degradation and cellular localization, but also by post-translational modifications (reviewed in [98]). Phosphorylation of several serine residues in p65 is critical to obtain full activation of NF- κ B. Therefore, we examined whether p65 phosphorylation is altered in anergic CD8⁺ T lymphocytes.

Degradation of I κ B α allows the IKK-mediated phosphorylation of p65 at Ser⁵³⁶ [27, 85]. Phosphorylation of p65 at Ser⁵³⁶ occurs only in the cytoplasm prior to nuclear translocation [27, 85]. Therefore, we analyzed the phosphorylation status of p65 in our cell populations. We observed that p65 is constitutively phosphorylated in unstimulated cells and anti-CD3 and anti-CD28 stimulation rapidly induced enhanced phosphorylation of p65 at 5 minutes post-stimulation in the cytosolic fraction of both naïve and anergic cells (Fig 10A, left panels). By 10 minutes after stimulation, phosphorylation decreased to below basal levels, and the kinetics were the same in both naïve and anergic T lymphocytes. Consistent with the literature [27, 85], p65 remained unphosphorylated at Ser⁵³⁶ in nuclear fractions isolated from naïve and anergic cells at all time points, indicating that the kinase that phosphorylates p65 at this residue in CD8⁺ T lymphocytes is cytoplasmic (Fig. 10A, right panels).

4.1.2 NF- κ B p65 phosphorylation at Ser²⁷⁶ is not affected in anergic cells

The catalytic subunit of PKA (cPKA) can bind to the I κ B α -NF- κ B complexes in the cytoplasm [83]. Subsequent to I κ B α phosphorylation and degradation the NF- κ B p65 subunit can be phosphorylated by cPKA at Ser²⁷⁶ in the cytoplasm [83, 205]. p65 can also interact with, and be phosphorylated by MSK1 at Ser²⁷⁶ in the nucleus both in vitro and in vivo [86, 87]. Either cytoplasmic phosphorylation by PKA or nuclear phosphorylation by MSK1 appears to be required for the association between p65 and the transcriptional co-activators CBP/p300 and enhancing transcriptional activity. However, Ser²⁷⁶ phosphorylation is not involved in promoting p65 nuclear translocation [86]. We therefore considered that misregulation of Ser²⁷⁶ phosphorylation might be a good

candidate to cause the abnormal NF- κ B activation in anergic T lymphocytes. To characterize the phosphorylation status of p65 at Ser²⁷⁶ we purified and stimulated T lymphocytes with anti-CD3 and anti-CD28 and fractionated the cells into cytoplasmic and nuclear fractions. Stimulation of T lymphocytes resulted in phosphorylation of p65 at Ser²⁷⁶ only in the nuclear fraction in both naïve and anergic lymphocytes at 5 and 10 minutes post-stimulation (Fig. 10B, right panels). No phosphorylation of p65 at Ser²⁷⁶ was observed in the cytoplasmic fractions (Fig. 10B, left panels), suggesting that phosphorylation of this residue in T lymphocytes may occur mostly via the nuclear/MSK1 mechanism. These data show that p65 phosphorylation at Ser²⁷⁶ is not affected in anergic T lymphocytes, leading us to analyze a third phosphorylation event involved in the activation of p65, phosphorylation at Ser³¹¹.

4.1.3 NF- κ B p65 is not phosphorylated at Ser³¹¹ in anergic cells

A third phosphorylation site in p65 is at Ser³¹¹. PKC ζ directly phosphorylates p65 at Ser³¹¹ in nuclear fractions of mouse embryonic fibroblasts and this residue is required for the recruitment and interaction of the acetyltransferase CBP with the NF- κ B subunit p65 [92]. As with Ser²⁷⁶, phosphorylation at Ser³¹¹ regulates gene transactivation without impacting nuclear localization [92]. Once we determined that phosphorylation of p65 at Ser⁵³⁶ and Ser²⁷⁶ were not affected in anergic cells, we wanted to analyze the phosphorylation pattern of p65 at Ser³¹¹ in anergic and naïve T lymphocytes. Fractionated naïve and anergic CD8⁺ T lymphocytes were analyzed by immunoblotting with an antibody against this phosphorylated residue, p-NF- κ B-p65 (Ser³¹¹). As is shown in Figure 11A, p65 was phosphorylated at Ser³¹¹ in nuclear fractions of naïve cells at 5 and

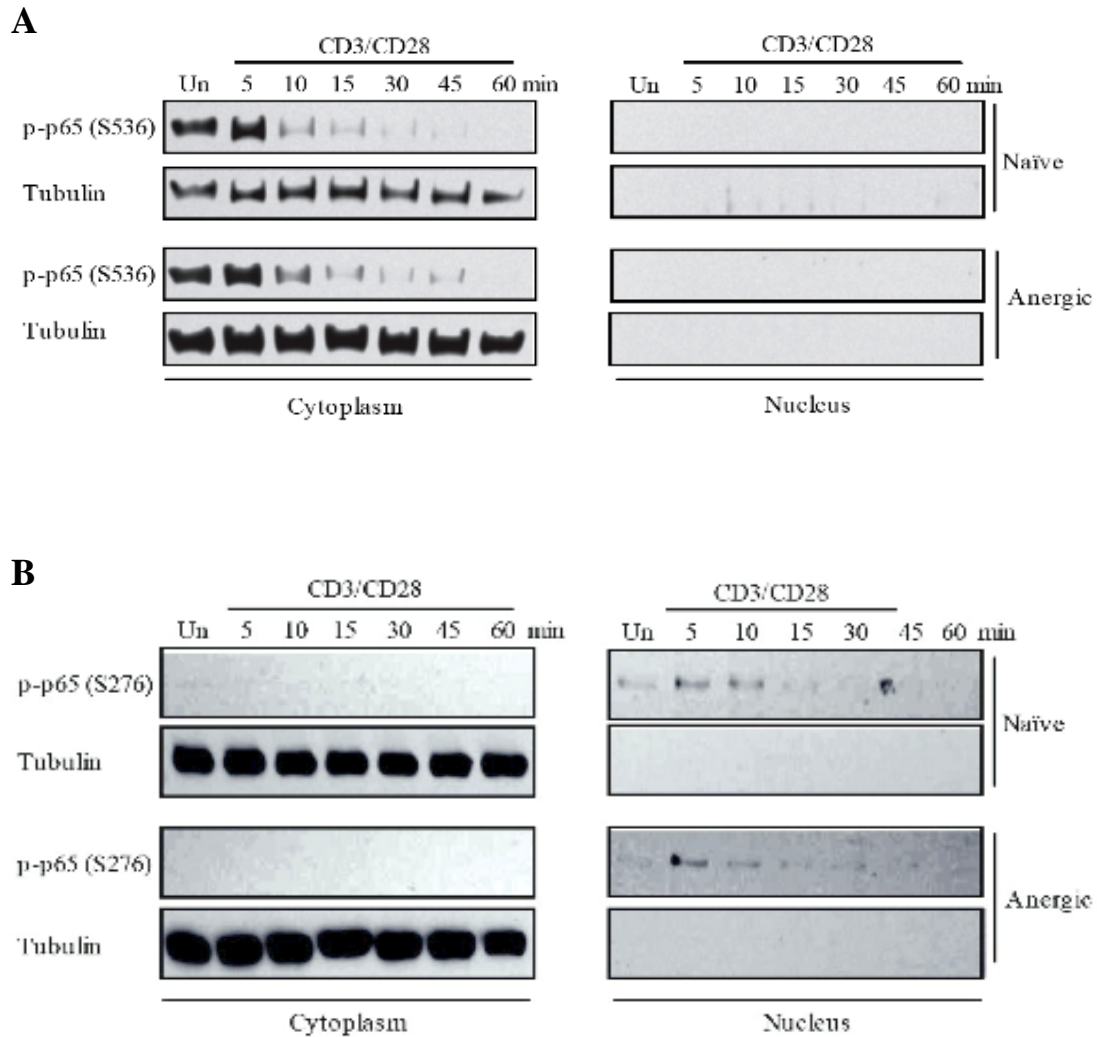


Figure 10. NF- κ B p65 is phosphorylated at Ser⁵³⁶ and Ser²⁷⁶ in both naïve and anergic lymphocytes. Naïve and anergic T lymphocytes were purified and stimulated with anti-CD3 and anti-CD28 for the indicated time points. Stimulated T lymphocytes were fractionated into cytoplasmic and nuclear fractions and analyzed for different proteins. Cytoplasmic and nuclear fractions were resolved by SDS-PAGE, and proteins were then analyzed by western blot using anti-p-p65 (S536) antibody (A) or anti-p-p65 (S276) antibody (B). Blots were re-probed with anti-tubulin antibody as a loading control for cytosolic fractions and to confirm the purity of nuclear fractions. All data are representative of three independent experiments.

