ROLE OF I KAPPA B KINASE ALPHA AND I KAPPA B KINASE BETA IN THE DEVELOPMENT AND FUNCTION OF B AND T LYMPHOCYTES

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For My Family

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by

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Transcription factor NF-κB plays a key role in regulating the expression of genes involved in the control of the inflammatory and immune response. NF-κB binds to a group of inhibitory proteins, IκBs, in the cytoplasm of non-stimulated cells. Activation of NF-κB is regulated by the IκB kinase complex that phosphorylates IκB proteins, leading to their poly-ubiquitination and degradation. The released NF-κB molecules translocate into the nucleus and activate gene transcription. IκB kinase complex contains two catalytic subunits, IκB kinase α (IKKα) and IκB kinase β (IKKβ), and a regulatory subunit, IκB kinase γ (IKKγ) or NEMO. To evaluate the functions of IKKα and IKKβ in the development and function of the immune system, transgenic mice expressing dominant negative forms of IKKα and IKKβ specifically in their B cells or T cells were generated.

Phenotypic analysis of transgenic mice expressing dominant negative IKKβ in the B cells revealed that the proliferation of B cells from these mutant mice in response to B cell mitogens was reduced due to impaired cell cycle progression. Accordingly, *in vitro* secretion of immunoglobulins by the mutant B cells in response to these mitogens was also decreased. In addition, these mice displayed selective defects in the production of specific immunoglobulin subclasses in response to type 2 but not type 1 T cell independent antigens. Moreover, the levels of certain immunoglobulin subclasses were reduced in mutant mice challenged with a T cell dependent antigen. These results indicate that IKKβ is critical for the proliferation of B cells and the control of some aspects of the humoral response.

Transgenic mice expressing one or both of the dominant negative IKK specifically in T cells exhibited distinct phenotypes in thymocyte proliferation, cytokine production, and cell survival. Proliferation of thymic T cells from IKKβ mutant mice and IKKα/β mutant mice was markedly reduced due to impaired cell cycle progression. In addition, inhibition of both IKKα and IKKβ appeared to suppress the expression of multiple cytokines by thymocytes. Furthermore, apoptosis of the double positive thymocytes induced by the administration of anti-CD3 antibody was significantly reduced in transgenic mice expressing dominant negative IKKβ, but increased in mice expressing only dominant negative IKKα. These results indicate that IKKα and IKKβ play different roles in regulating the activation and survival of T cells.

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1. Ren, H., Schmalsteig, A., Yuan, D., and Gaynor RB. (2001) IKKβ is critical for B cell proliferation and antibody response. (Submitted)
2. Ren, H., Schmalsteig, A., van Oers, N. S. C., and Gaynor RB. (2001) IKKα and IKKβ have distinct roles in regulating *in vivo* T cell function. (Submitted)

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7-AAD	7-aminoactinomycin D
aly	alymphoplasia
APC	antigen presenting cell
BCR	B cell antigen receptor
BrdU	5-bromo-2-deoxyuridine
Btk	Bruton's tyrosine kinase
C	constant region
CD40L	CD40 ligand
CHUK	conserved helix-loop-helix ubiquitous kinase
COX	cyclooxygenase 2
CPS	capsular polysaccharide
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T lymphocyte antigen-4
D	aspartic acid
DAG	diacylglycerol
DmIKKβ	Drosophila IKKβ
DN	dominant negative
DNP-HES	dinitrophenyl-hydroxyethyl starch
$E3RS^{I\kappa B}$	pIκBα-E3 receptor subunit
EMSA	electrophoretic mobility shift assay

EST	expression sequence tag
FLU-BA	fluorescein-Brucella abortus
G	glycine
H	heavy chain
HA	hemagglutinin
HIV	human immunodeficiency virus
HLH	helix-loop-helix motif
HTLV-1	human T cell leukemia virus type 1
ICAM	intracellular adhesion molecule
IFN	interferon
Ig	immunogobulin
IKAP	IKK complex associated protein
IκB	inhibitor of nuclear factor κ B
IKK	IκB kinase
IL	interleukin
iNOS	inducible form of nitric oxide synthase
IP	incontinentia pigmenti
IP3	inositol-1, 4, 5-triphosphate
IRAK	IL-1 receptor associated kinase
ITAM	immunoreceptor tyrosine-based activation motif
kDIF	keratinocyte differentiation-inducing factor
KIR	killer inhibitory receptor

L	light chain
LPS	lipopolysaccharide
LZ	leucine zipper motif
MALDI	mass accuracy matrix-assisted laser deposition and ionization
MAPK	mitogen activated protein kinase
MAP3K	MAP kinase kinase kinase
MHC	major histocompatibility complex
MIP-1	macrophage inflammatory protein-1
NEMO	NF-κB essential modulator
NES	nuclear export sequence
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor κ B
NIK	NF-κB inducing kinase
NLS	nuclear localization signal
PEST	proline (P), glutamic acid (E), serine (S), threonine (T) rich sequence
PDGF	platelet derived growth factor
PH	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinasae
pIκBα	phosphorylated IκBα
PIP	phosphatidylinositol –3-phosphate
PIP2	phosphatidylinositol-3, 4-biphosphate
PIP3	phosphatidylinositol-3, 4, 5-triphosphate

PKC	protein kinase C
PLCγ	phospholipase Cγ
PMA	phorbol-12-myristate-13-acetate
PP2A	protein phosphatase 2A
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTK	protein tyrosine kinase
PTPase	phospho-tyrosine phosphatase
RA	rheumatoid arthritis
Rag	recombinase activating gene
RHD	Rel homology domain
RPA	ribonuclease protection assay
S	serine
SH2	Src homology domain 2
SHIP	SH2 domain-containing inositol phosphatase
slimb	supernumerary limbs
TCR	T cell antigen receptor
TD	T cell dependent (antigen/response)
TDT	terminal deoxynucleotidyl transferase
TG	transgenic
Th	helper T cells
TI	T cell independent (antigen/response)
TIR	Toll/IL-1 receptor domain

TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TNP	trinitrophenyl
TRAF	TNF receptor associated factor
V	variable region
VCAM	vascular cell adhesion molecule
WT	wild-type
xid	X-linked immunodeficiency
ZAP-70	ζ associated protein-70

CHAPTER I. INTRODUCTION

A. General introduction of NF-κB and its activation

NF-κB and its cellular functions

The nuclear factor κB (NF-κB) was first identified in B cells as a molecule that bound to the intronic enhancer of the immunoglobulin κ gene and activated κ gene transcription (1). This eukaryotic transcription factor was later found in virtually all cell types. It binds to a conserved 10 base pair cis element: 5'-GGGGYNNCCY-3', the κB site, which is present in the promoters and enhancers of numerous genes (2). These include genes of cytokines, acute phase response proteins and cell adhesion molecules (3-5) that are involved in the immune and inflammatory response. NF-κB can also activate transcription of oncogenes such as c-myc (6-8), and human immunodeficiency virus (HIV) (9), indicating that it has an important role on the regulation of cell growth and survival.

In most cells, NF-κB exists in the cytoplasm in an inactive form bound to the inhibitor, IκB (10). Cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) lead to the release of NF-κB from IκB and the translocation of NF-κB to the nucleus where it increases transcription of target genes. In addition to proinflammotory cytokines, activation of NF-κB can be induced by a vast number of agents including bacterial and viral products such as lipopolysaccharide (LPS) and the Tax protein of the human T cell leukemia virus (HTLV-1), calcium ionophores, phorbol esters, ultra-violet radiation, etc. (5, 10, 11).

As an inducible transcription factor, NF-κB communicates with both the cytoplasm and the nucleus and carries messages directly to its nuclear targets. Therefore, it is a key element coordinating the cellular responses to infection, stress and injury. Activation of the NF-κB transcription factors does not require *de novo* protein synthesis. This permits the rapid and efficient activation of target genes. As such, NF-κB is utilized in immune, inflammatory, and acute phase responses where rapid activation of host defense genes following exposure to pathogens is critical for the survival of an organism. Many viruses have NF-κB binding sites and proteins that activate the NF-κB pathway to enhance viral replication. NF-κB is also involved in the regulation of cellular proliferation and apoptosis. Accordingly, deregulation of NF-κB activity is directly associated with cellular transformation. The NF-κB homologs in *Drosophila*, Dorsal and Dif, are involved in the formation of embryonic polarity and insect immunity, indicating an evolutionary role of NF-κB in development and immune system (12).

The regulation of the NF-κB pathway has been elucidated by the discovery and characterization of kinases responsible for the signal induced phosphorylation of IκB proteins. The diversity of the NF-κB/Rel and the IκB proteins is important in understanding the regulation of the NF-κB pathway.

NF-κB/Rel protein family and IκB protein

NF-κB/Rel proteins are members of an evolutionary conserved family of proteins (3, 4, 10, 11), some of which regulate body pattern formation and immune function in insects

(13). As shown in Figure 1, there are five members of NF-κB/Rel protein in mammalian cells: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), Rel B, and c-Rel (14). Rel proteins identified in insects include relish, dorsal and dif (15-18). Each member in this family is characterized by the Rel homology domain (RHD) of approximately 300 amino acids that is located in the N-terminus of the protein. The RHD domain mediates DNA binding, dimerization, and interaction with IκB proteins. NF-κB1 and NF-κB2 are synthesized as precursor p105 and p100 protein that contain multiple copies of the ankyrin repeat at the C-terminal region. Processing of these proteins leads to production of the p50 and p52 subunits, respectively (19). The p65, c-rel and RelB also contain a C-terminal transactivation domain that increases transcription of viral and cellular genes.

The RHD domain mediates protein-protein interaction in two critical ways: First, it mediates the dimerization of Rel proteins. NF-κB dimers such as p50/p65, p50/c-rel, p65/p65 and p65/c-rel are transcriptional active due to the DNA binding capability of RHD domain and the transactivation property of p65 and c-rel subunits. Second, the RHD domain interacts with the ankyrin repeats present in p105, p100, and the *Drosophila* protein relish. This interaction masks the nuclear localization signal (NLS) present in the carboxy portion of the RHD (14, 19, 20), retaining the NF-κB/Rel proteins in the cytoplasm. More importantly, RHD domain interacts with the ankyrin repeat motifs in the IκB proteins, which are the inhibitors of NF-κB. Binding of IκB proteins to the NF-κB dimers keeps the NF-κB proteins sequestered in the cytoplasm and thus prevents them from entering the nucleus in resting cells by masking the NLS of the Rel proteins (10, 22).

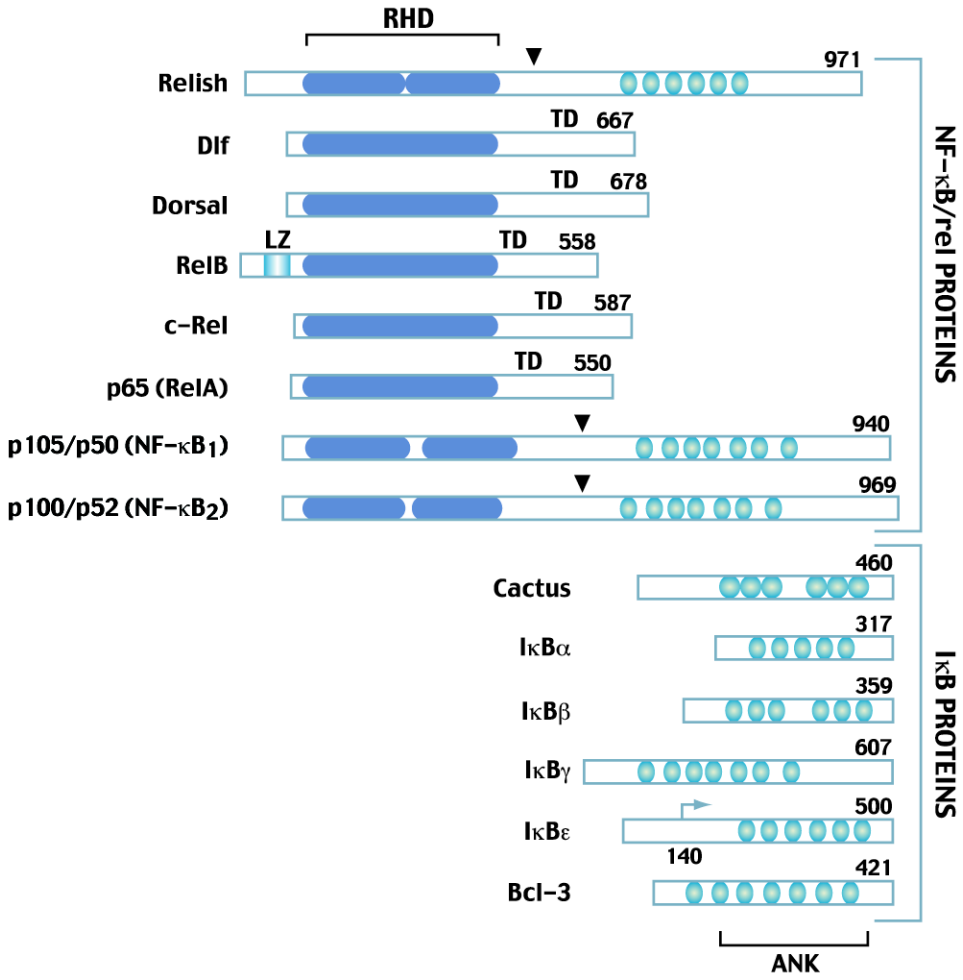


Figure 1. Schematic of members of the NF-κB/Rel and IκB protein family. The number of amino acids in each protein is shown on the right. The arrows point to the endoproteolytic cleavage sites of p105/p50 and p100/p52. RHD, rel homology domain; TD, transactivation domain; LZ, leucine zipper domain of RelB; IκBε has been proposed to be translated from either the first methoinine or from an internal methionine at position 140 (21).

The IκB proteins (Fig. 1) inhibit both NF-κB nuclear localization and DNA binding, and thus are key regulators of NF-κB activation. Members of IκB protein family include IκBα, IκBβ, IκBε, IκBγ and Bcl-3 in mammalian cells and cactus in *Drosophila* (14). The specific interaction of the ankyrin repeat motifs in these proteins with the RHD domain in the Rel proteins appear to be an evolutionarily conserved feature involved in the regulation of NF-κB pathway. This interaction not only prevents the Rel proteins from entering the nucleus by masking their NLS, but also blocks the DNA binding of these proteins. Recently, the three dimensional structures of IκBα in complex with the RHDs of the major NF-κB components, p50 and p65 was published. The N-terminus of the IκB core, containing 6-7 ankyrin repeats, was positioned next to the NLS and prevented its binding to the carrier proteins (karyopherins) which transport NF-κB to the nucleus (23, 24). Although the ankyrin repeats of IκB are necessary for NF-κB association, they are not sufficient for the inhibition of NF-κB DNA-binding, which also requires the carboxy-terminal acidic region of IκB (25-27). The region of IκB also contains a Pro, Glu/Asp, Ser, and Thr-rich PEST sequence (28, 29) that regulates the turnover of IκB proteins. The N-terminal region of IκB proteins contains two serine residues that are specifically phosphorylated upon cell stimulation, which leads to the ubiquitination and degradation of IκB by the 26S proteosome (14).

The activity of the multiple NF-κB dimers is likely regulated independently or coordinately by the different IκB proteins. IκBα is a 37-κDa protein that binds to NF-κB dimers containing p65 and c-rel subunits (28, 30). IκBα regulates the rapid but transient induction of NF-κB activity due to an autoregulatory feedback loop. The presence of κB

binding sites in the IκBα promoter results in up-regulation of transcription of IκBα that is induced by NF-κB nuclear translocation (31-34). IκBβ binds to the same rel subunits as does IκBα. The binding of IκBα or IκBβ may depend on the type of cellular stimulatory signal and the timing of the onset and duration of the response (35). In contrast to IκBα, the expression of IκBβ does not seem to be regulated by NF-κB activation. Therefore, the degradation of IκBβ results in persistent activation of NF-κB (35). It was noted recently that the presence of both NLS (36-38) and nuclear export sequence (NES) (39-41) in IκBα allows it to shuttle between the nucleus and the cytoplasm, and thus localize the Rel proteins by shifting the balance between their nuclear import and export (42). In contrast, the cytoplasmic retention of the Rel proteins by IκBβ, which lacks a NLS and NES, involves only the cytoplasmic sequestration but not shuttling (42). As the product of an alternatively spliced mRNA transcribed from the p105 gene, IκBγ is identical to the C-terminal region of p105. It inhibits p50 and p52 homodimers in mature B cell lines (43). The p105 and p100 are included in the IκB family because they both contain the C-terminal ankyrin repeat motif. They complex with p50, p65, and c-rel to cause their retention in the cytoplasm (44-47). IκBε associates exclusively with p65/p65 homodimer and p65/c-rel heterodimer (21). It is degraded slowly following cell stimulation and is involved in regulating the expression of specific genes such as IL-8 (48). Unlike other IκB proteins, Bcl-3 is located in the nucleus and binds specifically to p50 and p52 homodimers without masking their nuclear localization signal, leading to their transcriptional activation rather than repression (49-54).

The best-characterized NF-κB heterodimer is composed of p50 (NF-κB1) and p65 (RelA) that is present in most cell types and can be activated by multiple stimuli. The p50/p65 dimer is usually bound to IκBα, which is the major target of IκB kinase that regulates NF-κB activation.