10 minutes of post-stimulation. In contrast, phosphorylation of p65 at Ser³¹¹ was clearly impaired in the nuclei of anergic lymphocytes. No phosphorylation of p65 at Ser³¹¹ was observed in the cytoplasmic fraction of naïve and anergic T lymphocytes. Thus, the defect in NF- κ B activation in anergic T lymphocytes correlates with a failure in p65 Ser³¹¹ phosphorylation, which suggests that this post-translational modification may be important for the regulation of NF- κ B transcriptional activity in anergy.

4.1.4 NF- κ B p65 is not acetylated at Lys³¹⁰ in anergic cells

Phosphorylation of p65 at Ser²⁷⁶ and Ser³¹¹ is required for the recruitment of the transcriptional co-activators CBP/p300 [84, 92]. These transcriptional co-activators acetylate several transcription factors including the p65 subunit of NF- κ B. [89, 94, 118]. Acetylation by CBP/p300 is suggested to regulate p65 transcriptional activity and DNA binding [94]. Since we observed a difference in the phosphorylation status of p65 at Ser³¹¹ in our anergic cell population, we examined the acetylation status of p65 at Lys³¹⁰. We were interested in the acetylation at this residue due to its proximity to Ser³¹¹. T lymphocytes were stimulated with anti-CD3 and anti-CD28 antibodies, and nuclear fractions were analyzed by western blotting. We observed that p65 was acetylated at Lys³¹⁰ in nuclear fractions of naïve lymphocytes at 30 minutes of anti-CD3 and anti-CD28 stimulation (Fig. 11B, upper panel). In contrast, p65 was not acetylated at this residue in anergic lymphocytes (Fig. 11B, lower panel). Taken together, these results provide evidence of a functional defect in the activation of NF- κ B due to impaired phosphorylation of Ser³¹¹ and defective acetylation of Lys³¹⁰ in anergic CD8⁺ T lymphocytes.

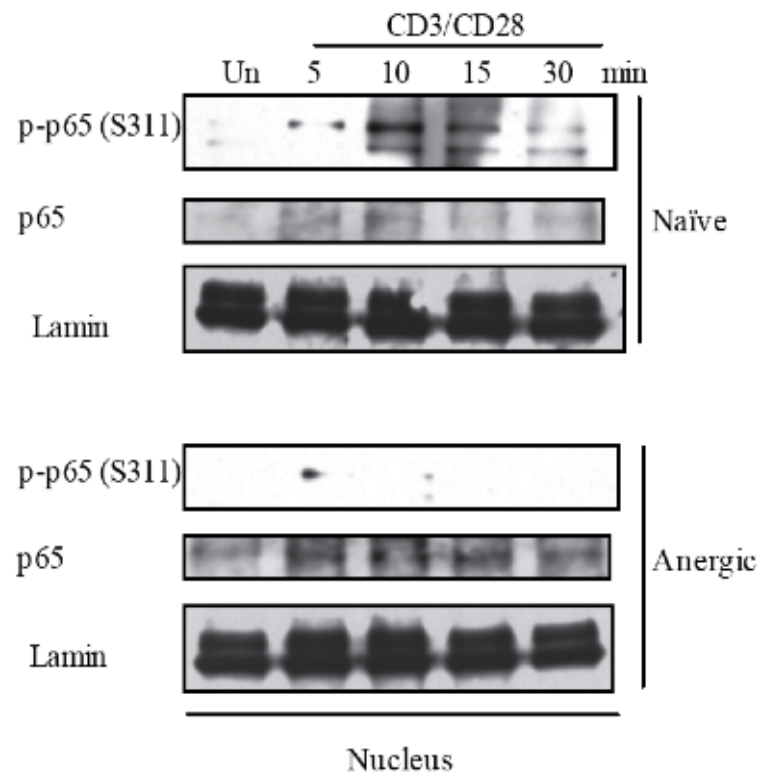
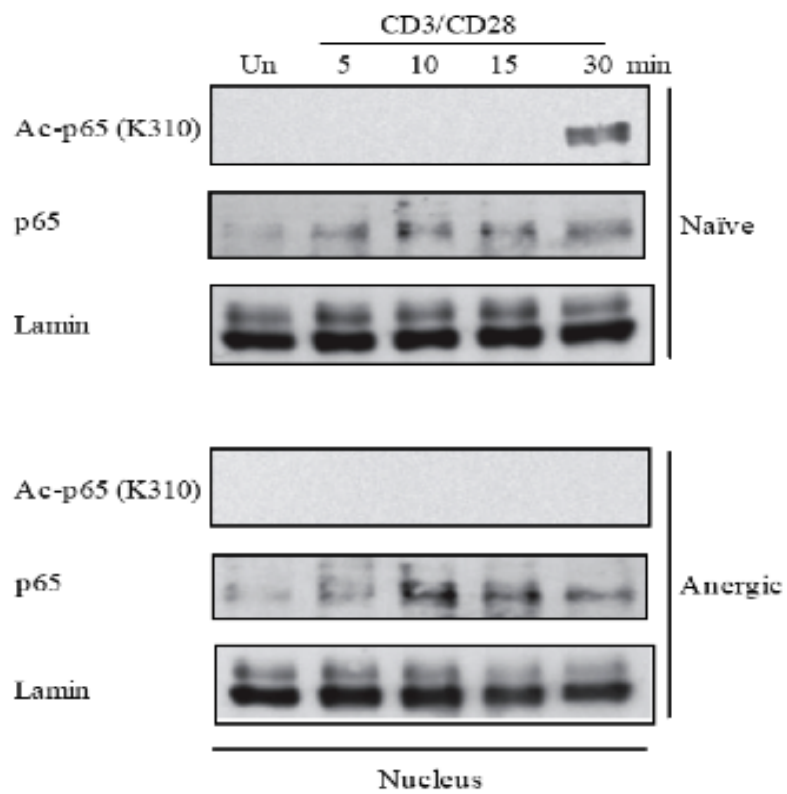
A**B**

Figure 11. Phosphorylation of p65 at Ser³¹¹ and acetylation at Lys³¹⁰ are defective in anergic lymphocytes. A. Purified naïve and anergic T lymphocytes were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated times. Cytosolic and nuclear fractions were purified and resolved by SDS-PAGE. Proteins were then analyzed by western blot using anti-p-p65 (Ser311) antibody. Blots were re-probed anti-total p65, and then with anti-tubulin antibody as a loading control for cytosolic fractions and to confirm purity of nuclear fractions. All data are representative of three independent experiments. B. After purification, T lymphocytes were stimulated for the indicated time points and nuclear fractions were collected and resolved by SDS-PAGE. Proteins were analyzed by western blot using anti-acetyl-p65 (Lys310) antibody. Blots were re-probed with anti-total p65, and then with anti-lamin A/C antibody as a loading control for nuclear fractions. All data are representative of three independent experiments.

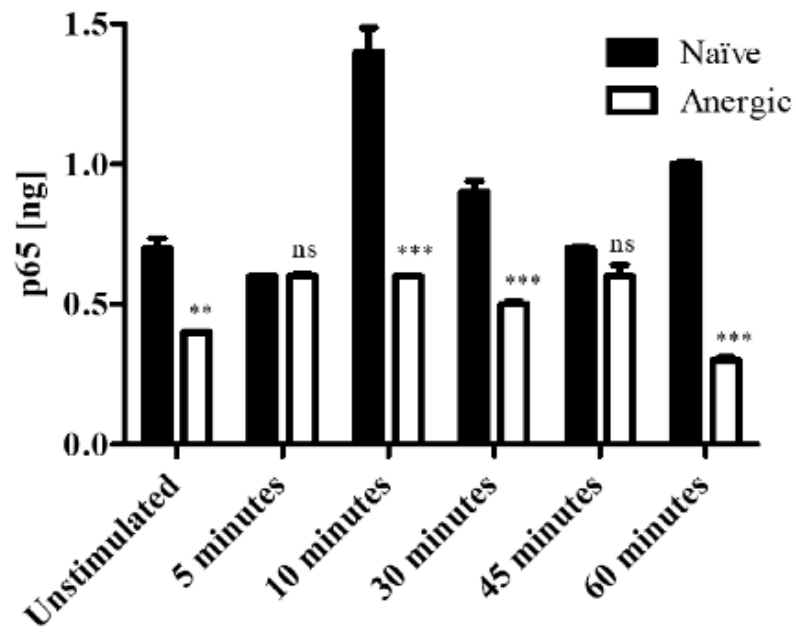
4.1.5 Analysis of NF- κ B DNA binding activity in anergic CD8⁺ T Lymphocytes

Defects in NF- κ B phosphorylation and acetylation could impact transcriptional activity in multiple ways: DNA binding, recruitment of positive or negative regulatory factors, protein stability, etc. Although the modifications at Ser³¹¹ and Lys³¹⁰ have not been implicated directly in DNA binding, it is possible that in combination with other differences not identified yet, they may still be important for general DNA binding or for target promoter selection. We therefore asked whether NF- κ B DNA binding is altered in anergic cells. Naïve and anergic 2C TCR transgenic T lymphocytes were stimulated *in vitro* with anti-CD3 and anti-CD28 antibodies for 5 to 60 minutes. Nuclear proteins were isolated and analyzed for κ B site binding activity. As seen in figure 12A, p65 binding to the NF- κ B consensus sequence is enhanced beginning at 10 minutes post stimulation in naïve cells. By contrast, binding of p65 to a κ B consensus sequence was not significantly increased after stimulation of anergic cells. Competition-binding assays using WT consensus oligonucleotides demonstrate the specificity of the assay, showing that p65 does not bind to the κ B consensus site when WT competitor oligonucleotides are added to the assay (Fig. 12B). Although this experiment was only performed once, and must therefore be considered preliminary, the results are consistent with a defect in NF- κ B binding activity in anergic T lymphocytes.

To analyze the binding of NF- κ B to bona fide target promoters in the context of chromatin, we plan to use the technique of chromatin immunoprecipitation (ChIP). Prior to working in primary T lymphocytes, we first began to optimize the ChIP procedure using the T lymphoma cell line EL-4. EL-4 cells grow constitutively, but only produce IL-2 after stimulation. Thus, they can serve as a model for IL-2 transcriptional regulation.

As shown in figure 13, NF- κ B is not bound to the IL-2 promoter in unstimulated EL-4 cells. However, after stimulation, DNA containing the IL-2 promoter coprecipitates with NF- κ B p65. The next step will be to use the optimized ChIP protocol to determine whether NF- κ B binding to the IL-2 promoter (as well as other selected target genes) is impaired in primary anergic CD8⁺ T lymphocytes.

A



B

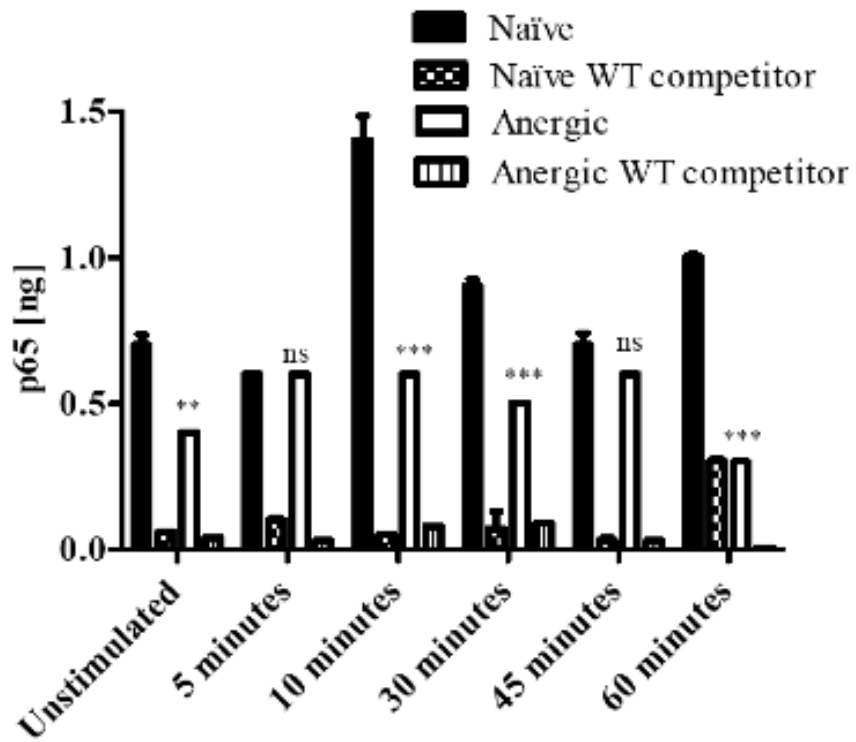


Figure 12. Anergic T lymphocytes display deficient NF- κ B dependent transcription and p65 binding activity. A. 5×10^6 purified naïve and anergic 2C TCR transgenic CD8⁺ T lymphocytes were stimulated for the indicated time points and nuclear extracts were prepared. Nuclear extract was added to plates coated with κ B consensus sequence oligonucleotides and then analyzed for NF- κ B p65 binding. ** $p < 0.001$, different from Naïve; *** $p < 0.0001$, different from Naïve. B. p65 binding specificity was analyzed. T lymphocytes were stimulated and analyzed as above. Binding was analyzed in the presence of excess WT competitor. Figure B represents the same experiment as A but competitor data is included. ** $p < 0.001$, different from Naïve; *** $p < 0.0001$, different from Naïve; ns, not significantly different from Naïve.

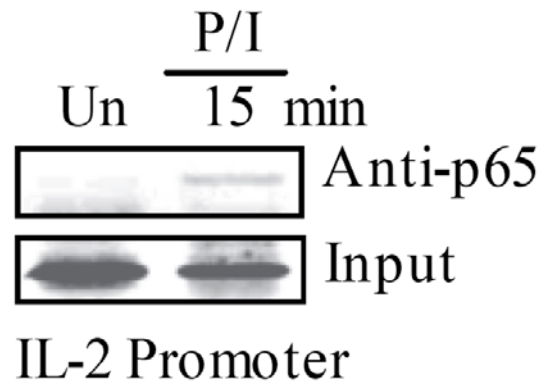


Figure 13. p65 is recruited to the IL-2 promoter in EL-4 cells stimulated with PMA and ionomycin. A. 3×10^7 EL-4 cells were stimulated with PMA and ionomycin (P/I) for the indicated time points. Chromatin was sheared and cross-linked. Cross-linked chromatin fragments were immunoprecipitated with anti-p65 antibody. Immunoprecipitated DNA was analyzed by PCR using primers specific for the *il-2* promoter. Data are representative of three experiments.