Inducible Activation of NF-κB pathway

Stimuli that activate the NF-κB pathway cause NF-κB to translocate to the nucleus, where it binds to the κB sites found in the promoters of of a variety of target genes. This process (Fig. 2) occurs through an evolutionally conserved pathway, which involves the coordinated action of a number of proteins (55). The key event in this process is the inducible degradation of the IκB protein, which leads to dissociation of NF-κB from IκB and subsequent NF-κB nuclear translocation and activation of gene expression by NF-κB. It occurs in three steps:

First, extracellular signals lead to the phosphorylation of two serine residues in the N-terminal region of IκB. The IκB proteins contain an N-terminal regulatory domain that has two invariant serine residues within a DSGψXS (ψ-hydrophobic amino acid; X-any amino acid) motif (55). These serine residues are phosphorylated very rapidly when the cells are treated with proinflammatory stimuli such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and bacterial lipopolysaccharide (LPS) (56-59). For IκBα, these residues are serine

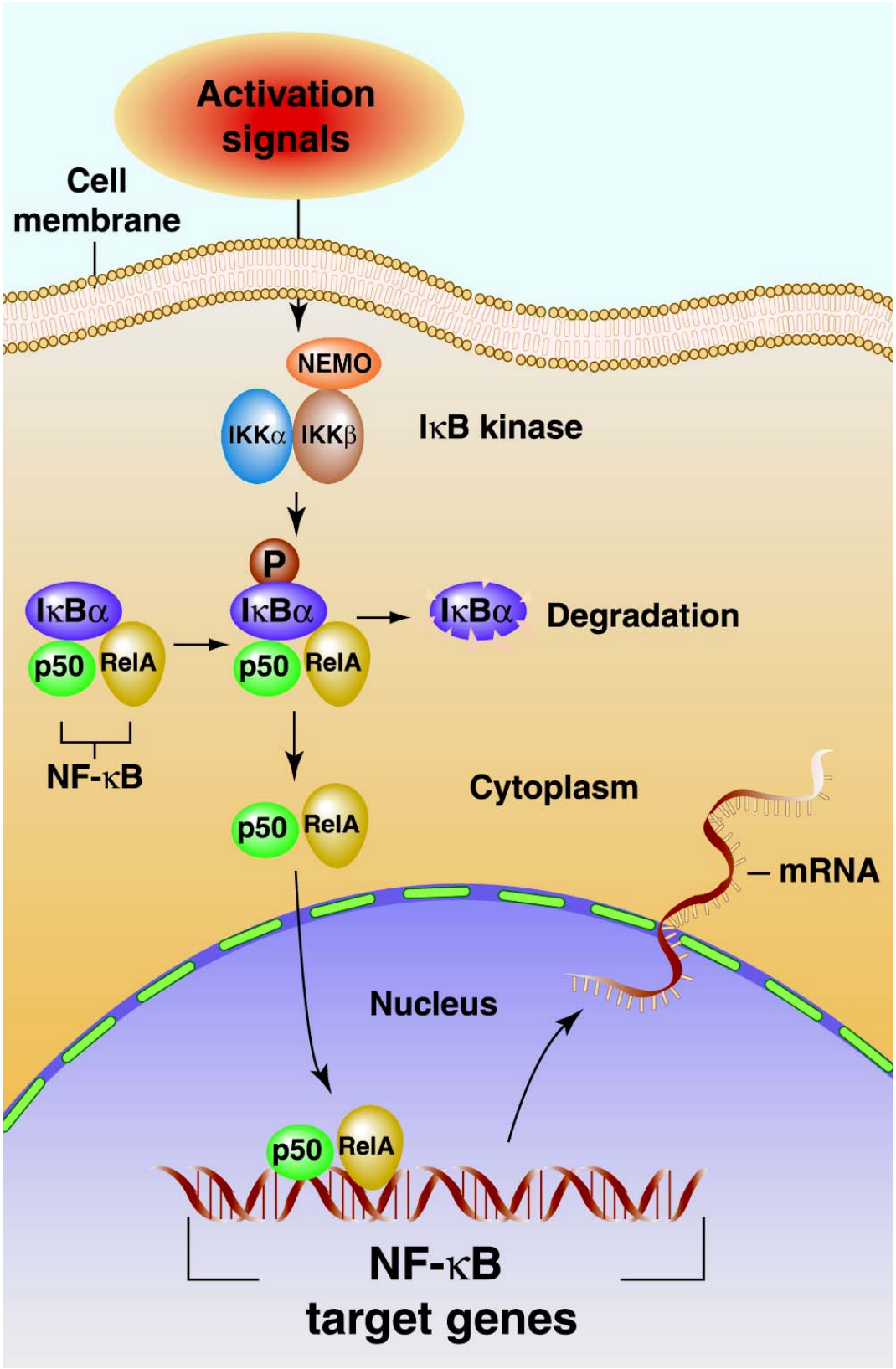

Activation signals
Cell membrane
NEMO
IKKα
IKKβ
IκB kinase
P
IκBα
p50
RelA
IκBα
p50
RelA
IκBα
Degradation
NF-κB
Cytoplasm
p50
RelA
mRNA
Nucleus
p50
RelA
NF-κB target genes

Figure 2. Schematic of NF-κB activation. Transcription factor NF-κB dimer (shown is p50/RelA heterodimer) binds to its inhibitor IκBα in the cytoplasm of resting cells. Extracellular activation signals stimulate the cell through cell surface receptors and induce the activity of IκB kinase (IKK), which is composed of two catalytic subunits IKKα and IKKβ and a regulatory subunit NEMO (IKKγ). Activated IKK complex in turn phosphorylates IκBα at serine residues 32 and 36, leading to its ubiquitination and degradation by the 26S proteosome. Once IκBα is degraded, p50/RelA dimer is allowed to translocate into the nucleus and activate NF-κB target gene transcription by binding to the promoter region of these genes such as IL-2, IL-2Rα gene, GM-CSF, and HIV provirus.

32 and serine 36. Phosphorylation of the N-terminus of IκB is carried out by an inducible IκB kinase complex that is the target of many signaling pathways.

Second, the phosphorylated IκB proteins undergo polyubiquitination in which multiple ubiquitin molecules are covalently attached to the IκB protein (60). Ubiquitination involves the sequential action of three components: a conserved ubiquitin-activating enzyme, E1, which links ubiquitin molecule to the enzyme through a high energy thioester bond; an ubiquitin-conjugating enzyme, E2, which interacts with E1 through a transacetylation reaction to transfer the ubiquitin chain to E2; and a protein-ubiquitin ligase, E3, which collaborates with E1 and E2 to attach ubiquitin molecules to the ε-amino group of lysine residues of the substrate, creating a reversible isopeptide bond. Transfer of additional ubiquitin molecules to the previously conjugated ubiquitin molecule results in the formation of a polyubiquitin chain, which serves as a recognition marker for the proteasome (61, 62). Although all three enzymes are required for polyubiquitination of a substrate, the E3 protein that brings the E2 and the substrate together to transfer ubiquitin confers substrate specificity (60).

Third, the polyubiquitinated IκB protein is recognized by the 26S proteosome and rapidly degraded (63-65).

A very important step in understanding the degradation of IκBα and other IκB proteins was the identification of an IκBα specific E3 that links ubiquitin with IκBα (66, 67). First, it was demonstrated that only phosphorylated IκBα (pIκBα) could be ubiquitinated in cell free extract. Using a series of phosphopeptides corresponding to the N-terminal regulatory motif in IκB proteins, it was elucidated that the recognition site for the IκBα

specific E3 is DS(PO_3)GXXS(PO_3) in which both serine residues are phosphorylated. This recognition site, also called the pIκBα degradation motif, is conserved in all IκB proteins, including the cactus protein in *Drosophila*. Mutations of any conserved residue in this site results in the stabilization of IκB and the inhibition of NF-κB activation (56, 58, 59, 68-70). Although a conserved lysine residue located 9-12 amino acids N-terminal to this recognition site is not required for the recognition of IκB by the E3 enzyme, it is the ubiquitin conjugation site for the IκB proteins. A large multi-protein complex that is associated with pIκBα:NF-κB in TNF-α stimulated Hela cells was isolated and shown to be sufficient to generate IκBα-ubiquitin conjugating activity *in vitro* following the addition of ubiquitin, purified E1 and UBC5C (a specific E2). The specific component of the E3 ligase that recognizes pIκBα was further purified from the complex using immunoaffinity purification with pIκBα and nanoelectrospray mass spectrometry. This E3 component was identified as the F-box/WD-domain protein β-TrCP, which was denoted $E3RS^{I\kappa B}$ (pIκBα-E3 receptor subunit). A homologous protein in *Drosophila*, known as the slimb (for supernumerary limbs) has been shown to be involved in the proteolysis of β-catenin, a protein involved in the Wnt/Wingless signaling (71). *Drosophila* embryos deficient in slimb were unable to activate the twist and snail genes that are regulated by Dorsal, the NF-κB homolog in *Drosophila* (72). This indicates that slimb is required for the ubiquitination and degradation of the cactus, which is required for the activation of Dorsal.

It should be noted that ultraviolet irradiation and certain chemotherapeutic drugs induce the proteosome-mediated degradation of IκBα without the phosphorylation-

dependent recognition of IκBα by $E3RS^{IκB}$ (73, 74). However, NF-κB activation induced by all the other known stimuli is dependent on the phosphorylation and ubiquitin-mediated degradation of IκB proteins.

NF-κB activation can be blocked by interference with any of the mutiple steps including IκB phosphorylation (57-59), binding of $E3RS^{IκB}$ to pIκB (67), polyubiquitination of IκB (57, 75), and proteasome-mediated degradation of IκB (63-65, 76). However, the various enzymes involved in IκB polyubiquitination and degradation are constitutively active *in vivo*. IκB phosphorylation is the only step that is regulated by the activation of an inducible multi-subunit protein kinase, the IκB kinase.

B. The IκB kinase complex

The IκB kinase (IKK) complex is able to phosphorylate the N-terminal serine residues of IκB proteins, leading to their polyubiquitination and degradation. This kinase complex is the only enzyme that is markedly stimulated when cells are stimulated by NF-κB inducers such as TNF-α, IL-1 and bacterial LPS in the NF-κB pathway (77, 78). IKK is also the target of lymphocyte antigen receptor signaling (79-88). Therefore, the IKK complex is the key regulator of the NF-κB pathway.

Identification of IKK complex and its components

Considerable efforts were made to identify the IκB kinase complex. It was not until 1997 that the components of this complex were identified and characterized by several

groups. NF-κB inducing kinase (NIK) is the first kinase identified that was linked to the IκB kinase complex. NIK is a member of the MAP kinase kinase kinase (MAP3K) family. It interacts with TNF receptor associated factor 2 (TRAF2) and TRAF6 to mediate NF-κB activation in response to both TNF-α and IL-1 (89-91). Using a yeast two-hybrid screening system, IKKα was found to be associated with NIK (90). IKKα was previously known as CHUK (conserved helix-loop-helix ubiquitous kinase) (92), a serine-threonine kinase of unknown function. Regnier *et al.* later found that IKKα was associated with IκBα in cytokine stimulated cells. Catalytically inactive mutants of IKKα were found to inhibit TNF-α, IL-1, TRAF and NIK induced NF-κB activation (90). Independently, IKKα was purified and its protein sequence identified from a TNF-α stimulated Hela extract (77). IKKα specifically phosphorylated the serine residues 32 and 36 on IκBα. Finally, using TNF-α treated Hela extracts, Mercurio *et al.* identified two protein subunits of 85- and 87-κD that correlated with the IκBα kinase activity. These subunits were present in an IKK signalsome complex that migrated on gel filtration chromatography at 500-700kD. The amino acid sequence of these proteins, which are designated IKKα and IKKβ, was then determined by high mass accuracy matrix-assisted laser deposition and ionization (MALDI) peptide mass mapping and nanoelectrospray tandem mass spectrometry (78). Another group identified IKKβ as an IKKα related kinase by screening the National Center for Biotechnology information DNA database of expressed sequence tags (EST) (93). These studies revealed that IKKα and IKKβ are highly related serine kinases with 52% amin acid identity. Both of these kinases contain an N-terminal kinase domain and a C-terminal protein interaction

region containing helix-loop-helix and leucine zipper motif, which facilitates their heterodimerization *in vivo*. Both IKKα and IKKβ specifically phosphorylate the serine residues 32 and 36 on IκBα and serine residues 19 and 23 in IκBβ. IKKβ was found to have a 20 fold higher kinase activity for IκB as does IKKα.

Following the identification of IKKα and IKKβ, another component of the IKK complex, the NF-κB essential modulator (NEMO) or IKKγ, was identified through both genetic complementation (94) and biochemical purification and microsequencing of IKKα associated proteins (95, 96). IKKγ is a 50/52-kD doublet containing two coiled-coil motifs and a leucine zipper motif at its C-terminus that is critical for the regulation of the IKK complex. IKKγ preferentially interacts with IKKβ and is essential for the NF-κB activation induced by TNF-α, IL-1 and overexpression of MEKK-1 and NIK. Gel filtration chromatography revealed that IKKα, IKKβ and IKKγ tightly bind to each other and reside in a complex with a molecular weight of 900kD (77, 95, 97).

The major components of the IKK complex, IKKα, IKKβ and NEMO (IKKγ), and their secondary structure are shown in Figure 3. Identification of these components in the IKK complex made it possible to characterize these components. The fact that IκBα and IκBβ can be phosphorylated by recombinant IKKα and IKKβ demonstrates that both kinases can directly phosphorylate IκB proteins on the N-terminal regulatory serines (98, 99). Both recombinant and native IKK have a higher kinase activity for IκBα as compared to IκBβ (78, 97, 98).

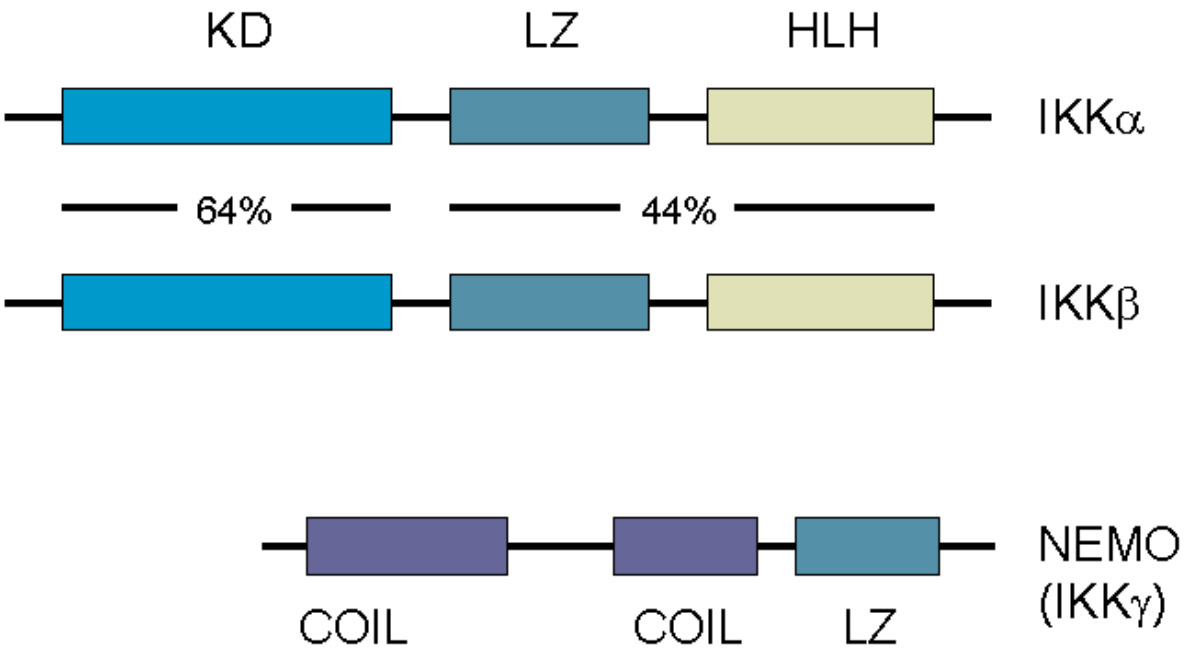


Figure 3. Schematic of the secondary structure of IKKα, IKKβ and NEMO. IKKα (85kD) and IKKβ (87kD) are catalytic subunits of the IKK complex with similar structure. The N-terminal kinase domain (KD) region and the C-terminal region containing leucine zipper motif (LZ) and helix-loop-helix motif (HLH) have 64% and 44% amino acid identity, respectively, between IKKα and IKKβ. KD contains two serine residues critical for the activation of IKKα and IKKβ. NEMO or IKKγ (50-52kD) is a regulatory subunit, with two coiled-coil (COIL) motif and a LZ motif.

Mutations in the leucine zipper motif of either IKKα or IKKβ prevent the dimerization of the catalytic subunits and abolish their kinase activity. These studies suggest that IKK subunits may assemble into different complexes that phosphorylate different substrates or respond to different stimuli. Fully active IKK complexes purified from mammalian cells consist of IKKα:IKKβ heterodimers associated with one or several IKKγ molecules (95). It was noted that IKKα and IKKβ were present in a 300kD complex in IKKγ deficient cells. Expression of IKKγ resulted in the generation of a 900kD complex and strongly enhanced the kinase activity of the complex. These results suggested that IKKγ is critical in assembling IKKα and IKKβ to respond to upstream activators (94). IKKγ may facilitate the interaction of IKK and its substrates IκBα or IκBβ by recruiting the latter into the IKK complex (100). The C terminal region of IκBα is involved in recognition by IKK and serves to facilitate IKK phosphorylation of the N-terminus of IκBα (78, 98, 101). IκBα or IκBβ complexed with the NF-κB heterodimer is considerably better substrates than their free forms, which is critical for the accumulation of newly synthesized IκB protein and termination of NF-κB activity.

A related kinase known as IKKε is involved in NF-κB induction by the phorbol ester PMA (102). The structure of IKKε is similar to that of IKKα and IKKβ. Recombinant IKKε is able to phophorylate serine 36 in IκBα. A dominant negative IKKε mutant in which a conserved lysine residue is substituted with alanine was able to block NF-κB reporter gene expression induced by PMA or via activation of the T cell receptor. However, this mutant was unable to block NF-κB activation in response to either TNF-α or IL-1 in Jurkat cells.

Thus, activation of the NF-κB pathway likely requires mutiple distinct IκB kinase complexes, which respond to both overlapping and discrete signaling pathways.

Regulation of IKK kinase activity

The activity of the IKK complex is regulated through both the regulatory subunit IKKγ and the catalytic subunits IKKα and IKKβ. IKKγ is indispensable for the activity of IKK complex. Although it was shown that IKKγ preferentially interacts with IKKβ (94-96), it can also be co-immunoprecipitated with IKKα from IKKβ deficient cells (103), indicating that it can interact with IKKα either directly or indirectly *in vivo*. A mutant IKKγ containing the C-terminal deletion did not affect the binding of IKKγ with IKKα and IKKβ, indicating that the interaction of IKKγ with the catalytic subunits of the IKK complex is most likely mediated by its N-terminal region containing the coiled-coil structure. The C-terminal half of IKKγ, however, is also required for NF-κB activation induced by TNF-α, IL-1, NIK and MEKK1. Bearing a LZ and zinc finger motif, the C-terminal region of IKKγ might function as a docking site for various signaling proteins, among which include upstream kinases that can activate IKK complex (95, 96) and RIP which mediates TNF-α induced NF-κB activation (104).

As with other members in the MEK kinase family such as MAP kinase kinase 1 (105, 106), activation of IKK depends on the phosphorylation of the MAP kinase activation motif that contains two serine residues: S177 and S181 in IKKβ and S176 and S180 in IKKα. Protein phosphatase 2A (PP2A) abolishes the kinase activity of the IKK complex purified

from TNF-α treated cells. In contrast, treatment of cells with okadiac acid, the PP2A inhibitor, enhances IKK activity and activation of the NF-κB pathway (77). Mutation of serine residues to alanines in the MAP kinase activation motif of IKKβ prevents its phosphrylation and activation and results in a potent dominant negative inhibitor of TNF-α induced RelA nuclear transloction. Conversely, the substitution of these serine residues with glutamic acid residues, which mimic phosphoserine, results in constitutive IKKβ activation (78, 107) and RelA nuclear translocation. Although some studies have demonstrated that IKKα mutants containing substitutions of serine residues 176 and 180 in the activation loop with alanine have no effect on the activation of IKK complex (107), other results indicate this mutant IKKα also blocks activation of the IKK complex and the NF-κB pathway. An IKKα mutant in which serine residue 176 is changed to alanine could not be phosphorylated by NIK and acted as a dominant negative inhibitor of NF-κB activation induced by IL-1 and TNF-α (108) and attenuated Tax-mediated NF-κB activation (109). An IKKα mutant containing a substitution of lysine residue with methionine inhibited the NF-κB reporter gene expression in response to overexpression of MEKK1 (110). It is likely that IKKα and IKKβ are both involved in the NF-κB activation induced by potent NF-κB inducers such as TNF-α, IL-1 and viral proteins such as HTLV-1Tax, although IKKβ is likely the more critical kinase involved in activating the NF-κB pathway in response to these stimuli.

In addition to the importance of the MAP kinase activation motif, the HLH motif in the C-terminal region of both IKKα and IKKβ is an intrinsic activator of both kinases. This motif likely directly interacts with the kinase domains of both IKKα and IKKβ to stimulate

their activity (107). An IKKβ mutant lacking its HLH motif was completely inactive when expressed in mammalian cells or insect cells. However, the coexpression of a polypeptide containing the HLH in the C-terminus of IKKβ restored TNF-α induced kinase activity. In contrast, the C-terminal serine rich region acts as a negative regulator of IKKα and IKKβ kinase activity (107). Phosphorylation of these serine residues after the signal-induced phosphorylation at the activation loop down-regulates IKK kinase activity. The accumulation of negative charges in the C-terminal region of IKKβ may weaken the interaction between the HLH motif and the kinase domain and thus reduce the kinase activity. This hypothesis is supported by the fact that substitutions of serine with glutamic acid to mimic phosphoserine in this region of IKKβ prevents TNF-α mediated increases in IKK activity, while substitutions of these serine residues with alanine results in prolonged IKK activity upon TNF-α stimulation.

In addition to regulation of IKK activity by the three components of this complex, signaling molecules upstream of the IKK complex are also involved in activating IKK. The NF-κB inducing kinase (NIK) was the first kinase that was proposed to activate IKK although the signaling pathway in which NIK operated was unclear. Studies using over-expression of dominant negative NIK mutants suggested that NIK activated IKK in response to stimulation of cells with TNF-α, IL-1 and HTLV-1 Tax protein (89, 91, 111). However, it was found that a TRAF2 mutant that did not interact with NIK retained its ability to activate IKK and the NF-κB pathway in response to stimulation of cells with TNF-α and IL-1 and other stimuli (112), suggesting that NIK may not be critical for activating the NF-κB

pathway. Another MAP3 kinase, MEKK1, has also been reported to be an upstream kinase that activates IKK (113-115). Although MEKK1 is not as potent as NIK to activate IKK (116), its activity can be stimulated by stimulation of cells with TNF-α (112). Unlike NIK and MEKK1, whose roles in IKK activation remain controversial, the HTLV-1 Tax protein directly interacts with IKK (117, 118) and persistently activate IKK (109, 119-122). Tax may cause IKK activation by increasing autophosphorylation potentially without the involvement of an upstream kinase. A conformational change in the IKK complex may directly alter its kinase activity via trans-autophosphorylation. Therefore, cellular or viral factors that interact with IKKγ or other parts of the complex may regulate the IKK activity by altering the conformation of the complex (55).

Biological functions of IKKα, IKKβ, and IKK γ

The presence of two distinct catalytic subunits tightly associated as a dimer that is associated with a regulatory subunit in the IKK complex makes the IKK a unique serine kinase. With a 52% identity in the amino acid sequence, both IKKα and IKKβ are able to phosphorylate N-terminal serine residues in IκBα and IκBβ. IKKα and IKKβ are both widely expressed in multiple tissues (123). The presence of these closely related, yet distinct kinases in the same IKK complex, prompted the question as to whether they serve distinct or redundant function. *In vitro* studies discussed above have shown differences in IKKα and IKKβ activity, suggesting that these kinases may have distinct functions. Phenotypic studies with IKKα$^{-/-}$ and IKKβ$^{-/-}$ mice provided direct evidence demonstrating the distinct biological functions of IKKα and IKKβ.

IKK$\alpha^{-/-}$ mice were generated by two groups and exhibited identical phenotypes (124, 125). These mice die shortly after birth with severe abnormalities in the skin and skeletal system. At embryonic day 18, IKK$\alpha^{-/-}$ fetuses had rudimentary protrusions instead of normal limbs. Their tails and heads are shorter than normal. In addition, their skin is taut and shiny, lacking folds and wrinkles seen on normal fetuses. Further examination revealed that the limbs and tail of mutant fetus were surrounded by a 10-fold thicker layer of skin, which prevented the emergence of limb outgrowths. The increased thickness of the skin is due to enhanced proliferation of the basal cells of the epidermis. In addition, there appears to be a block in keratinocyte differentiation such that the mutant mice are covered by a uniform layer of cells lacking the upper layer of keratinized cells, resulting in increased adhesiveness and stickiness of the skin. In addition to restraining the growth of bones by the skin, the abnormal epidermal differentiation may cause the defects in skeletal patterning by not providing morphogens and growth factors that normally control the differentiation of bones, which is one of the mesodermal derivatives (55, 126, 127). Only slight defects in NF-κB activation and IκBα degradation were observed in IKK$\alpha^{-/-}$ embryonic fibroblasts, thymocytes, or liver tissue upon stimulation with IL-1, TNF, or LPS. This indicates that IKKα is not essential for IKK activation by proinflammatory stimuli. Instead, it appears to be essential for the normal development of keratinocytes and skeletal morphogenesis. None of the currently available knockout mice, which are deficient in any of the known NF-κB subunits or components that regulate the activation of the NF-κB pathway, exhibit a similar phenotype as that of IKK$\alpha^{-/-}$ mice. Thus, it is unlikely that the developmental and morphogenetic defects in IKK$\alpha^{-/-}$ animals are caused by alterations in NF-κB activation. It was reported recently (128) that

IKKα controls the production of a keratinocyte differentiation-inducing factor (kDIF) that is essential for proper terminal differentiation of epidermal keratinocytes. More interestingly, the production of kDIF does not depend on IKK activity and NF-κB activation, indicating the unique function of IKKα (128).

In contrast to IKKα, the major function of IKKβ appears to be the phosphorylation of IκB in response to a variety of stimuli, leading to activation of NF-κB. This was clearly demonstrated by the phenotype of $IKK\beta^{-/-}$ mice. $IKK\beta^{-/-}$ embryos die at embryonic day 12.5 due to severe liver degeneration caused by TNF-α induced apoptosis of hepatocytes (103, 129, 130). This is strikingly similar to the major pathology previously observed in $RelA^{-/-}$ embryos, which die at E14.5 (131), and the $RelA^{-/-}p50^{-/-}$ embryos, which die at E12.5 (132). Indeed, mice that lack both RelA and TNF (133) or IKKβ and TNF receptor (TNFR) (129) are viable and have normal liver, indicating that liver degeneration in $RelA^{-/-}$ as well as $IKK\beta^{-/-}$ embryos are due to a lack of NF-κB activity that is normally stimulated by TNF-α during normal liver development. Correspondingly, $IKK\beta^{-/-}$ cells are unable to activate IKK or the NF-κB pathway in response to either TNF-α or IL-1, indicating the essential role of IKKβ in NF-κB activation.

Consistent with the *in vitro* results, which suggested that IKKγ is critical for the formation of an active IKK complex and thus NF-κB activation, IKKγ deficient mice also die of severe liver degeneration caused by TNF-α mediated apoptosis of hepatocytes (134). Combined with the results from $IKK\beta^{-/-}$ mice, these results indicated that both IKKβ and IKKγ are required for the activation of NF-κB pathway by proinflammotory cytokines.

Interestingly, the IKKγ gene is located on both the mouse and human X chromosome. Female mice heterozygous for the IKKγ deficiency exhibited a dermatopathy characterized by keratinocyte hyperproliferation, skin inflammation, hyperkeratosis and increased apoptosis (135). This phenotype is demonstrated to be a mouse model for the human hereditary disease familial incontinentia pigmenti (IP), an X-linked dominant disorder. In affected female patients, this disease causes highly variable degree of abnormalities of the skin, hair, nails, teeth, eyes and central nervous system. While male carriers of the disease usually die prenatally. It was shown recently that most cases of incontinentia pigmenti are due to mutations of NEMO/IKKγ locus (136, 137). The progression of this disease was thought to arise from IKKγ deficient cells triggering an inflammatory reaction as well as hyperproliferation of both mouse and human keratinocytes, resulting in hyperkeratotic lesions.

In conclusion, IKKα, IKKβ and IKKγ exhibit distinct biological functions (Table 1). It is now clear that IKKα is a bifunctional kinase that contributes to NF-κB activation induced by proinflammatory cytokines and is also essential for normal epidermal development. IKKβ and IKKγ are the essential catalytic and regulatory subunits, respectively, for the activation of NF-κB. Because NF-κB proteins are key activators of genes involved in innate immune and inflammatory responses, IKK should be considered as the master regulator of this highly important and evolutionally conserved defense system. Before we discuss the relationship between NF-κB activation and the immune system, it is necessary to discuss the major components and their function in the immune system.

Table 1. Biological functions of IKKα, IKKβ and IKKγ

	IKKα	IKKβ	NEMO (IKKγ)
role in IKK complex	catalytic subunit	catalytic subunit	regulatory subunit
cellular function	weak IκB kinase strong p100 kinase	strong IκB kinase	assemble IKK
necessity for NF-κB activation by proinf-lammatory cytokines	not required	required	required
viability of knockout mice	die within 1 hour after birth	lethal at embryonic day 12.5	lethal at embryonic day 12.5
cause of embryonic lethality	abnormal skin and skeleton ontogeny	TNF-α induced liver degeneration	TNF-α induced liver degeneration
others	required for p100 processing and B development	required for lympho-cyte development	X-linked gene, related to incontinentia pig-menti in humans

The content of this table is based on studies on IKKα, IKKβ and NEMO deficient mice (103, 124, 125, 129, 130, 134-137) as well as results from radiation chimeras reconstituted with IKKα or IKKβ deficient fetal liver cells (138-140). Please refer to the "recent studies" section in this chapter.

C. Overview of lymphocytes and their role in the immune system

The immune system is a remarkable defense system that provides the host with ability to make rapid and highly specific protective responses against pathogenic microorganisms. There are two classes of cell types in the immune system: the lymphocytes and the myeloid cells. Lymphocytes play a central role in host defense because they determine the specificity of immune response and orchestrate the effector function of the immune system. Lymphocytes are an enormously heterogeneous group of cells with each individual of them expresses a receptor specific for certain determinant on antigens. The heterogeneity of the antigen receptors enables lymphocytes to recognize a large number of antigens. Two broad classes of lymphocytes: B and T lymphocytes, have been defined by their distinct functions. B lymphocytes are precursors of antibody-secreting cells while T-lymphocytes are involved in important regulatory functions as well as effector functions during the immune response.

Development and function of B lymphocytes

B lymphocytes are derived from hematopoietic stem cells by a complex set of differentiation events that occur early on in the fetal liver or later in life in the bone marrow. As B cells differentiate from primitive stem cells, they proceed through stages that are marked by the sequential rearrangement of immunoglobulin (Ig) gene segments to generate a diverse repertoire of antigen receptors. The Igs are heterodimeric molecules consisting of a heavy (H) chain and a light (L) chain, both of which have variable (V) regions that contribute to the binding of antigen and the constant (C) regions. The genetic elements encoding the variable portions of IgH and L chains are not continuous in the genome. Therefore, they need

to be rearranged to construct an expressible V-region gene. This process involves random choice among a large set of potential variable (V), diversity (D), and joining (J) elements in a combinatorial manner to result in the generation of a very large number of distinct H and L chains. The B lymphocyte developmental program is characterized by two checkpoints: pro-B cells and pre-B cells. The pro-B cells are the first clearly identifiable members of the B lineage cells. They have begun the process of Ig gene rearrangement that is limited to heavy (H) chain genes and express the B cell marker CD45 (B220), the recombinase activating genes Rag-1 and Rag-2, and the terminal deoxynucleotidyl transferase (TDT). Successful completion of V_HDJ_H rearrangement and expression of μH chains signal the completion of the H-chain gene rearrangement and the onset of the L-chain gene rearrangement. This step marks the boundary between the pro-B cell and pre-B cell stages. Pre-B cells continue to express CD45 and Rag-1 and Rag-2 but not the TDT gene. They express genes that are required for the production of the surrogate light chains (VpreB and λ5) that can be expressed with the μH chain on the cell surface. Successful rearrangement of the L chain results in its pairing with the μH chain and expression of cell surface IgM. This marks the immature B cell stage. Further expression of the δH chain marks the maturity of B cells, which express IgM, IgD, B220 and CD23 on their cell surface. Figure 4 illustrates the organization and translocation of the Ig H chain gene.

Mature B cells can be activated in two ways: cross-linkage dependent activation and cognate activation. When B cells encounter antigens that bear multiple copies of an epitope that can bind to their surface Ig, the resultant cross-linking stimulates biochemical signals within the cell that include tyrosine phosphorylation and increased ionositol phospholipid

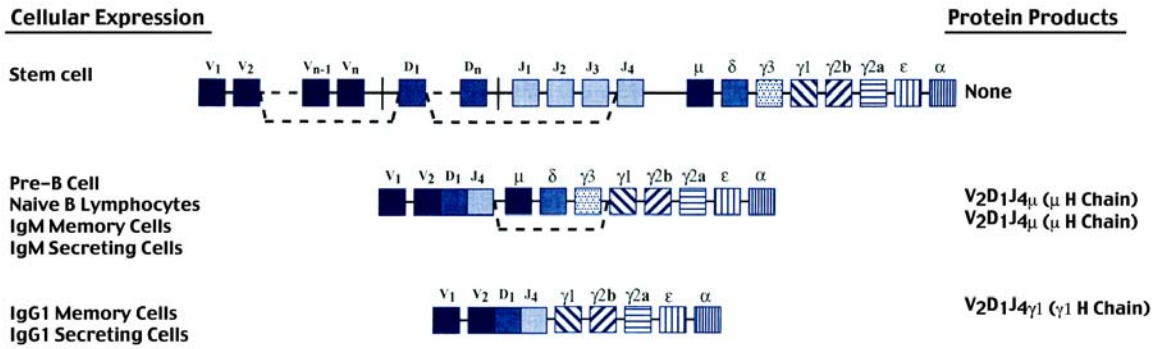


Figure 4. Organization and translocation of mouse IgH genes. IgH chains are encoded by four distinct genetic elements: Igh-V (V), Igh-D (D), Igh-J (J), and Igh-C (C) genes. The V, D, and J genes together specify the variable region of the H chain. The Igh-C gene specifies the C region. The same region can be found in association with each of the C regions (e.g., μ, δ, γ3, γ1, γ2b, γ2a, ε, and α). In the germline genome, the V, D, and J genes are far apart and there are multiple forms of each of these genes. During B cell development, a VDJ gene complex is formed by translocation of individual V and D genes so that they lie next to one of the J genes, with the excision of intervening genes. VDJ rearrangement occurs at the pro-B stage at which D and J segment recombined first and join with a V segment. Productive VDJ rearrangement leads to expression of pre-BCR on the surface of pre-B cells, which then start to rearrange their L chain and express surface Ig receptor. The VDJ complex is initially expressed with μ and δ C genes but may subsequently be translocated so that it lies near one of the other C genes such as γ1 and in that case leads to the expression of a VDJ γ1 chain. Such process is called isotype switching. (W. Paul, "Fundamental Immunology", 4th edition)

metabolism. These responses, together with the action of cytokines, contribute to B cell activation, growth, and differentiation. Cognate activation occurs in the context of interactions of B cells with helper T cells ($CD4^+$ T cell). When B cells encounter antigens that do not have repetitive epitopes and thus cannot crosslink the surface Ig, the antigen is instead bound by the surface Ig and endocytosed by the B cells. The antigen is then processed and presented as peptide in the context of the major histocompatibility complex (MHC) class II molecule on the surface of the B cells. The MHC class II/peptide acts as specific ligand for the T cell receptor (TCR) on $CD4^+$ T cells to mediate the interaction of B and T cells. Signals provided by the interaction of CD40 ligand (CD40L) on T cells and CD40 on B cells as well as cytokines produced by T cells act as co-stimulatory signals to activate B cells.

Activated B cells divide and differentiate into antibody secreting cells or memory cells. The former accounts for the primary response to an antigen and is largely controlled by the interaction with T cells and the cytokines produced including IL-4, IL-5, IL-6 and IL-12. These cytokines lead to faster and enhanced antibody production upon re-encountering the same antigen, which is dominated by Ig isotypes other than IgM, with greater magnitude and higher antibody affinity for the antigen. Differentiation of activated B cells into memory B cells occurs in the germinal centers of the spleen and lymph nodes where affinity maturation and isotype class switching occur.

Although in many physiologic situations, receptor cross-linking and cognate help activate B cells synergistically, the dominant type of response generated by B cells can sometimes be determined by the nature of antigens that B cells encounter. From this point of

view, the antigens that B cells encounter can be recognized as T cell dependent (TD) and T cell independent (TI) antigens. Most protein antigens are TD antigens. T cells that have the same antigen specificity are required to activate B cells that interact with such antigens. TI antigens fall into two categories: TI-1 antigens and TI-2 antigens, which activate B cells by different mechanisms. TI-1 antigens such as trinitrophenyl-LPS (TNP-LPS) (141-143) and fluorescein-brucella abortus (FLU-BA) (141, 143) contain an intrinsic activity that can directly induce B cell proliferation. These antigens at high concentrations act as polyclonal B cell activators. At low concentrations, these antigens only activate specific B cells that can bind to the antigen through their Ig receptors and increase the local concentration of the antigen. The TI-1 response has an important role in specific defense against some extracellular pathogens. TI-2 antigens such as TNP-Ficoll (143, 144), capsular polysaccharide (CPS) (145), and dinitrophenyl-hydroxyethyl starch (DNP-HES) (142) contain repetitive structures without intrinsic B cell stimulating activity. The repetitive structure of TI-2 antigen is able to crosslink the surface Ig receptor, leading to crosslinking dependent activation of B cells.

Development and function of T lymphocytes

T lymphocytes are derived from hematopoietic precursors. The maturation of T cells occurs in the thymus where both positive and negative selections occur, resulting in mature T cells expressing T cell receptor (TCR) that can recognize foreign antigens in the context of self-MHC molecules. Mature αβ T cells express either CD4 or CD8 molecules on their

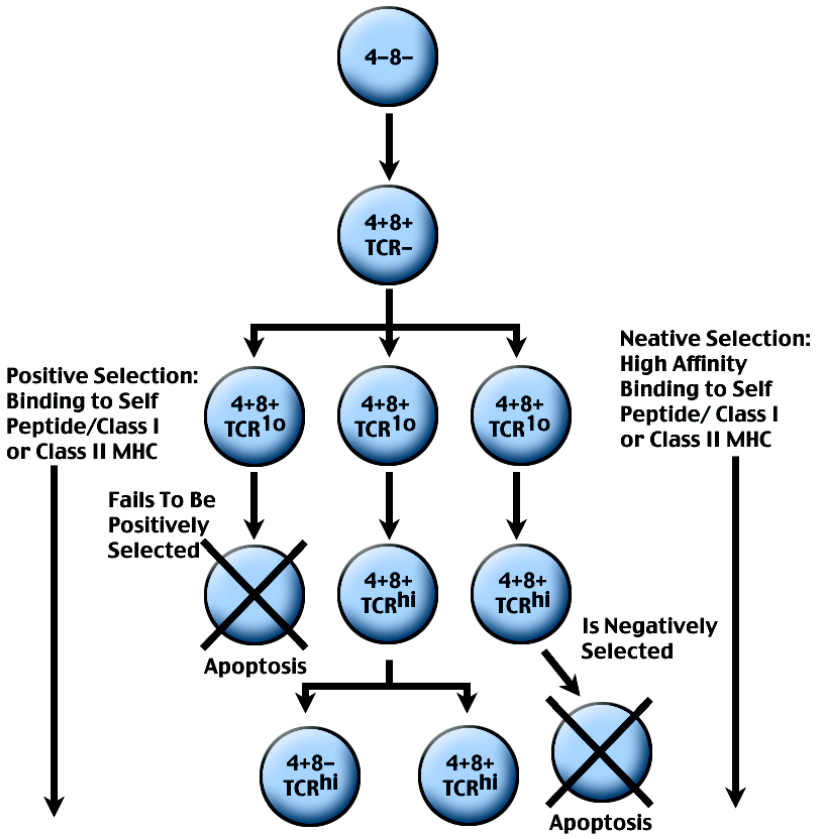


Figure 5. Schematic of the development of α/β T cells. Double negative ($CD4^-CD8^-$) T cell precursors first express CD44 and CD25. After the rearrangement of TCR β chain and successful expression of pre-TCR on the cell surface, T cells gain expression of CD4 and CD8 and start to rearrange the TCR α chain. The double positive ($CD4^+CD8^+$) T cells then express low levels of α:β TCR and the associated CD3 complex and are ready for selection. Thereafter, the degree of expression of TCRs increases and the cells differentiate into CD4 and CD8 cells and are then exported to the periphery. During the transition from the double positive stage to the single positive stage, most thymocytes die by failing to be positively selected or as a consequence of negative selection. Cells that recognize peptide/self MHC complex can survive and up-regulate their TCR expression. Positive selection by recognition of peptide/class I MHC or peptide/class II MHC complexes leads to the development of $CD8^+$ or $CD4^+$ T cells, respectively. Cells that recognize self-peptide/MHC complex with a high affinity are also eliminated via apoptosis. Positive and negative selection allows the establishment of a self-tolerant and self-MHC restricted T cell repertoire. Maturation of T cells takes place in the cortex of the thymus. Only mature single positive T cells are found in the medulla. (W. Paul, "Fundamental Immunology", 4th edition)

surface. These two types of T cells differ in the way they recognize antigen and their regulatory and effector functions.