4.2 DISCUSSION

Subsequent to I κ B degradation, p65 is phosphorylated at multiple residues, and these phosphorylation events are required for the proper activation and regulation of NF- κ B. Alterations to phosphorylation patterns of the NF- κ B proteins have not previously been studied in anergic T lymphocytes, and we hypothesized that altered phosphorylation of the NF- κ B subunits may be the cause of the defective NF- κ B activation observed in anergic CD8⁺ T lymphocytes. Therefore, we wanted to analyze phosphorylation of p65 at residues that are known to be involved in NF- κ B activation and regulation.

We began by examining the phosphorylation of Ser⁵³⁶. Here we show that p65 is phosphorylated at Ser⁵³⁶ in the cytoplasm upon CD3/CD28 stimulation with the same kinetics in both naïve and anergic lymphocytes. Phosphorylation at Ser⁵³⁶ has been shown to be required for optimal p65 transcriptional activity in several different cell types [27, 85]. In our system we observed that p65 is phosphorylated at Ser⁵³⁶ in both naïve and anergic T lymphocytes, which does not correlate with the defect in NF- κ B dependant luciferase activation that we observed in anergic T lymphocytes. Based on the time points tested herein, we suggest that in CD8⁺ T lymphocytes, p65 is exclusively phosphorylated at Ser⁵³⁶ in the cytoplasm. However, it remains possible that due to kinetics, nuclear Ser⁵³⁶ phosphorylation events may have been missed.

Another posttranslational modification important in NF- κ B activity is the phosphorylation of p65 at Ser²⁷⁶. Here we show that p65 is phosphorylated at Ser²⁷⁶ with similar kinetics in both naïve and anergic CD8⁺ T lymphocytes. Ser²⁷⁶ phosphorylation occurs only in nuclear fraction and correlates with I κ B α degradation and p65 nuclear translocation in both naïve and anergic T lymphocytes. Two kinases have been associated

with this phosphorylation event. The catalytic subunit of PKA (cPKA) has been reported to phosphorylate p65 at Ser²⁷⁶ in the cytoplasm, while in the nucleus, p65 can be phosphorylated by mitogen- and stress-activated protein kinase 1 (MSK1) [83, 86]. Phosphorylation at Ser²⁷⁶ causes a conformational change that allows for interaction of p65 with CBP and increases transcriptional activity [84]. Based on our data, we suggest that in CD8⁺ T lymphocytes, MSK1 might be the major kinase phosphorylating p65 at Ser²⁷⁶, since this phosphorylation correlates with p65 nuclear translocation and we only observed phosphorylation at this residue in nuclear fractions.

The roles played by kinases and p65 phosphorylation sites vary between cell types. Our data suggest that in CD8⁺ T lymphocytes, phosphorylation of p65 at Ser²⁷⁶ is not sufficient to enhance p65 transcriptional activity, since p65 is phosphorylated at this residue in both naïve and anergic lymphocytes. Although phosphorylation of p65 at Ser⁵³⁶ and Ser²⁷⁶ have been shown to be necessary for complete NF-κB activation in other systems, we show that they are not differentially regulated in anergic T lymphocytes relative to naïve T lymphocytes, suggesting that other posttranslational modifications may be regulating NF-κB activity in anergic CD8⁺ T lymphocytes.

An additional phosphorylation site that is important for the transcriptional activation of p65 is Ser³¹¹ [92]. Therefore, we analyzed the phosphorylation of p65 at this residue. We found that p65 is phosphorylated at Ser³¹¹ in nuclear fractions of stimulated naïve T lymphocytes, but not anergic T lymphocytes. Thus, we hypothesized that this phosphorylation occurs following NF-κB nuclear translocation. However, we cannot discard the possibility that p65 is phosphorylated at Ser³¹¹ in the cytoplasm with different kinetics than what we studied. In this context, it has been shown that p65 nuclear

translocation is not impacted in PKC ζ deficient embryonic fibroblasts [206]. In addition, recruitment of RNA Polymerase II was negatively impacted in cells expressing a mutant p65 (S311A) [92]. Since phosphorylation at this residue is abrogated in anergic T lymphocytes, we hypothesized that the defect in NF- κ B transactivation activity in anergic CD8⁺ T lymphocytes is related to the absence in p65 Ser³¹¹ phosphorylation.

The Ser³¹¹ residue is a part of a conserved PKC target sequence. PKC ζ , an atypical PKC isoform, was found to associate with and phosphorylate p65 at Ser³¹¹ in TNF- α stimulated fibroblasts, resulting in enhanced transcriptional activity [91, 92]. PKC ζ can phosphorylate Ser³¹¹ directly in vitro and this post-translational modification is essential for interaction with CBP, which increases NF- κ B transcriptional activity [92]. Consistent with this, overexpression of PKC ζ in a Jurkat cell line enhances nuclear p65 transcriptional activity [93]. However, others have shown that overexpression of PKC ζ does not enhance NF- κ B transcriptional activity in T lymphocytes [207]. The importance of phosphorylation at this residue is evidenced by PKC ζ ^{-/-} embryonic fibroblasts in which NF- κ B transcriptional activity was abrogated [91]. However, PKC ζ ^{-/-} mice undergo normal thymic development [91], and PKC ζ deficient T lymphocytes have no defects in IL-2 production and cellular proliferation when stimulated in vitro [206, 208]. Together these results suggest that the kinase phosphorylating p65 at Ser³¹¹ in T lymphocytes is not PKC ζ .

We propose that if as suggested above, PKC ζ is not the Ser³¹¹ kinase in CD8⁺ T lymphocytes, a different PKC isoform may be involved in this phosphorylation event. Of the known PKC isoforms, PKC α , β , δ , ϵ , λ , η , θ , and ζ are all expressed in T

lymphocytes [207, 209]. T lymphocyte activation is not defective in PKC β , ϵ , and λ knockout mouse models [210-212] and T lymphocyte proliferation and IL-2 production are enhanced in PKC δ deficient T lymphocytes [213]. Another PKC isoform that may be involved in Ser³¹¹ phosphorylation is PKC α . Studies with PKC α deficient T lymphocytes have shown reduced T lymphocyte proliferation, but this is accompanied by normal levels of IL-2 secretion [214]. Taken together, these data suggest that PKC β , ϵ , λ , δ , and α are unlikely to be essential for the normal activation of NF- κ B in CD8⁺ T lymphocytes, as defects in IL-2 expression are not seen in cells lacking these kinases.

Perhaps the most promising kinase that may be phosphorylating p65 at Ser³¹¹ is PKC θ . PKC θ is involved in phosphorylation of molecules involved in the NF- κ B pathway and is required for the activation of the IKK complex, which is required for the phosphorylation and degradation of I κ B α [215]. PKC θ deficient CD3⁺ T lymphocytes show defective IL-2 production and cell proliferation after anti-CD3 and anti-CD-28 stimulation [214, 216]. Given the context, it would be difficult to analyze whether PKC θ kinase is also important in the phosphorylation of p65 Ser³¹¹, because PKC θ is also required in the activation of the IKK complex. In the absence of PKC θ , phosphorylation and degradation of I κ B might be affected, altering the signaling downstream of these events. Another possibility could be a nuclear kinase, such as MSK1, which phosphorylates Ser²⁷⁶ in the nucleus [86]. Further studies are necessary to understand the regulation/phosphorylation of p65 at Ser³¹¹ and the kinase involved in this event.