The double negative ($CD4^-CD8^-$) T cell precursor cells do not express TCR or CD3. They begin to express TCR molecules through a complex rearrangement that is very similar to the gene rearrangement required for the production of Ig molecules in B cells. As they differentiate into $CD4^+CD8^+$ double positive cells that express low levels of TCR and CD3, they undergo both positive and negative selections. In positive selection, T cells with receptors that recognize self-MHC molecules survive. This results in the self-MHC restricted T cell population. In negative selection, T cells with receptors that bind to self-peptide/self MHC complexes with high affinity are deleted. This is the major mechanism through which the T cell compartment develops self-tolerance. These two selection processes result in the development of a population of T cells that are biased toward the recognition of foreign peptide in the context of self-MHC molecules. The development of single positive T cells from double positive T cells involves the interaction of TCR and CD4 (or CD8) with the MHC class II (or I) molecules on accessory cells. Figure 5 summarizes the major events during T cell ontogeny.

Activation of $CD4^+$ and $CD8^+$ T cells requires two signals. One is provided by the engagement of TCR with its cognate ligand, a peptide bound in the context of MHC or I molecules, respectively. The other is provided by an accessory cell-derived costimulatory activity, which usually results from the interaction of CD28 with its ligands B7.1 (CD80) or B7.2 (CD86) on antigen presenting cells (APC). Once activated, $CD4^+$ T cells and $CD8^+$ T cells become armed effector cells that have distinct immunological functions. $CD4^+$ T cells,

or helper T cells (Th cells), are sub-divided into Th1 cells and Th2 cells. Th1 cells secrete IFNγ and induce cellular immunity by enhancing the capacity of monocytes and macrophages to destroy intracellular microorganisms. In addition to IFNγ, Th1 cells activate macrophages by expressing the CD40 ligand, which synergize with IFNγ to enhance the killing of the engulfed bacteria. They also express Fas ligand and TNF-β that kill the chronically infected macrophages. Moreover, IL-3 and GM-CSF secreted by activated Th1 cells act on hematopoietic stem cells to give rise to new macrophages, leading to enhanced host defense against intracellular organisms. In contrast, Th2 cells induce humoral immunity by activating B cells that produce antibodies to eliminate extracellular pathogens and toxins produced by these pathogens. They activate B cells by providing both CD40 ligand and cytokines such as IL-4, IL-5 and IL-6, which drive the proliferation and differentiation of B cells into antibody secreting plasma cells. $CD8^+$ T cells, or cytotoxic T lymphocytes (CTLs), are able to kill viral or other intracellular pathogen infected target cells. CTLs induce apoptosis of target cells through polarized release of granules containing perforin and granzymes to the target cells but not adjacent normal cells. They also induce apoptosis of target cells by expressing Fas ligand. CTLs also secret IFNγ, which inhibits viral replication and activates macrophages.

Signaling pathways mediated by antigen receptors

B cell receptor (BCR) signaling is required for the survival and recirculation of naïve B cells (146) and antigen specific immune response. Antigen engagement of BCR can lead to different outcomes such as proliferation, differentiation, anergy and induction of apoptosis

(147). The type of antigen (148, 149), co-receptors on the B cell surface such as CD19 (150), CD40 (151), FcγRII (152), and adaptor proteins (153-155) that are involved in the signaling pathways can modulate the signal quantity and quality to determine the fate of B cells. Deregulation of B cell activation pathways can lead to immunodeficiency (156-159), autoimmune diseases (160, 161), leukemias (162) or lymphomas (163). Similarly, signaling mediated through the T cell receptor (TCR) is also critical in determining the ontogeny, activation and survival of T cells (164-166).

The earliest event in lymphocyte antigen receptor signaling is the activation of cytoplasmic protein tyrosine kinases (PTKs). Members in the Src kinase family such as Lck, Fyn and Lyn, which are anchored to the inner leaflet of the plasma membrane, initiate the receptor signaling by phosphorylating the tyrosines in the immunoreceptor tyrosine-based activation motif (ITAM) located in the cytoplasmic domains of the TCR and BCR. This allows members in the Syk/ZAP-70 kinase family to be recruited and activated by binding to the phosphorylated tyrosines in the ITAM through their SH2 (Src homology domain 2) (167). ZAP-70 (ζ associated protein-70) is the most well understood kinase in this family in T cells (168-175). It binds to the phosphorylated tyrosine in the ITAM of TCR ζ chain, leading to its activation (170, 176-181). Activation of PTKs such as Syk in B cells is mediated by the interaction of these kinases with ITAM motifs in the intracellular region of the Igα and Igβ chain of the B cell antigen receptor (BCR) complex. Activation of kinases in the Syk/ZAP-70 family allows amplification of the signal through activation of the substrates of these protein kinases, which include phosphatidylinositol 3-kinase (PI3K) and phopholipase Cγ (PLCγ) (147). Both acting on plasma membrane phospholipids, PI3K and PLCγ mediate lymphocyte

activation and differentiation signals. PI3K phosphorylates membrane phosphatidylinositol lipids on the D3 position of the inositol ring to generate phosphatidylinoditol-3-phosphate (PI(3)P), phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) or phosphatidylinositol-3, 4, 5-triphosphate (PI(3,4,5)P3). Both PIP2 and PIP3 can bind to kinases and adaptor proteins containing pleckstrin homology (PH) domains (182-186) and recruit them to the plasma membrane at sites of PI3K activation (187-190). PLCγ hydrolyzes PIP2 to produce soluble inositol-1, 4, 5-trisphosphate (IP3) and membrane-anchored diacylglycerol (DAG), which stimulates increased free Ca^{2+} in the cytosol by activating its release from the endoplasmic reticulum through IP3-gated calcium channels and the serine-threonine specific protein kinase C (PKC), respectively (191, 192). The Ras pathway is also activated down stream of antigen receptor engagement. This leads to activation of the MAP kinase (193-197).

Antigen receptor mediated activation also involves negative regulators that terminate the activation signal. In T cells, phospho-tyrosine phosphatase (PTPase) SHP-1 (198) and SHP-2 (199) negatively regulate the activation signal by dephosphorylating the PTKs. Csk, a PTK, inhibits the kinase activity of Src kinases by phosphorylating their inhibitory tyrosines (200). Cytotoxic T lymphocyte antigen-4 (CTLA-4) may serve in a negative-feedback loop to autoregulate T cell responses by recruiting the SHP-2 PTPase to the plasma membrane (199). Other molecules that may have inhibitory function include CD5 (201) and the killer inhibitory receptors (KIRs) (202, 203). In B cells, in addition to the inhibitory function of Csk and SHP-1 and -2, which act in similar way as they do in T cells, the SH2 domain-containing inositol phosphatase SHIP (204-206) and PTEN (phosphatase and tensin homolog deleted on chromosome ten) (207) dephosphorylate PIP3, preventing the elevation of PIP3

levels and its consequent second messenger function. These negative regulators control the threshold and the termination of receptor initiated signals, protecting the host from otherwise devastating lymphocyte responses.

NF-κB activation is involved in multiple downstream signaling pathways that are initiated by PI3K and PLCγ. In B cells, both Akt and Bruton's tyrosine kinase (Btk) that are activated by PIP2 and PIP3 can stimulate IKK to activate the NF-κB pathway. Akt mediates NF-κB activation induced by TNF, TCR and platelet derived growth factor (PDGF) (79-81), while Btk activates PLC-γ2 and couples BCR signaling to the IKK complex (82-84).

Recently, it was demonstrated that a PKC isoform, PKC-θ, mediates activation of the NF-κB pathway in lymphocytes in response to stimulation of the antigen receptor or phorbol esters. PKC-θ can activate the IKK complex through selective activation of IKKβ (85-88) when T cells are stimulated with anti-CD3 and anti-CD28. Mature T cells from mice lacking PKC-θ exhibit defects in NF-κB activation in response to TCR stimulation (208). PKC-θ and IKK associate in a complex that is recruited into membrane lipid rafts in response to TCR/CD28 or phorbol ester stimulation (209, 210). PKC-θ was also demonstrated to be critical in B-cell activation by mediating NF-κB activation in response to either BCR engagement and phorbol ester stimulation (211). However, activation of NF-κB in response to bacterial lipopolysaccharide (LPS) (212, 213) and CD40 ligands (214) is not dependent on PKC-θ in B cells. Neither is the activation of NF-κB in response to proinflammatory cytokines IL-1 and TNF-α (215).

Although the activation of NF-κB is required for the ontogeny and function of lymphocytes, the regulation of this pathway in different developmental stages of T and B cells and in immune response against a variety of antigens *in vivo* remains to be elucidated.

D. The role of NF-κB in immunity

NF-κB is a master regulator of immune and inflammatory responses

The evolutionarily conserved role of NF-κB in the immune system is reflected by the innate immunity in *Drosophila*. One of the mechanisms that contribute to microbial resistance in *Drosophila* is the rapid induction of anti-microbial peptide genes such as drosomycin, defensin and diptericin (12). The sequences in the promoter elements of these genes contain binding sites for NF-κB proteins such as dorsal, relish and dif (13, 17, 18, 216). Activation of these transcription factors is induced by signals transmitted from the transmembrane receptor Toll, which shares homology with the cytoplasmic TIR domain (Toll/IL-1 receptor domain) of the mammalian IL-1 receptor, resulting in the phosphorylation and subsequent degradation of cactus (IκB) (16, 217-221).

Insects and vertebrates use Toll-like receptors as pattern recognition receptors. More than 10 Toll-like receptors (TLR) were identified in mammals (222). Members in the TLR family discriminate between specific patterns of bacterial components. For example, Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) are involved in cell activation induced by bacterial peptidoglycan or lipoproteins (223, 224) and bacterial lipopolysaccharide (225-228), respectively. Signalling pathways initiated by the members of the TLR family is mediated by an adaptor protein MyD88 (222). MyD88 interacts with the

TIR domain and recruits IL-1 receptor associated kinase (IRAK) to the receptor. Activated IRAK associates with TRAF 6, leading to the activation of both JNK and NF-κB pathways (222).

In mammals, NF-κB regulates a wide range of genes involved in the much more advanced and complex immune system. Some of these genes amplify the inflammatory process by activating lymphocytes and myeloid cells and recruiting them to the sites of inflammation. These genes include those encoding cytokines IL-1β, TNF-α, IL-2, IL-6 and GM-CSF, Il-8, macrophage inflammatory protein (MIP)-1 (229-234), MHC class I genes, the α subunit of IL-2 receptor, and the adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1. NF-κB can also up-regulate the expression of iNOS (the inducible form of nitric oxide synthase) and COX2 (cyclooxygenase 2) to induce the production of the important inflammatory mediators nitric acid and prostanoids. Moreover, many of the products of NF-κB target genes, such as TNF-α and IL-1, can activate NF-κB, resulting in a positive regulatory loop, which is important in amplifying and propagating the inflammatory response.

NF-κB plays a critical role in the innate immunity and initiation of the adaptive immunity. The co-stimulatory molecules B7.1 and B7.2 induced by NF-κB are important in activating naïve T cells specific for pathogens. Inflammation mediated by NF-κB also has several generalized effects on the adaptive immune response. For example, interferons (IFNs) induced by viruses strongly up-regulate the expression of the MHC class I molecules, thus increasing the efficiency of presentation of viral peptides to the cytotoxic T cells (235); IL-6 induces the terminal differentiation of B lymphocytes into Ig producing plasma cells

(236); Expression of IL-2 and IL-2 receptor mediates the clonal expansion of activated T cells (229).

Being a master regulator in inflammatory response, NF-κB also plays a role in acute situations such as septic shock and in the development of chronic diseases such as rheumatoid arthritis (RA) and asthma. Septic shock develops when LPS or other microbial products induce massive production of various inflammatory cytokines, which lead to a lowered blood pressure and general organ failure. NF-κB may regulate important aspects of septic shock by controlling the expression of iNOS (237, 238). Increased expression of the NF-κB target genes iNOS and CoX-2 is likely involved in the pathogenesis of asthma (239-241). In addition, increased synthesis of the inflammatory mediators ICAM-1, VCAM-1 and E-selectin, which are also regulated by NF-κB, are associated with the pathogenesis of rheumatoid arthritis (RA) (242-244). Inhibitors of NF-κB activation such as glucocorticoids and salicylates are used to treat these diseases, indicating that the development of novel inhibitors of the NF-κB pathway may act as potent therapeutic reagents to treat these diseases.

The role of NF-κB transcription factors in the development and function of the mammalian immune system has been studied extensively using a variety of genetic approaches including knockout mice, transgenic mice, and stem cell reconstituted radiation chimeras (245, 246). Table 2 lists the major phenotype of mice bearing mutant NF-κB subunits or several components in the NF-κB pathway. These studies revealed that NF-κB is a key regulator of the development and function of the immune system.

Table 2. Phenotype of mice with mutations in various components of the NF-κB pathway

Mice	viability	immune development and function
NF-κB2$^{-/-}$ (p100/p50)	viable	reduced B cell number and impaired splenic structure, lack of marginal zone and germinal center, impaired TD response but normal TI response, mild defects in B cell mitogenic response
RelB$^{-/-}$	viable but increased mortality	multiorgan inflammation and myeloid hyperplasia, reduced number of thymic dendritic cells and medullary epithelial cells, impaired clonal deletion of autoreactive thymocytes
RelA$^{-/-}$ chimera	lethal viable	die at embryonic day 15 due to liver degeneration normal B and T cell development
NF-κB1$^{-/-}$ (p105/p50)	viable	normal B cell maturation, impaired B cell mitogenic response and antibody response, increased susceptibility to *Listeria m.*
c-rel$^{-/-}$	viable	normal B and T cell development, impaired mitogenic response of lymphocytes, cytokine production, and humoral response
p50$^{-/-}$RelA$^{-/-}$ chimera	lethal viable	die at embryonic day 13 due to liver degeneration impaired lymphopoiesis
NF-κB1$^{-/-}$NF-κB2$^{-/-}$	viable	impaired B and T cell development, bone thickening due to osteopetrosis
NF-κB1$^{-/-}$Rel-B$^{-/-}$	viable	die within 3-4 weeks due to myeloid hyperplasia, impaired B cell development
IκBα$^{-/-}$	viable	die 7-10 days after birth due to widespread dermatitis and granulopoiesis, increased expression of NF-κB target genes
aly (NIK mutant)	viable	impaired splenic structure and antibody response, lack of lymph node and peyer's patches

(For references, please refer to the text.)

Physiological roles of NF-κB/Rel subunits and IκB proteins

Each of the five members of the NF-κB/Rel proteins has been individually knocked out. Studies on these mice revealed distinct physiological role for each of these proteins (131, 247-253). Among the five members of NF-κB/Rel protein, NF-κB2 (p100/p52) and RelB have important developmental roles. NF-κB2 was demonstrated to be critical for the development and function of B lymphocytes (248, 249). Mice lacking NF-κB2 have a distorted splenic architecture characterized by diffuse B cell areas, a lack of discreet follicles and the absence of the perifollicular marginal zone and secondary germinal centers. The number of B cells in these mice is markedly reduced in spleen, bone marrow and lymph nodes. B cells from these mice exhibited mild proliferative defects and normal secretion of IgM and other Ig Isotypes in response to LPS, ligands of surface IgM and CD40 *in vitro*. In addition, the mutant mice have impaired antibody response to TD antigen but normal antibody responses to both TI-1 and TI-2 antigens, suggesting that the ability of NF-κB2 deficient T cells to help B cells elicit an antibody response against specific antigens is impaired. Thus, NF-κB2 is indispensable for the B cell development and T cell dependent humoral response.

RelB deficient mice have increased mortality due to multi-organ inflammation and myeloid hyperplasia (247, 253), which are mediated by T cells (254). These mice have reduced populations of thymic dendritic cells and medullary epithelial cells. They exhibit impaired clonal deletion of autoreactive thymocytes, resulting in pathogenic T cells that promote the inflammatory phenotype (246, 255). The development of multi-organ inflammation in RelB deficient mice can be blocked by depletion of T cells by crossing these

mice with Nur 77/N10 transgenic mice, which lack T cells (254). RelB deficient B cells exhibit proliferative defects (256), suggesting that RelB is required for both development of the hemotopoietic system and the functions of T and B lymphocytes.

Studies on RelA, NF-κB1 (p105/p50), and c-rel deficient mice reveal that these subunits play a more important role in lymphocyte function as opposed to lymphocyte development. The p65 knockout mice die prenatally due to severe liver degeneration (131), suggesting a critical role of p65 in preventing hepatocytes from undergoing apoptosis. In order to study the function of p65 in the immune system, fetal liver cells from p65 deficient embryo were transplanted into irradiated SCID mice (250). Both B and T cells developed normally in these irradiated hosts. However, these lymphocytes exhibited proliferative defects in response to mitogenic stimuli. B cells lacking RelA had reduced production of IgG1 and IgA, suggesting that RelA is dispensible for lymphocyte development but critical for their activation and function.

In contrast to NF-κB2 deficient mice, which have profound defects in B cell maturation and the formation of secondary germinal centers, NF-κB1 (p50) deficient mice (252, 257) exhibit no defects in the development of either T or B lymphocytes. However B cells from these mice exhibited selective proliferative defects in response to LPS and surface IgM crosslinking, decreased Ig secretion and isotype switching, dereased basal antibody levels, impaired humoral response to TD antigen, and increased susceptibility to infection with *Listeria monocytogenes*. Surprisingly, these mice exhibit enhanced resistance to murine encephalomyocarditis virus (EMC) due to up-regulation of the level of antiviral cytokine IFN-β, suggesting that p50 may act as a repressor on this cytokine. Gene disruption of c-rel

(251) revealed that this NF-κB subunit does not play profound role on the hematopoietic and lymphocyte development. However, c-rel is critical for the mitogenic response of both T and B cells, humoral response, and the production of cytokines such as IL-2, IL-3 and GM-CSF (258).

Generation of knockout mice lacking individual members of the NF-κB/Rel family reveal that each member has a unique role that cannot be fully complemented by the presence of other family members (19, 245). However, functional redundancy of these family members may mask some phenotypes of these mice. Due to these possibilities, groups have generated mice that lack multiple NF-κB/Rel subunits. For example, although neither p50 nor RelA have critical role in the development of lymphocytes, the fetal liver cells from p50/RelA double knockout embryos were not able to reconstitute the lymphocytes (132), suggesting that both p50 and RelA play a role in lymphocyte development and can be complemented by each other. Interestingly, the block of lymphocyte development in these mice can be rescued by co-transplantation of wild type bone marrow cells, indicating that the NF-κB activation in the surrounding, but not the developing, cells is required for normal lymphocyte development. Similarly, NF-κB1$^{-/-}$/NF-κB2$^{-/-}$ mice have severe developmental defects characterized by impaired lymphocyte maturation and bone development (259). NF-κB1$^{-/-}$/RelB$^{-/-}$ mice lack B cell population (260). These findings indicate the functional redundancy of these individual NF-κB subunits in lymphocyte development.

Studies with IκB deficient mice reveal that deregulation of NF-κB pathway in these mice causes severe immune and developmental defects. IκBα$^{-/-}$ mice die 7 to 10 days after birth due to severe and widespread dermatitis and extensive granulopoiesis (261, 262). As

expected, the nuclear levels of NF-κB are elevated in thymocytes and splenocytes. The expression of some NF-κB target genes such as GM-CSF, VCAM is also increased. Interestingly, IκBα /p50 double knockout mice exhibited a delayed onset of these defects, indicating that elevated NF-κB activity is responsible for these phenotype. Moreover, cultured IκBα deficient fibroblast cells have normal cytoplasmic levels of NF-κB subunits, suggesting that other IκB, most likely IκBβ, may be responsible for NF-κB cytoplasmic retention. The functional redundancy of IκBα and IκBβ is confirmed by studies using IκBβ knock-in mice (263), which demonstrated that expression of IκBβ as opposed to IκBα under the regulatory and promoter sequence of IκBα rescued the defects seen in IκBα$^{-/-}$ mice, indicating that IκBα and IκBβ share significant similarities in their biochemical activity. TNF-α treated IκBα deficient fibroblast cells exhibited prolonged NF-κB activity, indicating that unlike other IκB proteins, IκBα is able to restrict NF-κB activation to a limited time (262).

Recent studies on NF-κB and IκB kinases using genetic approaches

Although the roles of each subunit in the NF-κB/Rel- and the IκB- protein family in the immune system have become clearer, the mechanism by which the components of NF-κB pathway, especially IKK, regulate immune development and function remains to be elucidated. Since both IKKα and IKKβ deficient mice die before birth, it is impossible to use these mice as direct tools to study the role of these catalytic subunits of the IKK complex in the immune system. Studies utilizing fetal liver cells obtained from IKK mutant embryos

have been used to reconstitute lymphocytes in irradiated hosts. These studies revealed distinct roles of IKKα and IKKβ in the development and function of lymphocytes (138-140).

The phenotype of IKKα$^{-/-}$ mice indicated that IKKα appears to be dispensible for the induction of NF-κB DNA-binding activity in most cells types (124, 264). Instead, it is required for skeletal morphogenesis and the differentiation of epidermis, which however is independent of NF-κB activation (124, 125, 128). Since IKKα is a catalytic subunit in the IKK complex, the question arises whether IKKα play any role in regulating the NF-κB activity. This question remained intriguing until the discovery of the role of IKKα in the processing of NF-κB2, which is critical for the B cell development.

Due to the striking similarity between the NF-κB pathway in mammals and *Drosophila*, there should be a kinase that phosphorylates the IκB protein cactus, leading to its Slimb-mediated degradation by ubiquitin-conjugated proteosome machinery. Using a reverse genetic approach, Silverman *et al.* identified a cDNA sequence with homology to the kinase domain of the human IKKs from a *Drosophila* EST database (265). Surprisingly, instead of cactus, the protein encoded by this cDNA was able to phosphorylate relish in response to LPS stimulation. Phosphorylated relish undergoes proteolytic cleavage and nuclear translocation of its N-termianl region containing the RHD domain. Relish activates antibacterial genes such as Attacin, Cecropin, and Diptericin (265). Genetic screening of spontaneous mutations that interfere immunity against bacterial infection in *Drosophila* resulted in the discovery of the same gene that is required for the activation of relish and antibacterial immune response (266). This gene is designated DmIKKβ due to its homology

with IKKβ in mammals. DmIKKβ is not required for Toll receptor mediated-activation of the other two NF-κB subunits in *Drosophila*, Dif and Dorsal, which are essential for anti-fungal immunity. These results suggested that there must be another *Drosophila* IKK that performs this function. The discovery of DmIKKβ indicates that in addition to mediating NF-κB activation by targeting the degradation of IκB proteins, IKK can also regulate NF-κB activity by controlling the processing of NF-κB. Is there any kinase in mammalian cells with a similar function as DmIKKβ?

Interestingly, a spontaneous mouse mutant known as alymphoplasia (*aly*) causes similar phenotype as that seen in NF-κB2 (p100/p52) deficient mice, with impaired splenic structure and antibody response. The *aly* phenotype was attributed to a point mutation in the NF-κB inducing kinase (NIK) (267, 268). The similar phenotype between *aly* mice and the NF-κB2$^{-/-}$ mice implicated the possibility that NIK may be related to NF-κB2. Xiao *et al.* demonstrated NIK positively regulates p100 processing by inducing the site-specific phosphorylation and ubiquitination of p100, leading to the proteolytic degradation of its C-terminal region and generation of the p52 subunit (269). Senftleben *et al.* generated a set of chimeras using IKKα$^{-/-}$ and wild type fetal liver cells to reconstitute the immune system of lethally irradiated hosts (139). Analysis of these chimeras revealed that while B cells were present in the bone marrow of mice reconstituted with IKKα$^{-/-}$ cells, circulating mature B cells are absent due to an increased turnover rate of the mature B cells. Moreover, IKKα$^{-/-}$ B cells exhibited defective processing of p100, leading to accumulation of p100 and very low level of p52. Generation of chimeras using dominant negative IKKα (IKKα^{AA}) knock-in

mice that express IKKα with serine 176 and 181 substituted with alanines but not the wild type IKKα further confirmed that the kinase activity of IKKα is required for the processing of p100. In addition, recombinant IKKα had a much higher kinase activity for p100 than did NIK, suggesting the IKKα rather than NIK is the critical kinase that mediates the processing of p100 (139). Combined with previous data about up-regulation of NF-κB2 expression during B cell development (44, 270) and recent similar results (140, 271), these studies suggest that the defects in B cell maturation and the formation of secondary lymphoid organs in *aly* mice, IKKα$^{-/-}$ and IKKα^{AA} chimeras are due to the impaired p100 processing. NIK and IKKα regulate the NF-κB pathway predominantly through the processing of p100, but neither of them is required for NF-κB activation induced by proinflammatory cytokines such as TNF-α, IL-1, and bacterial LPS (272).

In contrast, IKKβ is clearly demonstrated to be the kinase responsible for activating NF-κB in response to proinflammatory cytokines or baterial LPS (103, 129, 138). Using adoptive transfer experiments, Senftleben *et al.* analyzed the capability of IKKβ$^{-/-}$ cells to reconstitute the immune system in irradiated recipient mice (138). They found that IKKβ$^{-/-}$ radiation chimeras did not have mature T and B cells and the number of granulocytes was higher in these mice as opposed to mice reconstituted with wild type cells. Massive apoptosis was found in the thymus of the IKKβ$^{-/-}$ radiation chimeras, which is most likely induced by TNF-α, whose levels were higher in the IKKβ$^{-/-}$ radiation chimeras than the wild-type chimeras. The inability of IKKβ$^{-/-}$ cells to reconstitute lymphocytes was due to TNF-α induced apoptosis of these cells was further suggested by the IKKβ$^{-/-}$/TNF-α$^{-/-}$ double mutant

radiation chimeras which had almost normal T and B lymphocytes. Interestingly, the defect in lymphopoiesis in IKKβ$^{-/-}$ cell-reconstituted chimeras could be rescued by the co-transfer of wild type bone marrow cells. These results are very similar to the results seen in previous adoptive transfer experiments, which demonstrated that co-transfer of RelA$^{-/-}$/NF-κB1$^{-/-}$ fetal liver cells with the wild type cells could rescue the defect in lymphoid development seen in RelA$^{-/-}$NF-κB1$^{-/-}$ double mutant chimeras (132). These results suggest that NF-κB activation in the hematopoietic microenvironment contributes to lymphopoiesis. TNF-α levels in IKKβ$^{-/-}$ radiation chimeras are higher than the wild type radiation chimeras. The increased number of granulocytes or the increased product from the host cells could be the two possible source of the increased TNF-α seen in IKKβ$^{-/-}$ chimeras. Transfer of wild type fetal liver cells with the IKKβ$^{-/-}$ fetal liver cells could reduce the number of granulocytes and thus reduce the level of TNF-α and other cytokine mediators of apoptosis. Due to these possibilities, it is likely that TNF-α mediated apoptosis is the primary contributor to the absence of lymphocytes in IKKβ$^{-/-}$ radiation chimeras. Therefore, IKKβ is not required for the development of T cells if the latter can be protected from TNF-α induced apoptosis. However, it is required for T cell proliferation in response a variety of T cell activators such as anti-CD3, PMA/ionomycin and concanavalin A (Con A) (138).

The role of NF-κB in the regulation of apoptosis has been controversial. There is ample evidence (273-277) supporting a role for NF-κB in preventing apoptosis in cell culture. *In vivo*, IKKβ$^{-/-}$ (103, 129, 130), IKKγ$^{-/-}$ (135, 278) and RelA$^{-/-}$ (131, 132) embryos die of severe liver degeneration and elimination of either TNF-α or the TNF-α receptor can

prevent their lethality (129, 133). In addition, mature T cells isolated from transgenic mice expressing a dominant negative IκBα in T lineage cells resulted in increased apoptosis upon stimulation with ConA and TCR crosslinker anti-CD3 (279). These results suggest that loss of NF-κB activity increases the susceptibility of hepatocytes and lymphocytes to apoptosis. However, there is also evidence demonstrating a pro-apoptotic role of NF-κB. For example, increased c-rel expression was associated with increased apoptosis in developing avian embryo and bone marrow cells (280). Additionally, a potent inhibitor of NF-κB, pyrrolidine dithiocarbamate, prevented apoptosis in the human promyelocytic leukemia cells HL-60 and thymocytes (281). Finally, double positive $CD4^+CD8^+$ thymocytes from T cell specific transgenic mice expressing a dominant negative IκBα protein demonstrated decreased susceptibility to apoptosis following the administration of anti-CD3 (282). It is possible that during physiological development, NF-κB activation prevents cells from undergoing apoptosis, when cells encounter exogenous stimuli, NF-κB can lead to activation-induced cell death. Recent studies on the $IKK\alpha^{-/-}$ radiation chimeras suggested an anti-apoptotic role of IKKα. Reconstituted $IKK\alpha^{-/-}$ B cells showed increased turn over rate and increased spontaneous apoptosis, indicating that IKKα may normally prevent spontaneous cell death (139, 140).

In conclusion, these studies have elucidated a role for both IKKα and IKKβ in regulating immune development and function. IKKα and IKKβ regulate NF-κB activity via different mechanisms and probably via different substrates. In *Drosophila*, processing of NF-κB mediated by IKK is a major mechanism in anti-bacterial immunity. Interestingly, this

pathway is involved in a specific aspect of adaptive immunity in mammals, namely B cell maturation and the formation of secondary lymphoid organs. These steps are regulated by p52, whose production depends on IKKα kinase activity. In contrast, IKKβ is an activator of NF-κB in response to infection and inflammation with a critical role in the activation of both T and B lymphocytes. Thus, the functional divergence of IKKα and IKKβ correlates with the advanced and complex immune system in mammals.

Thesis rational and objectives

The research presented in this thesis is focused on the role of the two catalytic subunits of IKK, IKKα and IKKβ, on the development and function of B and T lymphocytes.

Results from NF-κB and IKK knockout mice and studies using IKKα$^{-/-}$ and IKKβ$^{-/-}$ radiation chimeras elucidated that both IKKα and IKKβ have important and distinct roles in the development and function of lymphocytes. However, inconsistencies in the methods used to derive the hematopoietic chimeras may hamper the interpretation of such studies. Generation of radiation chimeras depend on repetitive injection of IKK$^{-/-}$ cells into new irradiated hosts, lack of consistency would make it hard to compare results from time to time; There may be different host responses to irradiation, the irradiation may not only kill the hematopoietic cells but also change the physiological condition of the remaining cells. In addition, defects in stromal cells derived from IKK$^{-/-}$ cells can be responsible for the effects on lymphocyte development. In fact, it is reported that the levels of circulating TNF-α in irradiated hosts are considerably higher than those detected in untreated normal mice. In addition, the level of serum TNF-α and the level of TNF-α mRNA in the livers of IKKβ$^{-/-}$

radiation chimeras are both elevated in comparison to the wild type radiation chimeras (138). Variable TNF-α levels can cause differential amount of apoptosis of developing lymphocytes, thus causing discrepancies in reconstitution. Therefore, there is significant limitations in the results from $IKK^{-/-}$ radiation chimeras used to study the role of IKK in the development and function of lymphocytes.

Several questions remain to be addressed about the role of IKKα in the development and function of lymphocytes. First, $IKK\alpha^{-/-}$ cells were able to give rise to a normal T cell compartment but not B cell compartment in irradiated hosts (139), suggesting that IKKα is not required for T cell development. However, the question remains whether IKKα is important for T cell function. Second, $IKK\alpha^{-/-}$ chimeras exhibited decreased basal serum immunoglobulin levels and impaired antigen-specific immune responses (140). It is not clear whether these defects are attributed to IKKα deficiency in B cells or T cells. Third, B cells lacking IKKα exhibited increased apoptosis (139, 140). How IKKα regulates B cell survival and whether IKKα has a role in regulating the survival of T cells remain to be determined.

The role of IKKβ in the development and function of lymphocytes has not been addressed due to the embryonic lethality of the $IKK\beta^{-/-}$ mice (103, 129, 130) and inability of $IKK\beta^{-/-}$ fetal liver cells to reconstitute lymphocytes in irradiated hosts (138). Although previous results suggested that IKKβ is not required for lymphocyte development but the mitogenic response of either B or T cells, the role of IKKβ in regulating cytokine production and T cell mediated immunity has not been addressed. In addition, it is not clear whether

IKKβ deficiency alters either B cell activation or humoral response including basal Ig production and the response to TD antigens.

In an attempt to answer these questions, transgenic mice were generated to inhibit the function of IKKα or IKKβ specifically in B or T lymphocytes. Phenotypic studies of these mice would help to reveal the role of IKKα or IKKβ in the development and function of B or T cells with intact development and function of the other lineage cells. Transgenic mice in which the function of both IKKα and IKKβ were inhibited in T cells were also generated. Comparison of the phenotype of these mice would elucidate whether IKKα and IKKβ regulate the development and function of T cells in a redundant, synergistic or antagonistic way.

CHAPTER II. GENERATION AND PHENOTYPIC STUDIES OF TRANSGENIC MICE EXPRESSING DNIKKβ IN THE B CELLS

A. Introduction

NF-κB comprises a family of transcription factors that play a critical role in the control of immune and inflammatory responses (14, 19). Members of the NF-κB family including p50, p52, p65, RelB and c-Rel are present predominantly in the cytoplasm where they are bound to a group of inhibitory proteins known as IκB (10, 14, 19, 20). In response to a variety of stimuli, including the cytokines TNFα and IL-1, the IκB proteins are phosphorylated leading to their ubiquitination and degradation by the 26S proteasome (10). This process results in the nuclear translocation of NF-κB and the binding of these proteins to promoter elements in a variety of genes and activation of their expression.

One of the major steps involved in the control of the NF-κB pathway is the phosphorylation of the IκB proteins by the IκB kinases (60, 77, 78, 90, 93, 97). The IκB kinase complex consists of three proteins IKKα (77, 78, 90), IKKβ (78, 90, 97) and IKKγ/NEMO (94-96, 283). IKKα and IKKβ are kinases that are each capable of phosphorylating IκB (77, 78, 90, 93, 97) while IKKγ/NEMO is a scaffold protein that is critical for IKKα and IKKβ kinase activity (94-96, 283). Treatment of B cells with a variety of agents, including cytokines and lipopolysaccharide in addition to the CD40 ligand on the surface of T cells, increases IKK kinase activity resulting in the phosphorylation and subsequent degradation of IκB and NF-κB nuclear translocation (60).

IKKα and IKKβ have a high degree of amino acid homology and a similar domain organization that includes an N-terminal kinase domain, a leucine zipper that facilitates their heterodimerization and homodimerization and a C-terminal helix-loop-helix domain (77, 78, 90, 93, 97, 107). However, IKKα and IKKβ appear to have different functions. Homozygous disruption of the IKKβ gene results in marked decreases in NF-κB activation and embryonic lethality in mice (103, 129, 130). These mice die of severe apoptosis of the liver due to their failure to activate NF-κB responsive genes that help to prevent apoptosis. In contrast, IKKα disruption plays only an auxiliary role on activation of the NF-κB pathway. Mice carrying homozygous deletions of this gene die shortly after birth due to severe skin and skeletal abnormalities (124, 125, 284). The ability of IKKα to regulate epidermal proliferation suggests that this kinase can likely activate signal transduction pathways other than those involved in activating NF-κB (128). Finally, homozygous disruption of IKKγ/NEMO leads to embryonic lethality due to hepatic apoptosis much like that seen in the IKKβ deficient mice (134, 135, 278). These results indicate that the IKK function is critical for a variety of biologic processes.

The NF-κB proteins are critical for the regulation of immune function (14, 19, 60). For example, they regulate the expression of a variety of genes encoding cytokines and cytokine receptors, chemokines, cell adhesion molecules and cell surface receptors that are critical for T and B lymphocyte function (5). Target inactivation of genes encoding individual NF-κB subunits demonstrates the importance of these proteins in regulating the immune system (245). Gene disruption of single NF-κB subunits in mice including p105/p50

(252, 257), p100/p52 (248, 249), c-Rel (251), RelA (250), and RelB (253) lead to reduced B and T cell proliferation and immune defects but no major defects in the maturation of T and B cells. However, mice lacking multiple NF-κB subunits including p105/p50 and p100/p52 (259), p105/p50 and RelB (260, 285), RelA and c-Rel (286), and p105/p50 and RelA (132) have more severe defects in B and T cell development than do mice with mutations of single NF-κB subunits. These results indicate that the NF-κB pathway is critical for the function of both B and T lymphocytes.

Because IKKβ-deficient mice die *in utero*, the role of IKKβ on B cell development and function has not been addressed (103, 129, 130). Transgenic mice containing dominant negative mutants in NF-κB regulatory proteins such as IκB have previously been useful in defining the role of the NF-κB pathway in the immune system (245). To investigate the role of a dominant negative IKKβ mutant on regulating B cell development and differentiation in transgenic mice, we generated and characterized transgenic mice expressing this protein under the control of the IgH promoter and enhancer. In this study, we demonstrate that inhibiting IKKβ function does not affect B cell development but results in marked defects in cell cycle progression, B cell proliferation, and in the humoral response to both T-independent and T-dependent antigens.

B. Materials and Methods

Generation of transgenic mice expressing DNIKKβ mutant in B cells

An expression vector containing the B cell specific immunoglobulin heavy chain (IgH) promoter and enhancer has been described (287). The rat insulin intron A and an SV40

polyadenylation signal were also inserted into this vector. A human IKKβ cDNA containing substitutions of serine residues 177 and 181 with alanine and an amino-terminal Flag-epitope (78) was inserted into a NotI site between the intron and the polyA elements. The linearized transgene IgH/DNIKKβ was microinjected into the pronuclei of ICR (CD-1) strain in the Transgenic Core Facility at University of Texas Southwestern Medical Center and mice were maintained in a specific pathogen free (SPF) colony. The presence of the transgene was confirmed by PCR and Southern blot analysis.

Purification of B cells from mouse spleen

To purify B cells from the spleens of 8-12 week old mice, the MACS magnetic cell sorting system (Miltenyi Biotec) was utilized. The splenocytes were incubated with anti-CD43 microbeads and separated into CD43 positive (non-B cells) and CD43 negative (B cells) according to the manufacturer's instructions. Purified B cells were either stimulated with B cell mitogens or used to make whole cell extract. To purify small resting B cells from the spleen, T cells in the unfractionated splenocytes were depleted by cytotoxic elimination using anti-CD90 antibody and Low-Tox-M rabbit complement (Cedarlane) and the remaining cells were fractionated through Percoll gradients (288). Small resting B cells which sediments between 75% and 100% Percoll were collected and used in in vitro cultures.