Multiple phosphorylation events are associated with CBP/p300 recruitment and p65 acetylation. Phosphorylation at Ser²⁷⁶ and Ser³¹¹ appears to enhance gene transcription without affecting p65 nuclear translocation or DNA binding [83, 86, 92],

suggesting regulation of recruitment or assembly of the transcriptional machinery. It has been suggested that CBP/p300 not only acetylates histones but also acetylates other proteins bound to chromatin [90]. In vivo over-expression of the HATs p300 and CBP increased p65 acetylation at Lys³¹⁰ and this modification increased p65 transcriptional activity [94]. Consistent with this, p65 Lys³¹⁰ acetylation was abrogated in p65 mutants where serine 276 or Ser 536 was replaced by alanine [89]. We found that in anergic CD8⁺ T lymphocytes, p65 is not acetylated at Lys³¹⁰, while in naïve cells p65 is acetylated at this residue after 30 minutes of stimulation. The timing of Lys³¹⁰ acetylation of p65 is in agreement with data showing that p65 acetylation occurs after p65 phosphorylation [89, 94].

p65 is regulated both positively and negatively by multiple acetylation events [94, 95, 118]. The acetylation event that has been studied the most is acetylation of Lys³¹⁰. We were interested in this particular event due to the proximity of Lys³¹⁰ to Ser³¹¹ and the defect we found in Ser³¹¹ phosphorylation. Acetylation at Lys³¹⁰ is essential for NF-κB transcriptional activity, but does not impact p65 nuclear translocation or DNA binding [94]. This acetylation event has previously been reported to be dependant in phosphorylation at Ser²⁷⁶ and Ser⁵³⁶ [89], both of which are unaffected in our anergic cell population, but a role for Ser³¹¹ specifically in acetylation at Lys³¹⁰ has not previously been reported. Our results correlate with our Ser³¹¹ phosphorylation data showing that a defect in phosphorylation at Ser³¹¹ may result in inhibition of acetylation at Lys³¹⁰ in anergic T lymphocytes. The absence of Lys³¹⁰ acetylation in our anergic T lymphocytes may be explained by the absence of phosphorylation at Ser³¹¹ and a lack of recruitment of CBP/p300 or by dysfunctional CBP/p300 molecules binding to the NF-κB complex.

Recent work shows that Lys³¹⁰ is also a methylation site and that phosphorylation of p65 at Ser³¹¹ blocks the action of methylases on this residue [217], suggesting that phosphorylation at Ser³¹¹ favors the acetylation of Lys³¹⁰ over methylation, thus regulating the transcriptional activation of p65.

The defect in NF- κ B activation could occur at many points. One of these points is binding of NF- κ B to gene promoters. Therefore, we analyzed NF- κ B p65 subunit binding to a κ B consensus oligonucleotide. We have preliminary results suggesting that NF- κ B binding activity is reduced in stimulated anergic T lymphocytes, compared with naïve T lymphocytes. In T lymphocytes two extensively studied NF- κ B targets are the IL-2 promoter and the I κ B α promoter. It has been shown that inhibitory p50-p50 homodimers bind to the IL-2 promoter in anergic CD4⁺ T lymphocytes, while in naïve cells transcriptionally active p65-p50 heterodimers bind to the IL-2 promoter [188]. The approach that we utilized only allowed us to examine one NF- κ B subunit at a time making it difficult to determine the actual composition of the NF- κ B dimers in our naïve and anergic cells. If only p50-p50 homodimers bind to DNA in anergic cells, then p65 binding to the consensus sequence under these conditions would be abrogated. It has been observed that p50-p50 homodimers out-compete p65-p50 heterodimers for binding in both unstimulated cells and in anergic cells [187]. We only performed this experiment one time, so it would be important to repeat it to confirm the data obtained. Although the evidence obtained from individual mutations in different posttranslational modifications regulating NF- κ B have not shown a defect in DNA binding, combination with other alterations that have not been identified might be causing a defect in DNA binding activity.

In this chapter, we show two functional defects in anergic T lymphocytes. We show that p65 phosphorylation at Ser³¹¹ and acetylation at Lys³¹⁰ are abrogated in anergic T lymphocytes. These findings suggest that p65 phosphorylation at Ser³¹¹ and acetylation at Lys³¹⁰ are required for proper NF- κ B activation in CD8⁺ T lymphocytes.

Chapter 5: Conclusions

5.1 Summary and General Discussion

Anergy is a form of hyporesponsiveness characterized by decreases in IL-2 production and T lymphocyte proliferation. Anergy has been studied in many systems and has been shown to present a variety of signaling defects in T lymphocytes. The variety of systems and models used to study anergy makes it unclear how specific defects seen in different signaling pathways may cooperate in the induction of the anergic phenotype. The majority of studies on anergy to date have been performed CD4⁺ T lymphocytes. Far less is understood about signaling alterations involved in anergy in CD8⁺ T lymphocytes. Thus, it is important to study the different activation pathways in CD8⁺ T lymphocytes. Our lab has previously showed that the NFAT signaling is affected in CD8⁺ T lymphocytes [184] and others have identified defects in MAPK pathways in anergic CD4⁺ and CD8⁺ T lymphocytes [180, 187]. In this work, I explored the regulation of NF- κ B activation in anergic CD8⁺ T lymphocytes.

T lymphocyte stimulation triggers the activation of three main signaling cascades; NF- κ B, NFAT, and AP-1. All of these signaling pathways are involved in the induction of IL-2 and T lymphocyte proliferation. As mentioned before, NF- κ B is a transcription factor involved in the regulation of several genes in the immune system as well as in some diseases such as cancer, making the study of this transcription factor of particular interest to me. I therefore chose to study the regulation of NF- κ B in anergic CD8⁺ T lymphocytes. The NF- κ B family is comprised of five subunits that can form homodimers

or heterodimers. The major form of NF- κ B present in activated T lymphocytes is p50-p65 heterodimers [187]. In resting T lymphocytes, NF- κ B is sequestered in the cytoplasm by I κ B molecules. Once the T lymphocytes are activated, I κ B α is phosphorylated and degraded. Degradation of I κ B α releases the NF- κ B dimers allowing for phosphorylation of p65 at Ser⁵³⁶ in the cytoplasm and translocation into the nucleus. In the nucleus, p65 is regulated by phosphorylations at Ser²⁷⁶ and Ser³¹¹, which have been suggested to allow the recruitment of HATs and the acetylation of p65 at multiple lysine residues, including Lys³¹⁰. Together these events allow for full activation of p65-containing NF- κ B complexes.

The primary goal of my thesis was to gain a better understanding of the regulation of one of the pathways implicated in IL-2 production and cell proliferation. In this work, I analyzed the NF- κ B signaling pathway in anergic CD8⁺ T lymphocytes. To address whether the NF- κ B pathway is impacted in anergic CD8⁺ T lymphocytes, I analyzed the activation of NF- κ B using NF- κ B-luc transgenic mice. I showed that NF- κ B activation was decreased in anergic CD8⁺ T lymphocytes, thus demonstrating a defect in the NF- κ B pathway. The defects associated with anergic CD8⁺ T lymphocytes observed by the luciferase assay led us to hypothesize that an alteration in the NF- κ B pathway is contributing to the inhibition of IL-2 production.

Given that I κ B regulation is a central event in NF- κ B activation, we decided to start analysis of the signaling pathway at this point. We hypothesized that the lack of I κ B synthesis might be causing the defect in NF- κ B activity. I analyzed I κ B expression and found that I κ B α was degraded following T lymphocyte stimulation in both naïve and anergic lymphocytes. Interestingly, in contrast to naïve T lymphocytes, I found that I κ B α

was not resynthesized in anergic CD8⁺ T lymphocytes. Since IκBα is a transcriptional target of NF-κB, I hypothesized that NF-κB nuclear translocation, DNA binding, or chromatin remodeling is affected in anergic T lymphocytes. I also analyzed IκBβ expression and found that this protein was degraded at least as rapidly in anergic as in naïve CD8⁺ T lymphocytes. My results therefore indicate that the defect in NF-κB activation in anergic CD8⁺ T lymphocytes is not due to impaired IκB degradation.