Flow cytometry analysis

Splenocytes from either wild-type or transgenic littermates were placed in RPMI media, washed twice and resuspended in buffer containing PBS with 1%FBS. Approximately

$5x10^5$ cells were incubated with fluorescent antibodies: PE-αB220, FITC-αThy1.2, FITC-αIgM, FITC-αIgκ, FITC-αIgD, Biotin-αIgM, Biotin-αB220, FITC-αCD21, PE-αCD23, APC-Streptavidin, and PE-Streptavidin (Pharmingen). Fluorescence analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson).

Immunoprecipitation and Western blot analysis

To prepare whole cell extracts from either unfractionated splenocytes, purified B cells or non-B cells, the cells were lysed in TNE buffer (1% Triton X-100, 10mM Tris-HCl (pH8.0), 150mM NaCl, 1mM EDTA) containing a protease inhibitor cocktail (Roche). Cell lysates containing 10μg of protein were incubated with anti-Flag antibody (Sigma) followed by incubation with protein G sepharose beads (Sigma) and immunoblotting using an IKKβ rabbit polyclonal antibody (sc-7607) (Santa Cruz).

RT-PCR analysis of IKKβ mRNA isolated from B cells.

B cells were purified from the splenocytes of both transgenic and wild-type mice using the MACS magnetic sorting system. Total RNA was extracted from these cells and analyzed by semi-quantitative RT-PCR analysis. The oligonucleotide primers used to amplify a 341bp homologous fragment from both mouse IKKβ and human DNIKKβ included the sense primer: 5'-gtgtcagctgtatccttc-3' and the antisense primer: 5'-gctccacagcctgctcc-3' with the sense primer end-labeled with [γ-^{32}P]ATP. Oligonucleotide primers for amplifying GAPDH mRNA have been described previously (289). The PCR products were analyzed by digestion with EcoRI, which cuts the cDNA fragment amplified

from mice expressing human DNIKKβ to generate two fragments of 176bp and 165bp. Following gel electrophoresis and autoradiography, the intensity of the species was measured by PhosphorImager analysis (Cyclone, Packard) and compared with that of the 341bp mouse fragment.

Stimulation of primary B cells and electrophoretic mobility shift assays

Magnetically purified B cells were incubated with either RPMI alone or RPMI containing the $F(ab')_2$ fragment of anti-IgM (10μg/ml, Jackson ImmunoResearch), LPS (10μg/ml, Difco) or PMA (50ng/ml, Sigma) and ionomycin (200ng/ml, Sigma) for 45 minutes to 2 hours, respectively. The cytoplasmic and nuclear extracts of the non-stimulated and stimulated B cells were prepared and analyzed according to published methods (290, 291).

To detect NF-κB binding, a ^{32}P-labeled oligonucleotide probe containing the class I MHC κB site (292) or NF-Y binding site was incubated with the nuclear extracts. The binding reaction contained 60,000 cpm of the radiolabeled probe, 4 to 5 μg of nuclear extract, 500ng of poly(dI-dC) (Pharmacia), 10 μg BSA, 20mM HEPES (pH7.9), 1mM EDTA, 1% NP-40, 5% glycerol, 5mM DTT in a final volume of 20 μl. Reactions were incubated at room temperature for 30 minutes and subjected to electrophoresis on a 5% native gel in 0.5x TBE buffer. For super-shift assays, 5μg of goat polyclonal antibody directed against p65 or normal goat sera was added to the binding reactions and incubated for 30 minutes on ice before the samples were subjected to gel electrophoresis. The gels were dried and exposed to X-ray film and quantified by PhosphorImager analysis.

In vitro proliferation assay and immunoglobulin production of primary B cells

Small resting B cells were prepared from the spleens of two transgenic mice and two wild-type littermates. The cells were pooled and resuspended in RPMI and plated in 96-well plates in quadruplicate at 10^5 cells/well for each condition. LPS (10μg/ml), anti-IgM F(ab')$_2$ (10μg/ml), anti-CD40 (5μg/ml), IL-4 (100U/ml), IL-5 (0.1%) (293), and IFNγ (10ng/ml) (R&D systems) were added to the cells in each well, cultured for 3 days, and pulsed with [^{3}H]-thymidine (1mCi/well) overnight. The [^{3}H]-thymidine incorporated was quantified by scintillation counting and the secreted Ig in the culture supernatant in day 6 cultures was measured by ELISA using class specific antisera.

Cell cycle analysis

Cell cycle analysis of B cells was performed using a BrdU Flow Kit (Pharmingen). Resting B cells were cultured in RPMI media supplemented with 10% FBS in the presence of LPS (10μg/ml), anti-IgM F(ab')$_2$ (10μg/ml), or anti-CD40 (5μg/ml) for 60 hours. Cells were then pulsed with BrdU for 40 minutes and processed for BrdU and 7-AAD staining according to the manufacturer's instructions. Flow cytometry analysis was performed using a FACSCalibur (Becton Dickinson).

Semi-quantitative RT-PCR analysis

To compare the relative mRNA levels of μM, μS, γ3 and γ2a in stimulated B cells from mutant and normal mice, semi-quantitative RT-PCR analysis was performed as described (294). 1x10^6 resting B cells were stimulated with either LPS (10μg/ml) alone or

with both LPS and INF-γ (10ng/ml) for a period of 3-4 days. Total RNA was prepared from the cells using an RNeasy Kit (Qiagen). Equal portions of total RNA from each sample were reverse transcribed and titrations were performed so that PCR products corresponding to the μM, μS, γ3 and γ2a transcripts were within linear range. These same dilutions of the cDNA samples were used as templates in the RT-PCR reactions. For μM the sense primer was: 5'-ggtatgcaaaatccactacggaggc-3' and the antisense primer was: 5'-gataaaagctggagggcaac-3'; for μS the same sense primer was used and the antisense primer specific to the μS exon: 5'-gacatgatcagggagacattgtac-3' was used; for γ2a and γ3 a sense primer with the sequence 5'-tatggactactggggtcaag-3' was used while the antisense primer for γ 2a was: 5'-ggccaggtgctcgaggtt-3' and the antisense primer for γ3 was 5'-aatagaacccagactgcagga-3'. One of the primers was end-labeled with [γ^{32}-P] ATP and the PCR products were subjected to electrophoresis on a 1% agarose gel and quantified by PhosphorImager analysis.

In vivo response to T-independent and T-dependent antigens

Immunization of mice with T-independent and T-dependent antigens was performed as previously described (295). Briefly, littermates of 9-11 week old mice were injected intraperitoneally with the type 2 T-independent antigen TNP-Ficoll (Solid Phase Sciences) at 40 μg/mouse or the type 1 T-independent antigen TNP-LPS (Sigma) at 20μg/mouse in 100 μl PBS. Sera were collected from the tail vein at day 0 (before injection) and day 14. TNP-specific IgM, IgG3, and IgG2a were measured by ELISA. To determine the antibody response to the T-dependent antigen, TNP-KLH (295), 100μg of this antigen was dissolved

in 100µl PBS with RIBI adjuvant (Corixa) and was injected intraperitoneally into each littermate. Sera were collected at day 0 (before injection) and 14 and TNP-specific IgM, IgG1, and IgG2a were measured by ELISA.

ELISA

ELISA was performed as described previously (295). To determine the concentrations of total IgM, IgG3, IgG2a and IgG1 in the culture supernatant or the sera of naive mice, 96-well MicroTest III flexible plates (Becton Dickinson) were coated with AffiniPure goat anti-mouse IgG and IgM antibodies (Jackson ImmunoResearch). TNP-BSA (295) was used to detect TNP-specific immunoglobulin in the sera of immunized mcie. Immunoglobulin levels in the mouse sera were determined at 3 dilutions of each serum in duplicate. The baseline level of TNP-specific Ig in the sera from mice prior to immunization was subtracted from the levels in the TNP-immunized mice in order to determine TNP-specific responses. Isotype standards (IgM, IgG1, IgG3 and IgG2a), HRP conjugated goat anti-mouse IgM, IgG3, IgG2a, IgG1 and Ig(H+L) antibodies and the substrate ABTS were obtained from Southern Biotechnology Associates and the plates were read on a Universal Microplate Reader (Bio-Tek Instruments) at 405nm.

C. Results

DNIKKβ protein is expressed in B cells of IgH-DNIKKβ transgenic mice

In an attempt to inhibit inducible NF-κB activity specifically in B lymphocytes, we generated transgenic mice that expressed a dominant negative form of human IKKβ

(A176/181) (78) that was inserted between the B cell specific immunoglobulin heavy (IgH) promoter and enhancer (287, 296) (Figure 6A). The IKKβ cDNA contained an amino terminal Flag-epitope to facilitate its detection in murine B cells. Previous studies have demonstrated that an IKKβ protein in which serine residues 177 and 181 in the MAP3 kinase activation loop were substituted with alanine have a dominant negative phenotype which inhibits NF-κB activation in response to treatment with proinflammatory cytokines such as TNFα and IL-1 (78, 90, 97, 119, 297). Since mouse and human IKKβ have greater than 90% amino acid identity, we assumed that this dominant negative IKKβ mutant should be able to inhibit the function of endogenous mouse IKKβ and thus alter NF-κB activation in mouse B lymphocytes.

The Flag-tagged IKKβ dominant negative construct shown in Figure 6A was microinjected into pronuclei of the murine CD-1 strain. Southern blot analysis indicated that three founders designated A, B, and C had an integrated transgene although only founder C transmitted the transgene (Figure 6B). Next we addressed whether the dominant negative IKKβ protein was expressed in splenocytes isolated from transgenic mice derived from founder C. These cells were either unfractionated or fractionated into either B lymphocytes or non-B cells which contained predominantly T cells, macrophages and myeloid cells. Western blot analysis performed with a monoclonal antibody directed against the Flag-epitope indicated that there was more DNIKKβ protein present in B lymphocytes from the transgenic mice (Figure 6C, lane 3) than in the same number of cells from unfractionated splenocytes (Figure 6C, lane 2). There was no detectable DNIKKβ expression in non-B cells isolated from this transgenic mouse (Figure 6C, lane 4).

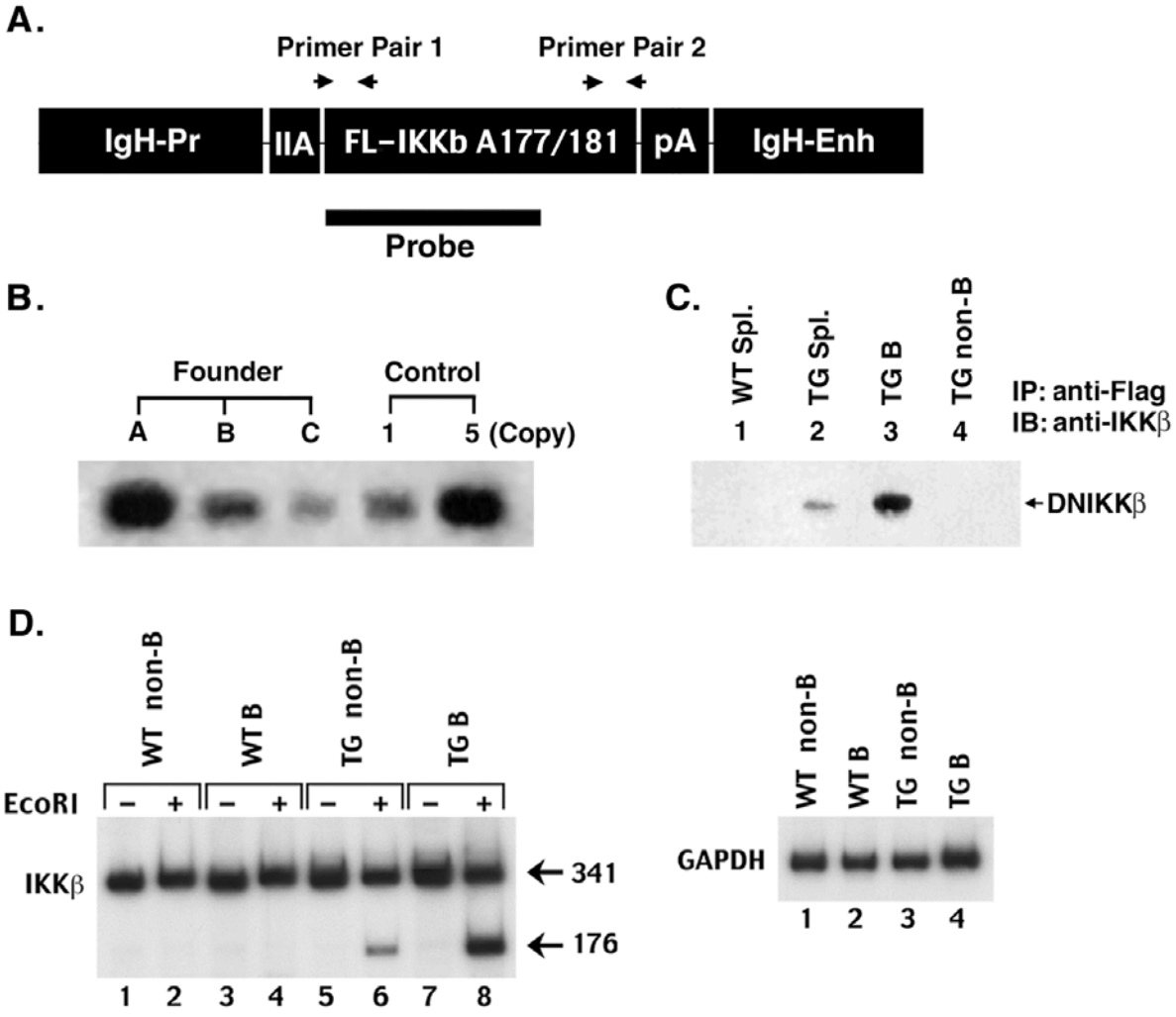

A.
Primer Pair 1
Primer Pair 2
IgH-Pr
IIA
FL-IKKb A177/181
pA
IgH-Enh
Probe
B.
Founder
Control
A
B
C
1
5 (Copy)
C.
WT Spl.
TG Spl.
TG B
TG non-B
1
2
3
4
IP: anti-Flag
IB: anti-IKKβ
←DNIKKβ
D.
WT non-B
WT B
TG non-B
TG B
EcoRI
– + – + – + – +
IKKβ
← 341
← 176
1 2 3 4 5 6 7 8
GAPDH
WT non-B
WT B
TG non-B
TG B
1 2 3 4

Figure 6. Generation of transgenic mice expressing a dominant negative IKKβ protein in B lymphocytes. (A) A cDNA encoding a Flag-tagged dominant negative form of human IKKβ (FL-IKKβA176/181) in which serine residues 177 and 181 were changed to alanine was inserted between the B cell specific promoter region (IgH-Pr) and enhancer region (IgH-Enh). A rat insulin intron A (IIA) and an SV40 polyadenylation signal (pA) were inserted at the 5' and 3' ends of the DNIKKβ cDNA, respectively. Two pairs of PCR primers that hybridized to the 5' and 3' portions of the FL-IKKβ A177/181 cDNA were used for genotyping. (B) Southern blot analysis of tail DNA from three founders (A, B, C) and a control plasmid DNA was performed using a ^{32}P-labeled 1.4kb probe which hybridizes to the DNIKKβ cDNA as indicated in part A. Control DNA whose intensity corresponded to that of 1 and 5 copies of the IKKβ trangene in the mouse genome is shown. The copy numbers of founders A, B and C were estimated as 6, 2 and 1, respectively. (C) The cell lysates of unfractionated splenocytes from wild-type (lane 1) and transgenic mice (lane 2), or purified splenic B cells (lane 3) and non-B cells (lane 4) from the transgenic mice were immunoprecipitated with the M2 monoclonal antibody directed against the Flag epitope. The immunoprecipitates were then subjected to SDS PAGE and immunoblotted with anti-IKKβ antibody. (D) Splenocytes were separated into B cells and non-B cells by magnetic sorting. The mRNA level of IKKβ in B cells (purity>95%, as indicated by flow cytometry analysis of B220^{+} cells) and non-B splenocytes in the wild type and transgenic mice was analyzed by RT-PCR. Identical oligonucleotide primers with one of them end-labeled with ^{32}P were used to amplify a 341bp fragment (upper arrow) of both DNIKKβ (human) and endogenous IKKβ (mouse). The PCR products which were either digested with EcoRI (lanes 2, 4, 6, 8) or non-digested (lanes 1, 3, 5, 7) were subjected to electrophoresis on a 5% native gel. The intensity of the 176bp fragment (lower arrow, lanes 6 and 8) generated from EcoRI digestion of the human fragment in lane 8 was about 1.5-2 fold of that of the 341bp fragment as measured by PhosphorImager analysis. RT-PCR of GAPDH (lower panel) indicated that equivalent amount of template cDNA was used in the PCR reactions.

In an attempt to compare the expression level of DNIKKβ with that of the endogenous IKKβ in the B cells of the transgenic mice, we used RT-PCR analysis to assay the levels of IKKβ mRNA (Figure 6D). We chose to amplify a 341bp fragment, which includes sequences encoding the leucine zipper motif of IKKβ and contains an EcoRI site in the human but not the mouse sequence. The 176bp fragment generated by EcoRI digestion of the ^{32}P-labeled PCR product represents the mRNA level of DNIKKβ expressed in transgenic B cells. Thus we could compare the relative levels of the expression of DNIKKβ and the endogenous murine IKKβ gene. PhosphorImager analysis demonstrated that there was 1.5-2 fold more of the fragment from the human DNIKKβ than from the endogenous murine IKKβ (Figure 6D, lane 8). Residual DNIKKβ mRNA was also detected in the splenocytes of the transgenic mice in which the majority of B cells have been removed and this is most likely due to residual B cell contamination in this fraction of cells (Figure 6D, lane 6). The DNIKKβ transgenic mice were housed in a specific pathogen free colony and used in the phenotypic studies described below.

Reduced NF-κB DNA binding in B lymphocytes of DNIKKβ transgenic mice

Next we addressed whether DNIKKβ expression altered NF-κB activation in response to various agents that are known to stimulate this pathway. Previous studies have demonstrated that lipopolysaccharide (LPS), anti-IgM, and PMA and ionomycin potently stimulate NF-κB DNA binding properties in B lymphocytes (298-300). To analyze the effects of DNIKKβ expression on NF-κB DNA binding, we performed electrophoretic

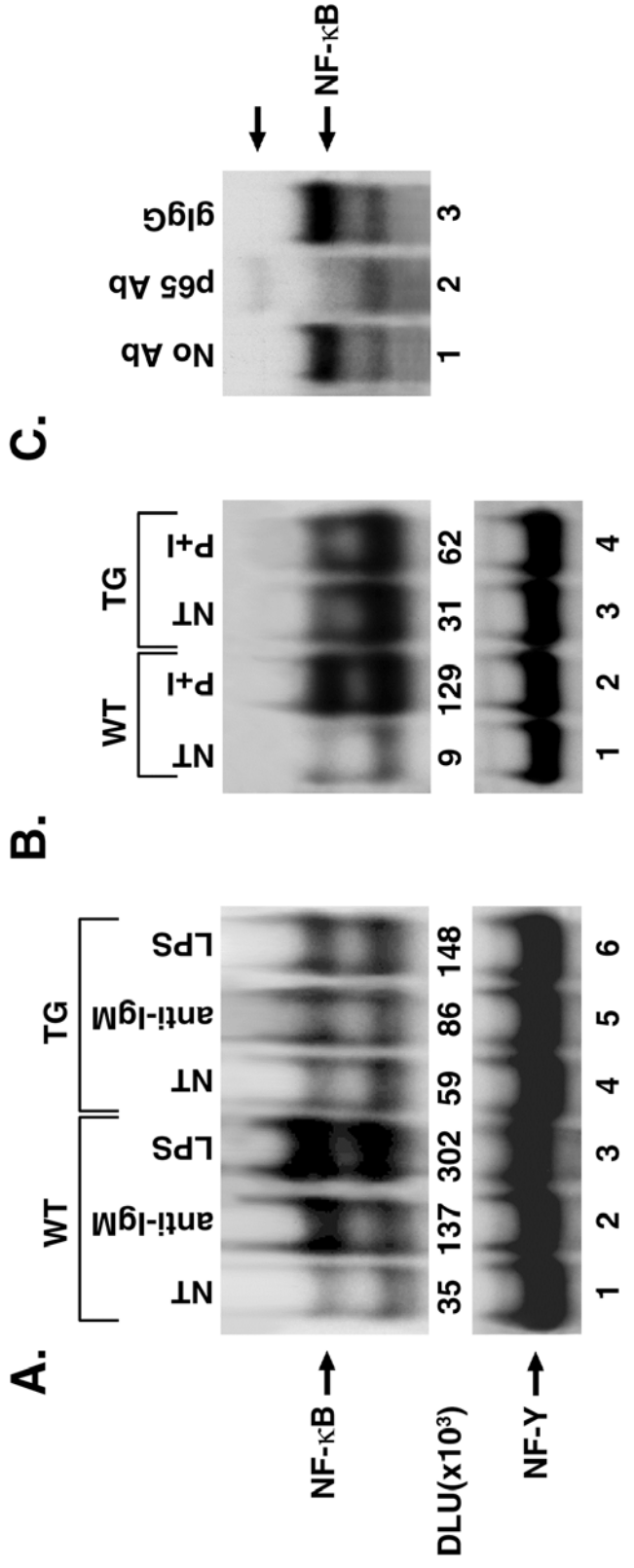

A.
WT
TG
NT
anti-IgM
LPS
NT
anti-IgM
LPS
NF-κB
35 137 302 59 86 148
1 2 3 4 5 6
DLU(x10³)
NF-Y
1 2 3 4
B.
WT
TG
NT
P+I
NT
P+I
NF-κB
9 129 31 62
C.
No Ab
p65 Ab
IgG
1 2 3
NF-κB

Figure 7. NF-κB binding activity is reduced in the B cells of IgH/DNIKKβ transgenic mice. (A) Magnetically purified B cells from the spleens of transgenic mice and their wild-type littermates were either not treated (lanes 1 and 4) or stimulated with either anti-IgM (10μg/ml) (lanes 2 and 5) or LPS (10μg/ml) (lanes 3 and 6) as described in the Materials and Methods. Nuclear extracts were prepared from treated and untreated cells and subjected to EMSA using a radiolabeled NF-κB probe (top panel) or an NF-Y probe (bottom panel) as a control. (B) Purified B cells were either not treated (lanes 1 and 3) or stimulated with PMA (50ng/ml) and ionomycin (200ng/ml) (lanes 2 and 4) and DNA binding activity was analyzed by EMSA. PhosphorImager quantification of NF-κB binding is indicated in the bottom of each lane. (C) Nuclear extract prepared from anti-IgM treated wild-type B cells was subjected to a super-shift assay with no added antibody (lane 1) or either 5 μg of either anti-p65 antibody (lane 2) or normal goat IgG (lane 3).

mobility shift assays (EMSA) using nuclear extracts prepared from both non-stimulated and stimulated B lymphocytes isolated from wild-type and DNIKKβ transgenic mice. In B cells isolated from wild-type mice, treatment with either the $F(ab')_2$ fragment of anti-IgM, LPS, or PMA and ionomycin strongly induced NF-κB DNA binding activity (Figures 7A and B). In contrast, there was significantly less NF-κB DNA binding in B cells isolated from the transgenic mice (Figures 7A and B). There was little difference in the DNA binding properties of nuclear extracts prepared from B lymphocytes isolated from the wild-type and transgenic mice using the control NF-Y probe (Figures 7A and B). The p65 antibody, but not the normal goat IgG, resulted in a supershift of the NF-κB DNA binding protein complex indicating the presence of p65 in this complex (Figure 7C). These results indicated that NF-κB activation was significantly inhibited in B lymphocytes isolated from the DNIKKβ transgenic mice.

Normal B cell development in DNIKKβ transgenic mice.

Since the dominant negative IKKβ inhibited NF-κB activation in the B cells of the transgenic mice, we asked whether the expression of DNIKKβ altered B cell development in the transgenic mice. The DNIKKβ transgenic mice have spleens of normal size and normal architecture as demonstrated by hematoxylin and eosin staining (data not shown). To confirm that B lymphocyte development in these mice was normal, the surface markers on the splenocytes isolated from these mice were analyzed by flow cytometry. A representative analysis is shown in Figure 8. The DNIKKβ transgenic mice have similar percentages of B and T cells as compared with wild-type mice as reflected by similar B220 and Thy1.2 surface

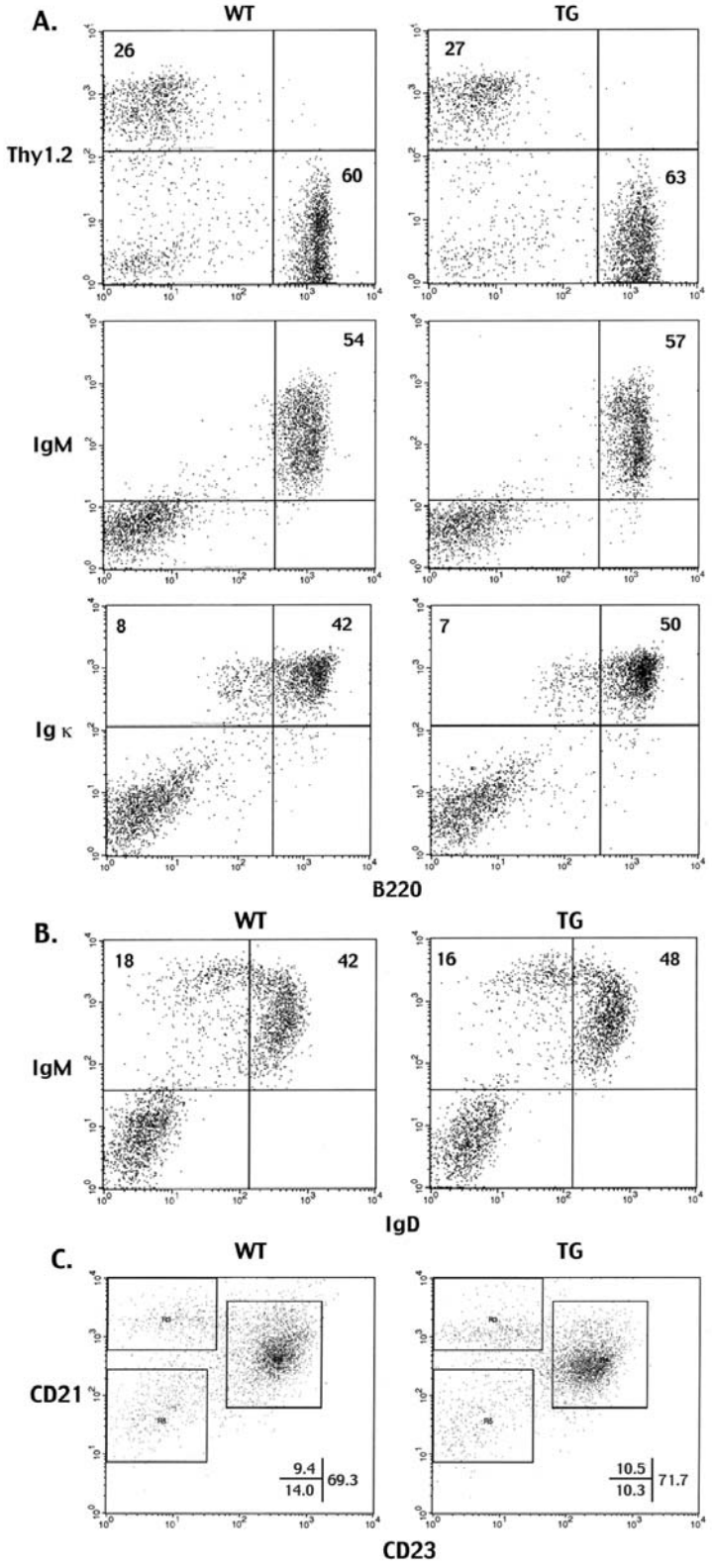

A.
WT
TG
26
27
Thy1.2
60
63
54
57
IgM
8
42
7
50
Ig κ
B220
B.
WT
TG
18
42
16
48
IgM
IgD
C.
WT
TG
CD21
9.4
14.0
69.3
10.5
10.3
71.7
CD23

Figure 8. Normal B cell repertoire in IgH/DNIKKβ transgenic mice. Splenocytes from wild-type (WT) and transgenic (TG) mice were stained with labeled antibodies and analyzed by flow cytometry. The percentage of cells in the relevant quadrants or regions are shown. Surface expression of either (A) Thy 1.2, IgM, and Igκ (Y axis) and B220 (X axis) or (B) IgM and IgD was analyzed. (C) Equivalent numbers of splenocytes were obtained from IgH/DNIKKβ transgenic mice and control littermates and stained with biotinylated αB220, FITC-conjugated αCD21, PE-conjugated αCD23, followed by APC-conjugated streptavidin. The B220 positive population was gated and analyzed for surface expression of CD21 and CD23. The percentage of marginal zone B cells ($CD21^{high}CD23^{low}$), follicular B cells ($CD21^{+}CD23^{+}$) and newly formed B cells ($CD21^{low}CD23^{low}$) are shown at the lower right corner of each panel in their corresponding positions.

expression (Figure 8A). The percentage of B cells expressing surface IgM and Igκ was also similar in the transgenic and wild-type mice (Figure 8A). Since surface IgD expression reflects the state of maturation and activation of B cells, we also compared the surface expression of IgM and IgD (Figure 8B). There were comparable percentages of IgD^{high}/IgM^{high} (upper right) and IgD^{low}/IgM^{high} (upper left) populations of cells in transgenic and wild-type mice indicating that the dominant negative IKKβ protein does not significantly alter the maturation and activation of B cells.

A recent study of p50 deficient mice indicated that they had impaired development of marginal zone B cells and that the loss of these cells may in part be responsible for defective B cell proliferation in response to LPS and the compromised humoral response to the type 2 T-independent antigen TNP-Ficoll (301, 302). We asked whether the inhibition of endogenous IKKβ function in B cells from the transgenic mice affected the accumulation of this subset of B cells. As shown in Figure 8C, the B cell population defined by staining with CD21 and CD23 ($B220^{+}CD21^{high}CD23^{low}$) in our transgenic mice was similar to that of wild-type mice, indicating that inhibition of the NF-κB pathway in B cells does not affect the development of marginal zone B cells. In summary, our results are consistent with previous studies of p50, c-rel, and RelA-deficient mice demonstrating that inhibition of NF-κB function does not cause marked defects in B lymphocyte development (250-252).

B cells from DNIKKβ mice exhibit proliferative defects

Although B cell development is normal in the DNIKKβ transgenic mice, previous data demonstrating that NF-κB is involved in regulating B cell function prompted us to analyze

the B cell proliferative responses in these mice. Resting B cells isolated from the splenocytes of wild-type and transgenic mice were stimulated over a 72-hour period with either LPS, anti-CD40 or anti-IgM and their proliferation was monitored by [^{3}H]-thymidine incorporation. A representative experiment is shown in Figure 9. Stimulation of transgenic B cells with LPS resulted in only ~30% of the proliferation seen in the wild-type B cells (Figure 9A). In contrast, B cell proliferation induced by either anti-CD40 or anti-IgM was less significantly compromised in the DNIKKβ transgenic mice, with a [^{3}H]-thymidine incorporation of 70% and 55% of normal levels, respectively (Figure 9B). Furthermore, the proliferation defects seen with anti-CD40 and anti-IgM were partially compensated by the addition of IL-4, which further enhances B cell proliferation (Figure 9B). These results indicate that IKKβ is involved in the B cell proliferative responses induced by LPS as well as anti-CD40 and anti-IgM.

Impaired cell cycle progression in B cells from DNIKKβ mice

It was important to address whether the decreased [^{3}H]-thymidine incorporation in LPS, anti-IgM and anti-CD40 treated B lymphocytes isolated from the transgenic mice was due to either defective cell cycle progression or increased cell death. B cells were isolated from transgenic and wild-type splenocytes and cultured with either LPS, anti-IgM or anti-CD40 for 60 hours and pulsed with bromodeoxyuridine (BrdU) for 40 minutes. Flow cytometry analysis was then performed to determine the amount of BrdU incorporation into newly synthesized DNA and 7-AAD was used in order to detect total cellular DNA content. Wild-type mice exhibited a marked increase in the number of cells present in the S phase in

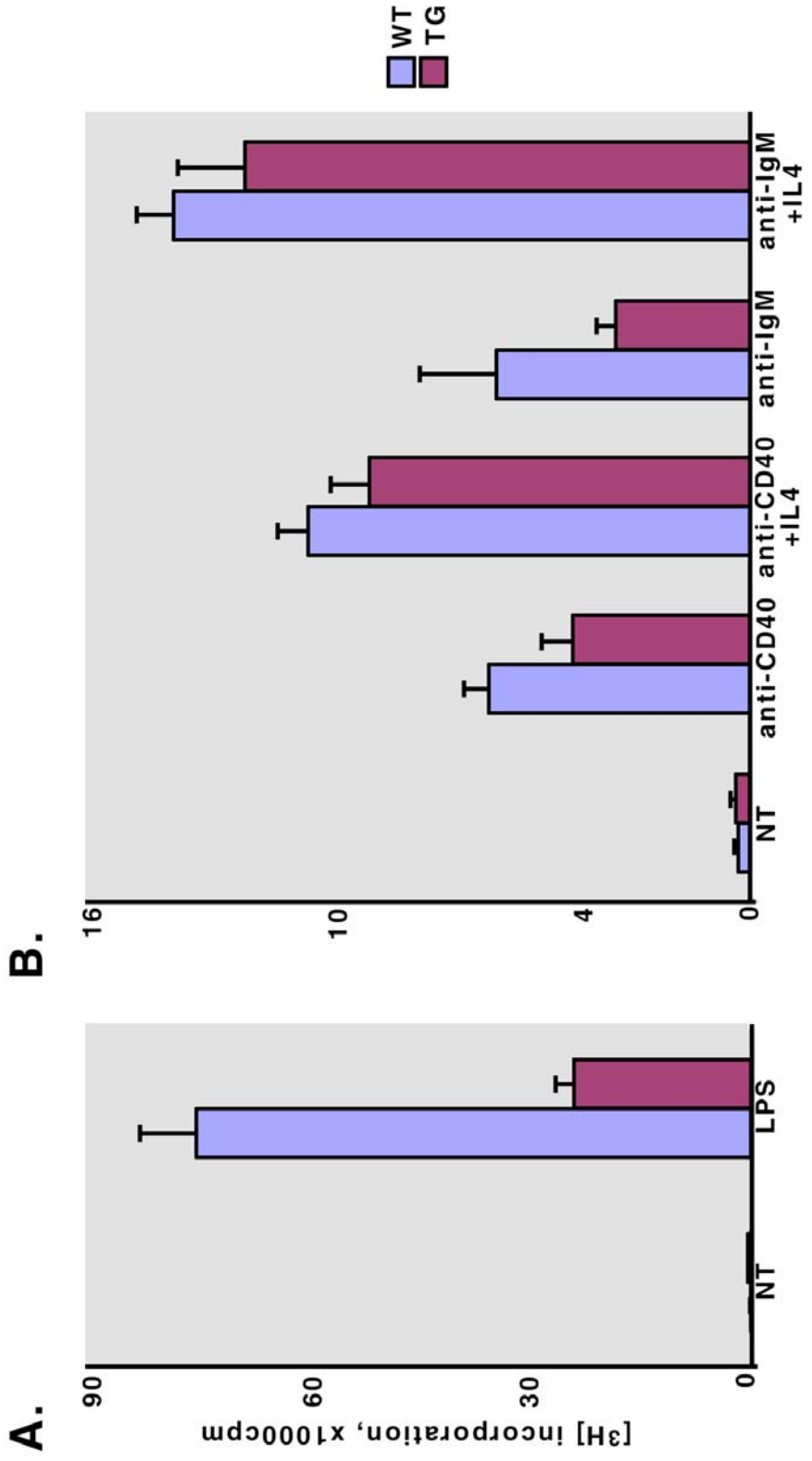

A.
[3H] incorporation, x1000cpm
90
60
30
0
NT
LPS
B.
16
10
4
0
WT
TG
NT
anti-CD40
anti-CD40 +IL4
anti-IgM
anti-IgM +IL4

Figure 9. B cells from IgH/DNIKKβ transgenic mice exhibit proliferative defects *in vitro*. Small resting B cells isolated from transgenic mice and their littermates were incubated with RPMI only or in the presence of either (A) LPS (10μg/ml) or (B) anti-CD40 (5μg/ml), anti-CD40/IL4 (100U/ml), anti-IgM (5μg/ml) or anti-IgM/IL-4 (100U/ml) for 3 days and pulsed with [^{3}H]-thymidine (1μCi/well) overnight. The mean of the incorporated [^{3}H]-thymidine with standard deviation in four wells is shown and the results are representative of three independent experiments.