The degradation of IκB showed that all signaling events upstream of IKK activation were likely intact. The next major step after IκB degradation is the translocation of NF-κB into the nucleus. Even though I did not observe defects in IκB degradation in anergic T lymphocytes, I hypothesized that p65 nuclear translocation may be impacted in anergic T lymphocytes. Although IκBα and IκBβ degradation was normal in anergic T lymphocytes, other IκB isoforms such as IκBε [71] could be retaining NF-κB in the cytoplasm in anergic T lymphocytes. Also, a defect in the transport of NF-κB into the nucleus by importin α molecules [218] could be causing the retention of this transcription factor in the cytoplasm in anergic T lymphocytes. I therefore studied NF-κB p65 subunit translocation into the nucleus. My results showed that p65 translocated into the nucleus with similar kinetics in both naïve and anergic T lymphocytes; moreover, the use of two different techniques confirmed the results obtained. These results indicate that two key events in the regulation of NF-κB, IκB degradation and NF-κB nuclear translocation are normal in anergic T lymphocytes.

NF-κB subunits are extensively modified by phosphorylation, acetylation, ubiquitination, etc. These modifications can positively or negatively regulate NF-κB

activation, and most have been shown not to affect nuclear localization. Since I κ B degradation and NF- κ B p65 subunit nuclear translocation are not defective in anergic T lymphocytes I hypothesized that posttranslational modifications may be responsible for the NF- κ B transcriptional activation defect observed in anergic CD8⁺ T lymphocytes. To test this hypothesis I initially analyzed two well-characterized sites in p65 that have been shown to be phosphorylated very quickly after I κ B degradation: Ser⁵³⁶ and Ser²⁷⁶. I showed that p65 was rapidly phosphorylated at Ser⁵³⁶ in the cytoplasm and at Ser²⁷⁶ in the nucleus with similar kinetics in both naïve and anergic T lymphocytes following activation. These data show that phosphorylation at these two residues is not sufficient to increase NF- κ B activity; since we found that these residues are phosphorylated similarly in naïve and anergic T lymphocytes.

Since I did not find a defect in the phosphorylation of p65 at Ser⁵³⁶ and Ser²⁷⁶, I analyzed another phosphorylation event that is associated with increased NF- κ B transcriptional activity: phosphorylation of p65 at Ser³¹¹. Phosphorylation of Ser³¹¹ is required for recruitment of the HATs CBP/p300 and RNA polymerase to NF- κ B target promoters [92], and thus is critical for NF- κ B transactivation function. I found that p65 was phosphorylated at Ser³¹¹ in naïve cells, with kinetics that correlated with p65 nuclear translocation, but that p65 phosphorylation at Ser³¹¹ was inhibited in anergic CD8⁺ T lymphocytes. These data correlate with a defect in NF- κ B activation in anergic T lymphocytes and suggest that phosphorylation at this residue is might be required for activation of T lymphocytes in our system. This result is important because it provides a potential molecular mechanism for NF- κ B activation defect in anergic CD8⁺ T lymphocytes.

Although HAT recruitment is generally described in terms of histone acetylation and chromatin remodeling, CBP/p300 have been also shown to acetylate transcription factors such as NF- κ B p65 subunit at multiple sites. One site that was of particular interest was Lys³¹⁰. Acetylation at this residue has been related to increased p65 transcriptional activity, and its proximity to Ser³¹¹ (and the availability of antibodies to Lys310-acetylated p65) made it an attractive candidate for study. The finding that p65 is not phosphorylated at Ser³¹¹ in anergic T lymphocytes led us to hypothesize that this could result in the absence of CBP/p300 recruitment and subsequent Lys³¹⁰ acetylation of p65. To test this hypothesis, I analyzed acetylation of p65 at Lys³¹⁰. I found that p65 was acetylated in naïve T lymphocytes with delayed kinetics relative to phosphorylation at Ser³¹¹. However, p65 was not acetylated at this residue in anergic T lymphocytes. This result also correlates with the defect in NF- κ B activation and suggests that acetylation at Lys³¹⁰ might be required for proper NF- κ B activation in our system. This result is also important because for the first time we show that acetylation of the NF- κ B p65 subunit is defective in anergic T lymphocytes.

Putting together the results of all my studies on the NF- κ B pathway, I now propose a model for NF- κ B activation in anergic CD8⁺ T lymphocytes (Fig. 14). After TCR activation, I κ B α is phosphorylated by the IKK complex, leading to the ubiquitination and degradation of I κ B α . This allows the phosphorylation of p65 at Ser⁵³⁶ in the cytoplasm and the translocation of p65 to the nucleus in both naïve and anergic CD8⁺ T lymphocytes. Once in the nucleus, p65 is phosphorylated at Ser²⁷⁶, again both in naïve and anergic cells. In the nucleus of naïve cells, p65 is also phosphorylated at Ser³¹¹

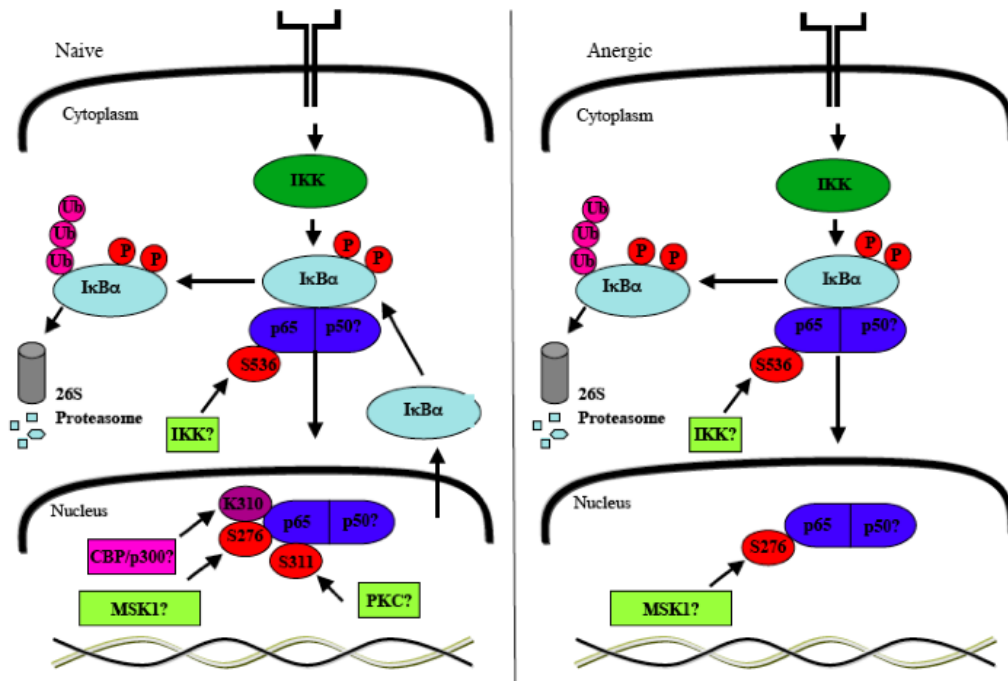


Figure 14. Schematic model of NF- κ B signaling pathway in naïve and anergic CD8⁺ T lymphocytes. In naïve cells (left panel), stimulation of the TCR causes the IKK mediated phosphorylation of I κ B α which is subsequently ubiquitinated and degraded. This allows the phosphorylation of p65 at Ser⁵³⁶ and translocation into the nucleus. Once in the nucleus, p65 is phosphorylated at Ser²⁷⁶ and Ser³¹¹ and acetylated at Lys³¹⁰. In anergic cells (right panel), degradation of I κ B, phosphorylation at Ser⁵³⁶ and Ser²⁷⁶, and nuclear translocation occur normally. By contrast, p65 is not phosphorylated at Ser³¹¹ nor acetylated at Lys³¹⁰ in anergic T cells.

and acetylated at Lys³¹⁰, whereas in anergic cells p65 is not phosphorylated or acetylated at these residues. The defects in Ser³¹¹ phosphorylation and Lys³¹⁰ acetylation, perhaps in combination with other alterations, are thus hypothesized to result in impaired NF- κ B transactivation function.

A logical first step in trying to understand how the altered post-translational modifications affect NF- κ B function was to analyze the DNA binding activity of the NF- κ B subunit p65. Preliminary data from a transcription factor ELISA indicated that p65 DNA binding may be defective in anergic CD8⁺ T lymphocytes but this result remains to be confirmed. The defects in NF- κ B phosphorylation and acetylation that I discovered here have not previously been found to cause a DNA binding defect. However, as mentioned earlier, there are other posttranslational modifications as well as other proteins that associate with NF- κ B subunits and regulate their function. Combined with the defects presented in this work, alterations in these other regulatory factors might alter DNA binding properties, and this is a potential direction to future studies.

5.2 Future Directions

To continue with this project we would like to analyze whether the defects we identified in this work actually affect NF- κ B function in our system. As mentioned earlier all these posttranslational modifications have been studied in other systems such as embryonic fibroblasts. We would like to analyze whether mutant p65^{S311A} or p65^{K310R} will lead to a defect in NF- κ B function in T cells similar to what we present in this work. For that, we would generate mutants and analyze whether these mutants will cause a defect in NF- κ B transcriptional activity in T lymphocytes. To analyze these mutants, first

we have to block the expression of endogenous p65 in T lymphocytes. For that, we would transfect specific p65 siRNA into hematopoietic stem cells. Once we have blocked the expression of endogenous p65, we would analyze whether mutant p65^{S311A} or p65^{K310R} will lead to a defect in NF-κB function by adoptive transfer. In this case, hematopoietic stem cells from a donor mouse, previously infected with retroviruses containing the p65^{S311A} or p65^{K310R} mutations, would be transferred into an irradiated mouse. In this model, hematopoietic cells from the acceptor animal are inactivated, thus allowing the donor cells to repopulate the organism. This process would allow for the generation of T cells with these mutations, and the effects on expression of NF-κB target genes could be tested after TCR stimulation.

Although data from others suggest that PKCζ is not the kinase phosphorylating p65 at Ser³¹¹ in T lymphocytes, we still have to test it in our system. For that, performing an *in vivo* kinase assay would help to confirm or disprove that PKCζ is the kinase involved in phosphorylating this residue. For that, T lymphocytes would be co-transfected with HA-p65 or HA-p65^{S311A} and PKCζ, and then labeled with [³²P] orthophosphate. In this way, samples can be analyzed by autoradiography to determine whether PKCζ is phosphorylating p65 at Ser³¹¹. If a kinase is able to phosphorylate HA-p65, but not HA-p65^{S311A}, that would be evidence that it is the endogenous Ser³¹¹ kinase. We can also perform western blot to confirm that PKCζ is the kinase phosphorylating Ser³¹¹. For that, we can immunoprecipitate our samples with anti-HA and then analyze by western blot with antibodies against phosphorylated p65. We can also obtain PKCζ^{-/-} T lymphocytes and analyze if p65 is phosphorylated at Ser³¹¹ after TCR stimulation. If p65 is not phosphorylated at this residue, that would indicate that PKCζ is necessary for this

phosphorylation. However if p65 is phosphorylated at Ser³¹¹ in PKC ζ ^{-/-} cells, that would indicate that PKC ζ is not the kinase involved in this event.

I earlier suggested that the kinase phosphorylating p65 at Ser³¹¹ in our system could be PKC θ or MSK1. To test whether either of these kinases is involved in this phosphorylation event, *in vivo* kinase assays would be performed, as described for PKC ζ . We can also obtain PKC θ ^{-/-} or MSK1^{-/-} T lymphocytes and analyze if p65 is phosphorylated at Ser³¹¹. If known kinases are not involved in the phosphorylation of p65 at this residue, we could use immunoprecipitation to find kinases that bind to p65, and then determine their identities by mass spectrometry or protein sequencing. Once we identify the kinase, we would analyze its functionality in our anergic T lymphocytes. For that, we could generate a constitutively active kinase (e.g. PKC ζ , PKC θ , or MSK1) and overexpress it in or cells to see if it restores p65 Ser³¹¹ phosphorylation and NF- κ B function in anergic T cells.

This work did not address other posttranslational modifications affecting p65. As is shown in figure 4, several phosphorylation and acetylation events negatively or positively regulate p65. In this work, I only analyzed the most well studied modifications. Analysis of additional posttranslational modifications will provide us with a better understanding of the regulation of p65. Phosphorylation of p65 at Ser²⁰⁵, Ser²⁸¹ and Ser⁵²⁹ are known to increase transcriptional activity of p65 [80, 117], while phosphorylation at Thr⁴³⁵, Ser⁴⁶⁸ and Ser⁵⁰⁵ decrease transcriptional activity of p65 [111, 113, 115]. Likewise, acetylation of p65 at Lys¹²² and Lys¹²³ results in decreased DNA binding and transcriptional activity [109], while acetylation of p65 at Lys²¹⁸ and Lys²²¹ increases DNA binding and p65 transcriptional activity [98, 109]. To test these posttranslational

modifications affecting NF- κ B p65, I would follow a similar approach as I did with the phosphorylations and acetylation presented in this work.

Following translocation into the nucleus, p65 binds to the IL-2 and I κ B α promoters, among others. To investigate whether endogenous NF- κ B associates with the IL-2 and I κ B α promoters in anergic CD8⁺ T cells in vivo, a ChIP assay should be performed. EMSA and transcription factor ELISA analyses have shown that NF- κ B dimers bind to the CD28RE site [189]. These dimers are composed of p50, p65, and c-Rel subunits, which form p50-p65 or p50-c-Rel heterodimers [189]. In naïve CD4⁺ T lymphocytes p50-p65 heterodimers bind DNA, while in anergic CD4⁺ T lymphocytes the inhibitory p50-p50 homodimers are bound to DNA [188]. Thus, it would be interesting to analyze whether p50-p50 homodimers are binding to the IL-2 and I κ B α promoters in our system. To analyze the NF- κ B subunits binding to the promoters of interest in vivo we can use ChIP. Here, we would perform individual ChIP for the different NF- κ B subunits. First we would analyze p65 binding to the IL-2 and I κ B α promoters. If we find p65 binding we might expect p65 homo- or heterodimers. If we do not find p65 binding, but p50 binding, we might expect p50 homo- or heterodimers. Performing individual ChIP could help us to find out the actual composition of NF- κ B in naive and anergic T lymphocytes. Then, we can perform sequential ChIP. This technique allows one to analyze various proteins binding to a region of the genome. For example, we could immunoprecipitate our samples first against p65 and then against p50, or vice versa, and analyze using the same sets of PCR primers. It would also be interesting to study what is the posttranslational modification status of p65 when bound to the proposed promoters. The presence of p65 acetylation and histone acetylation at the NF- κ B binding site at the

IL-2 and I κ B α promoters could be determined by ChIP assay. To analyze this, the ChIP assay has to be performed with antibodies specific for the different post-translational modifications required, such as Ser³¹¹ and Lys³¹⁰.

Chromatin remodeling by acetylation and deacetylation plays an important role in the initiation of gene transcription. Histone acetylation is a positive regulator of transcription, while histone deacetylation is a negative regulator of gene transcription. CBP/p300 is a complex of histone acetyltransferases (HATs) involved in the acetylation of histones, while HDAC1 and HDAC-3 are histone deacetylases (HDACs) that are involved in deacetylation of histones. Recent findings show that CBP/p300 in addition to acetylating histones, acetylates the NF- κ B subunit p65 [98]. Both HATs and HDACs are involved in acetylation/deacetylation of NF- κ B. It has been suggested that PKA and MSK-1 mediated phosphorylation of p65 causes a conformational change in p65 that is responsible for its interaction with CBP/p300 [84, 86]. Previous studies have shown that p50 homodimers are associated with the negative regulator HDAC-1 that represses NF- κ B dependent gene transcription, while p65/p50 heterodimers containing phosphorylated p65 associate with CBP/p300. The p-p65/CBP/p300 complex displaces the repressive p50/p50/HDAC1 complex bound to the κ B enhancers allowing for activation [90]. It has been also shown that acetylated p65 can be deacetylated by HDAC-3. This deacetylation allows for new synthesized I κ B α molecules to bind to NF- κ B, remove it from the DNA and export it back into the cytoplasm [98]. Association of NF- κ B with CBP/p300 and HDAC-1/3 can be identified by co-immunoprecipitation analysis.

To date no data on the epigenetic regulation by NF- κ B in anergic CD8⁺ T lymphocytes have been published. Some data are available regarding epigenetic

regulation by NF- κ B in EL-4 thymoma cells and in CD4⁺ T cells, which showed that anergic CD4⁺ T cells exhibited little or no chromatin remodeling after 96 hours of stimulation [138]. Data from the same group suggested that methylation might be affecting IL-2 CpG content in the DNA, which would affect transcription from the IL-2 promoter. It would be interesting to analyze patterns of chromatin remodeling and DNA methylation in our system. To analyze chromatin remodeling, a DNase sensitivity assay or a nuclease accessibility analysis can be performed. In this case nuclear extracts from naïve and anergic T lymphocytes would be treated with DNase or other nucleases. DNase is known to cut only relaxed DNA. In this way we can test whether chromatin in the IL-2 or I κ B α promoters are being remodeled, and the kinetics of the remodeling. To study methylation at the IL-2 and I κ B α promoters, DNA would be cleaved with methylation sensitive restriction enzymes such as BamHI or HindIII, followed by PCR using primers for the IL-2 and I κ B α promoters. Methylated regions prevent digestion by the enzyme, allowing for amplification, whereas in unmethylated regions the DNA is cleaved, blocking amplification. With this method we can analyze which promoters are methylated in our system.

A major event in the NF- κ B signaling pathway is the translocation of NF- κ B subunits into the nucleus where they can impact gene transcription. Here we showed that p65 translocates into the nucleus in both naïve and anergic T lymphocytes. A question to be addressed in a future work is whether other NF- κ B subunits translocate into the nucleus and if so, whether this occurs with the same kinetics as p65 or in CD8⁺ anergic T lymphocytes relative to naïve T lymphocytes. To test this, I would use the same approach that I used to study p65. Naïve and anergic T cells would be fractionated into cytoplasmic

and nuclear fractions and then analyzed by western blot using antibodies specific for the different NF- κ B subunits. We can also analyze nuclear translocation by immunofluorescence using antibodies specific for the different NF- κ B subunits. T lymphocytes. By analyzing nuclear translocation of other NF- κ B subunits we might find other defects associated to anergic T lymphocytes. We may find that c-Rel is not moving into the nucleus in anergic T lymphocytes or that p50 is moving into the nucleus in anergic T lymphocytes preferentially, comparing with naïve T lymphocytes. Such data could therefore reveal another component of the NF- κ B defect.

5.3 Application

As a result of this project we have identified defects in NF- κ B phosphorylation and acetylation as a potentially important component of non-responsiveness in CD8⁺ T lymphocytes. Since this is a model system in mice, we would like to explore places where the problem is important. Here are some places where improper regulation of T lymphocyte tolerance can cause problems: infectious diseases, cancer and tumor immunology, and transplantation. CD8⁺ T lymphocytes are important in the immune response against cancer cells. It has been proposed that T lymphocytes that are specific for certain tumors become unresponsive mainly because of the production of transforming growth factor- β (TGF- β) by the tumor cells [219]. Testing whether the non-responsive T lymphocytes have a similar defect in NF- κ B would be important to restore their function to generate T lymphocyte associated therapies. If phosphorylation of p65 at Ser³¹¹ were defective in anergic tumor-specific T cells, one reason for this defect could be

improper regulation of the yet-to-be-defined kinase phosphorylating this residue in our anergic cells. If that is the case, the generation of a constitutively active form of the kinase might help to restore activation of NF- κ B and revert the anergic state. In real life, this can be done using gene therapy. Solid tumors contain tumor infiltrating T lymphocytes that are normally unresponsive. We could collect the infiltrating T lymphocytes and infect them *in vitro* with genetically engineered retroviruses containing a copy of the constitutively active kinase gene. Retroviruses integrate their genetic material into the patients chromosomal DNA, causing in this way the generation of a functional protein. Once it is confirmed that the kinase gene is incorporated into the chromosomal DNA of the T lymphocytes, we could inject the T lymphocytes back into the tumor and see if they mount an immune reaction against the tumor cells. Another possibility could be the presence of a phosphatase that might be dephosphorylating p65 Ser³¹¹ and thus generating a defect in NF- κ B activation. One way to revert this condition would be the use of inhibitors specific for that phosphatase.

It has been observed that 15% of patients affected by *Mycobacterium tuberculosis* present anergic T lymphocytes in peripheral blood. These T lymphocytes are unresponsive to a protein derived from tuberculin, compared to normal T lymphocytes that are responsive and proliferate normally [191]. It would be important to analyze which factors are causing unresponsiveness in T lymphocytes associated with *M. tuberculosis*. If the NF- κ B pathway would be affected in a similar way as shown in this work, it would be essential to restore T lymphocyte function and reverse this condition. As discussed above, if that is the case, the generation of a constitutively active form of

the p65 Ser³¹¹ kinase, or the use of specific phosphatase inhibitors, might help to restore activation of NF- κ B and revert the anergic state.

Rejection of transplants and graft vs. host disease (GVHD) are caused by T cell activation, and preventing them now requires systemic general immunosuppression. A much-desired approach would be to induce tolerance. Transplantation possesses several limitations such as the absence of MHC-matched donors and GVHD. Actual therapies include the inhibition of costimulatory molecules such as CD28 or B7.1/B7.2. In autoimmunity or transplantation, it would be critical to induce T lymphocyte anergy or tolerance to reduce GVHD. To treat graft vs. host disease, where anergy has to be induced in T lymphocytes, an inverse approach from the presented above has to be used. In this case, inhibitors that act on MSK1 might be used to generate a defect in the downstream signaling pathway and therefore inhibit activation of NF- κ B and induce anergy. In this way, drugs that inhibit the activation of MSK1 can be used to decrease p65 phosphorylation and NF- κ B activation. Since MSK1 is not the only kinase phosphorylating p65, it would be important to study other kinases phosphorylating this NF- κ B subunit to generate a drug cocktail that might inhibit a variety of kinases. Alternatively, a phosphatase that dephosphorylates MSK1 or p65 itself might be transfected in T lymphocytes to generate anergy.

Data presented here are only the beginning of a series of studies to understand the signaling events involved in anergy and tolerance induction in T lymphocytes. In the future, we would like to continue with our work. Our results could be applied to the development of new treatments for conditions characterized by the presence of unresponsive T lymphocytes or in conditions where induction of anergy is required.

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