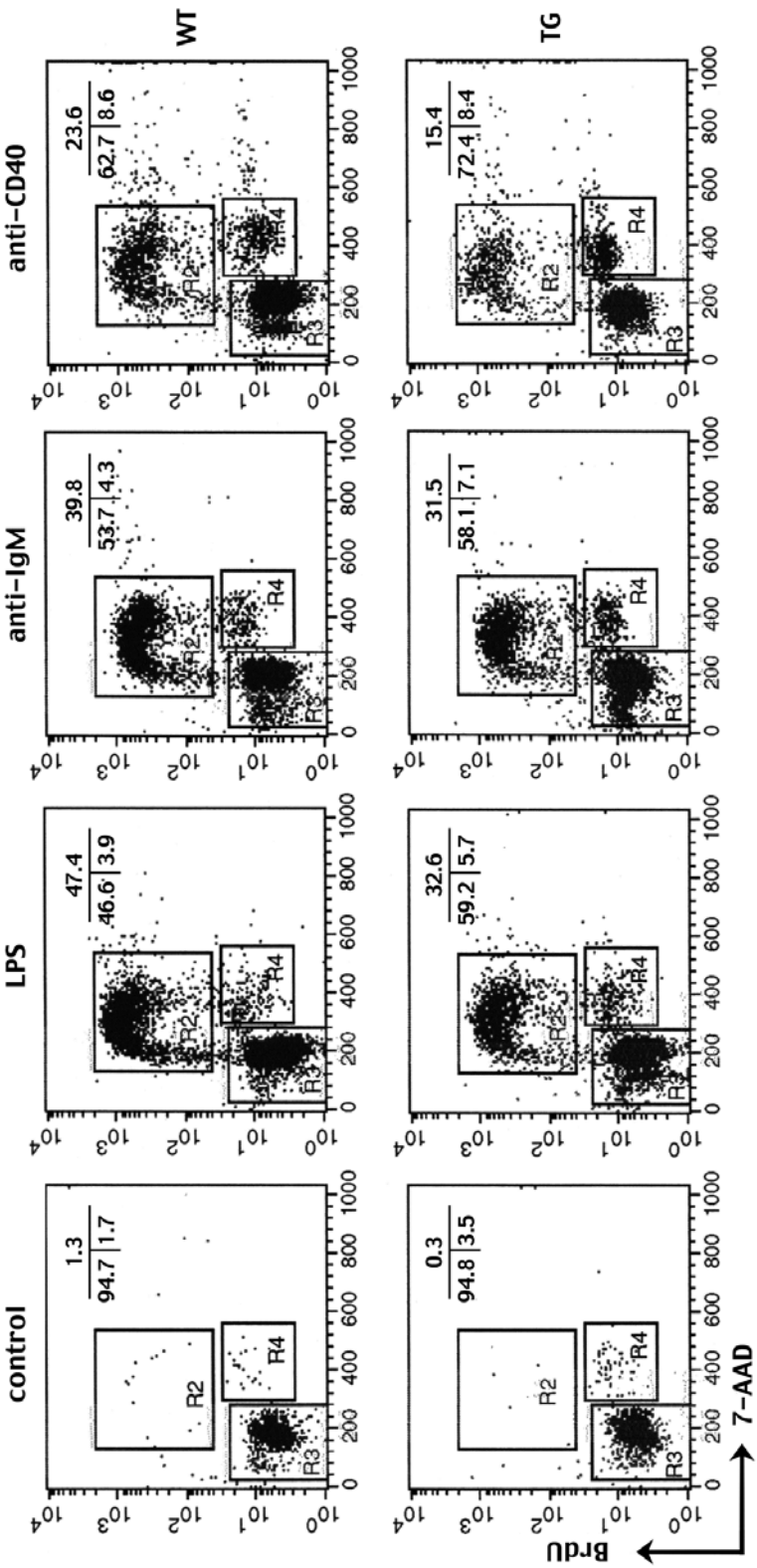

control
LPS
anti-IgM
anti-CD40
WT
TG
1.3
94.7 | 1.7
47.4
46.6 | 3.9
39.8
53.7 | 4.3
23.6
62.7 | 8.6
0.3
94.8 | 3.5
32.6
59.2 | 5.7
31.5
58.1 | 7.1
15.4
72.4 | 8.4
R2
R3
R4
BrdU
7-AAD

Figure 10. Impaired cell cycle progression in B cells from IgH/DNIKKβ transgenic mice. The cell cycle profile of B cells that were stimulated for 60 hours in culture either with or without LPS, anti-IgM or anti-CD40 was obtained using a BrdU Flow kit. Cultured cells were pulsed with BrdU for 40 minutes and stained with anti-BrdU to identify cells synthesizing DNA and 7-AAD to stain for the total DNA content in the cells. The percentage of cells in the S phase (R2), G0/G1 phase (R3) and G2/M phase (R4) are shown in the upper right corner of each panel in the corresponding positions.

response to treatment with either LPS, anti-IgM or anti-CD40 (Figure 10). The percentage of cells in the S phase is shown on the top line within each panel and represents the cells shown in the R2 grid (Figure 10). In contrast, there was a reduction in the number of transgenic B cells in the S phase following treatment with these agents (Figure 10). This decrease in the percentage of B cells in the transgenic mice was associated with a corresponding increase in the percentage of B lymphocytes in the G0/G1 phase of the cell cycle as compared to wild-type mice. These results are consistent with the [^{3}H]-thymidine incorporation assays which indicate that B cell proliferation induced by LPS is more dependent on NF-κB than that induced by anti-IgM and anti-CD40.

Both quiescent and activated B cells lacking the p50 NF-κB subunit have a higher rate of apoptosis (303, 304). Annexin V staining of untreated or LPS, anti-IgM or anti-CD40 treated B cells indicated that there were similar numbers of apoptotic B cells isolated from wild-type and transgenic mice (data not shown). These results indicated that the decreased proliferation of transgenic B cells in response to a variety of different mitogens is not due to an increased amount of apoptosis.

Reduced Ig secretion in vitro in B cells from DNIKKβ mice

Next, we determined whether inhibition of IKKβ activity altered *in vitro* immunoglobulin secretion and class switching of B cells. Resting B cells isolated from wild-type or DNIKKβ transgenic mice splenocytes were cultured in the presence of either LPS, LPS and IFNγ or anti-CD40, IL-4 and IL-5 over a 6-7 day period. IgM (Figure 11A), IgG2a and IgG1 (Figure 11B) and IgG3 (Figure 11C) levels in the culture supernatants were

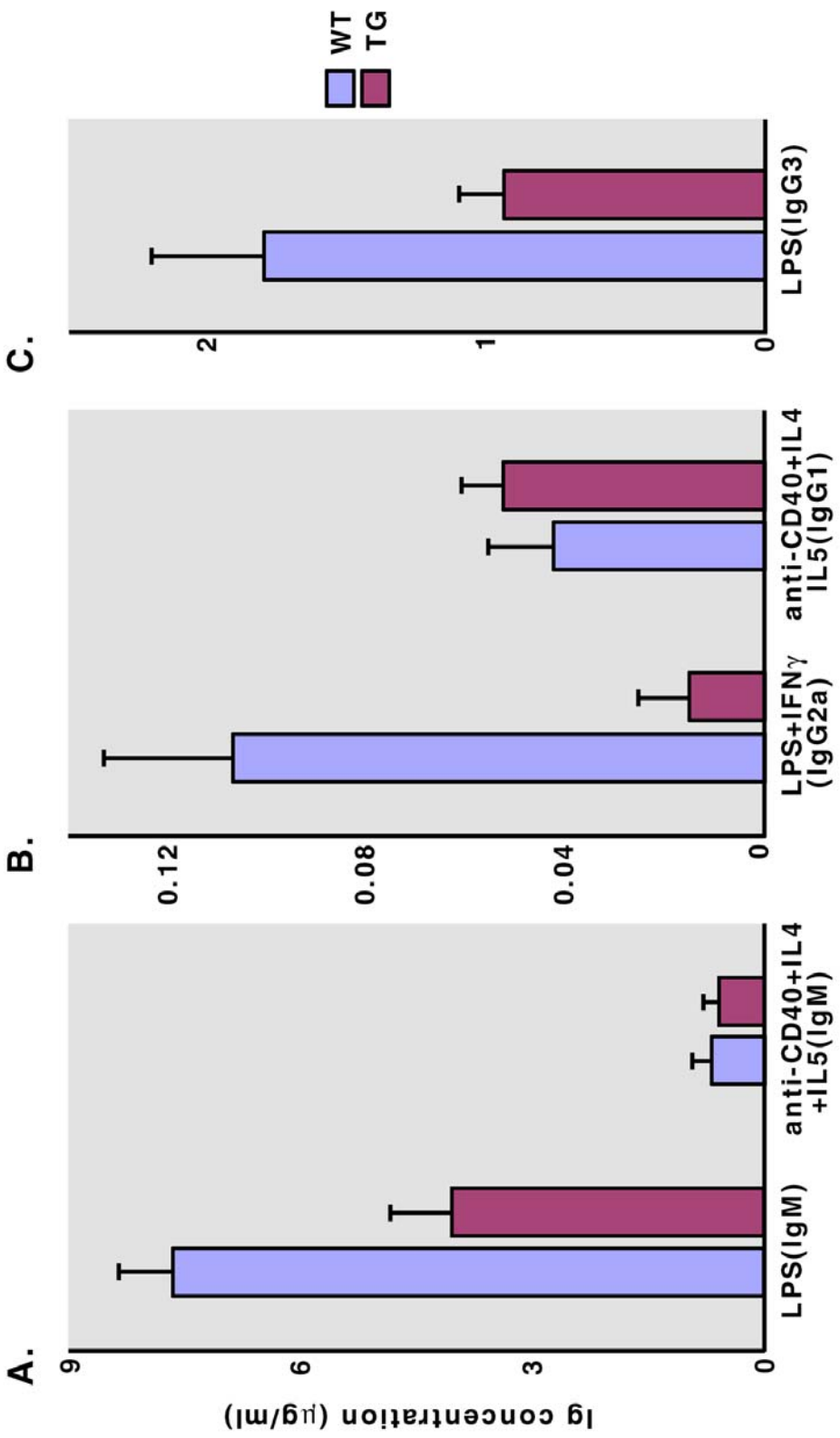

A.
B.
C.
WT
TG
Ig concentration (μg/ml)
0
3
6
9
0
0.04
0.08
0.12
0
1
2
LPS(IgM)
anti-CD40+IL4
+IL5(IgM)
LPS+IFNγ
(IgG2a)
anti-CD40+IL4
IL5(IgG1)
LPS(IgG3)

Figure 11. Defects in immunoglobulin production in B cells from IgH/DNIKKβ transgenic mice. Small resting B cells from transgenic mice and their wild-type littermates were stimulated *in vitro* with either LPS, LPS and IFN-γ or anti-CD40/IL4/IL5 for 6 days. The concentration of (A) IgM (B) IgG2a and IgG1 and (C) IgG3 in corresponding culture supernatants were measured by standard ELISA. The mean of each immunoglobulin concentration in four wells is shown which represent the results of three independent experiments.

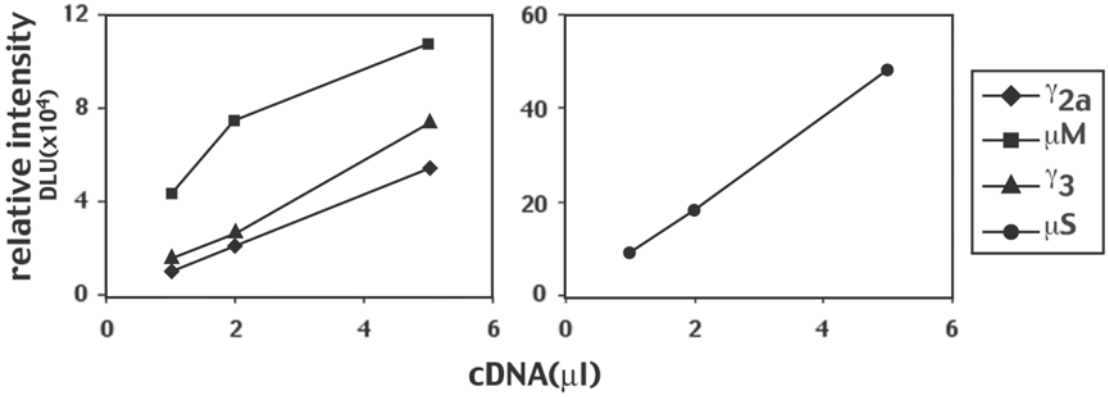
measured by ELISA. Total IgM and IgG3 secretion was reduced approximately 50% in B cells from the transgenic mice as compared to wild-type mice in the LPS-stimulated culture (Figures 11A and C), while IgG2a secretion was reduced to approximately 20% of that seen in wild-type B cells in the presence of LPS and IFNγ (Figure 11B). The synthesis of IgM and IgG1 in response to treatment with anti-CD40 and both IL-4 and IL-5 was also assayed in transgenic and wild-type B cells. In the presence of IL-4 and IL-5, anti-CD40 stimulation induced similar levels of IgM (Figure 11A) as well as IgG1 (Figure 10B), indicating that signaling through CD40 and switching to IgG1 is much less dependent on NF-κB activation than is LPS signaling.

Defects in Ig secretion are due to altered cellular proliferation

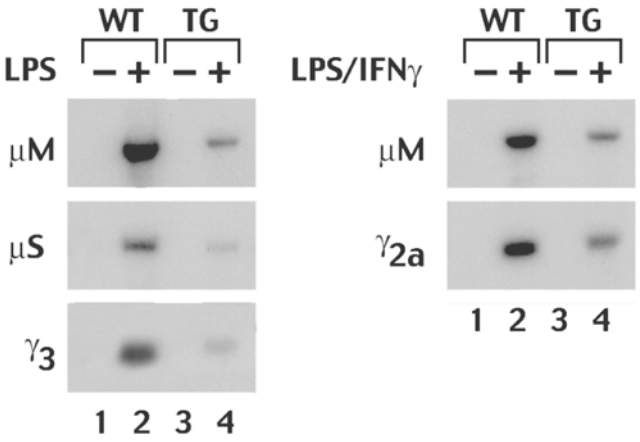
The decreased amounts of IgM, IgG3 and IgG2a in the cultures of the DNIKKβ B cells in response to LPS-treatment could be due to a decreased number of immunoglobulin secreting cells or to a decreased capacity for immunoglobulin secretion from individual cells. When B cells were counted following stimulation with either LPS or LPS and IFNγ, there were 2.9 times more wild-type cells than transgenic cells following LPS stimulation and 4.6 times more wild-type cells than transgenic cells in the presence of LPS and IFNγ. These results suggested that proliferative defects were largely responsible for the decreased immunoglobulin levels present in the transgenic mice.

In an attempt to further distinguish between these two possibilities, we performed semi-quantitative RT-PCR to compare the synthesis of mRNA for the secretory forms of μ, γ3, and γ2a heavy chain from the B cells of wild-type and the DNIKKβ mice (Figure 12).

A.



B.



C.

	WT	TG
μS / μM	0.43	0.37
γ3 / μM	0.45	0.45
γ2a / μM	0.9	1.1

Figure 12. *In vitro* synthesis of secreted IgM, IgG3 and IgG2a is not reduced in DNIKKβ B cells. (A) Various concentrations of cDNA were used in 30 cycle PCR reactions using ^{32}P-labeled primers. The relative intensity of the specified bands was quantified using PhosphorImager analysis. Due to variations in the abundance of each mRNA, different dilutions of the cDNA samples were used to identify the linear range of cDNA where the amount of the template cDNA correlates with the intensity of the products. In these experiments 1, 2, 5, and 10μl of cDNA was diluted 1 to 100 (γ2a, μM), 1 to 200 (γ3), or 1 to 10,000 (μS) to obtain the titration curve. (B) Purified resting B cells from wild-type (WT) and transgenic (TG) mice were cultured in the presence of LPS or LPS and IFNγ. The relative abundance of each mRNA was tested by semi-quantitative RT-PCR with μM, μS and γ3 mRNA assayed in cDNA prepared from cells cultured in the presence and absence of LPS and μM and γ2a mRNA assayed in cDNA prepared from cells cultured in the presence of absence of LPS and INFγ. The diluted cDNAs within the linear range of the assay as determined in part A were used as templates in the PCR reaction. The PCR reactions were subjected to electrophoresis on an agarose gel and autoradiography was performed. The size of amplified product of μM, μS, γ3, and γ2a were 1350, 978, 225, and 275 base pairs respectively. (C) The ratio of the intensities of the PCR products as determined by PhosphorImager analysis for secreted IgM (μS), IgG3 (γ3) and IG2a (γ2a) to that of μM from wild-type and transgenic mice is indicated.

The levels of mRNA from each immunoglobulin isotype should correlate with the level of secreted immunoglobulin protein. First, it was important to demonstrate that the RT-PCR was performed in the linear range relative to RNA abundance for μM, μS, γ2a and γ3 (Figure 12A). Next, we analyzed RNA prepared from resting and LPS-stimulated B lymphocytes isolated from wild-type and DNIKKβ transgenic mice (Figure 12B). Since surface IgM (μM) expression is relatively constant after B cell stimulation and roughly correlates with the cell number (293, 305), the abundance of mRNA corresponding to each of the secreted immunoglobulins in the B cells can be adjusted for cell number by estimating the ratio of its intensity to that of μM. This analysis demonstrated that there were similar ratios of μS/μM, γ3/μM, and γ2a/μM mRNAs in the LPS and LPS and IFNγ-stimulated B lymphocytes isolated from the transgenic and normal mice (Figure 12C). These results indicate that when corrected for differences in cellular proliferation the synthesis of IgM, IgG3 and IgG2a from transgenic B cells is similar to that from wild-type B cells. Thus, the signals for isotype switching to γ3 and γ2a are not reduced in B cells isolated from transgenic mice that are activated by polyclonal stimulators such as LPS.

Normal basal Ig level and impaired humoral response in DNIKKβ mice

The restriction of the DNIKKβ defect to B lymphocytes allowed us to determine whether *in vivo* B cell responses were compromised in the presence of T cells that had intact NF-κB function. To determine whether inhibition of the NF-κB pathway in B cells plays a role in regulating basal antibody production, we analyzed serum immunoglobulin levels in naive mice. Although there was a somewhat lower level of serum IgM in the transgenic mice,

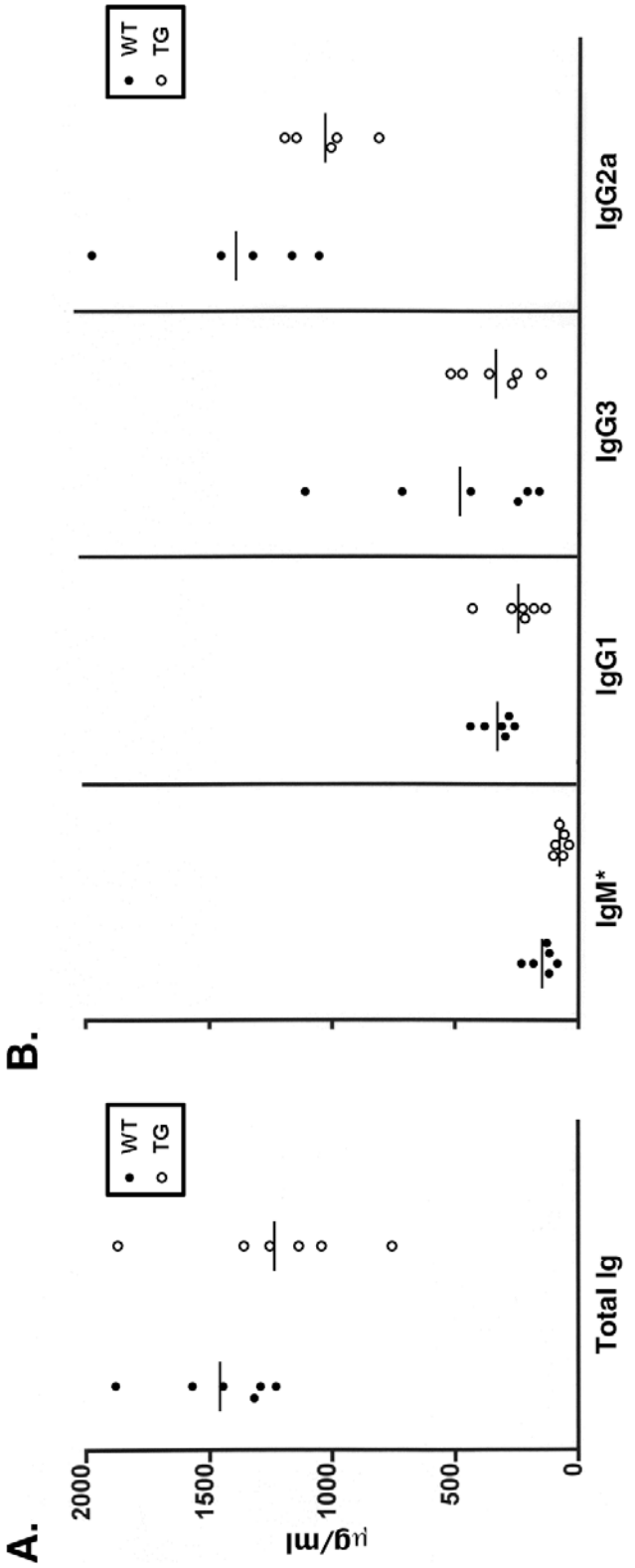

A.
B.
WT
TG
μg/ml
2000
1500
1000
500
0
Total Ig
IgM*
IgG1
IgG3
IgG2a

Figure 13. Basal immunoglobulin levels in IgH/DNIKKβ transgenic mice are not significantly altered. (A) Total immunoglobulin level and (B) the levels of several immunoglobulin isotypes in the sera of naive mice were measured by ELISA. The average values from normal and transgenic mice were statistically analyzed using Student t Test. Only the basal IgM level but not the total immunoglobulin level or other isotype levels of the transgenic mice was shown to be significantly lower than that of the normal mice ($P < 0.05$, indicated by *).

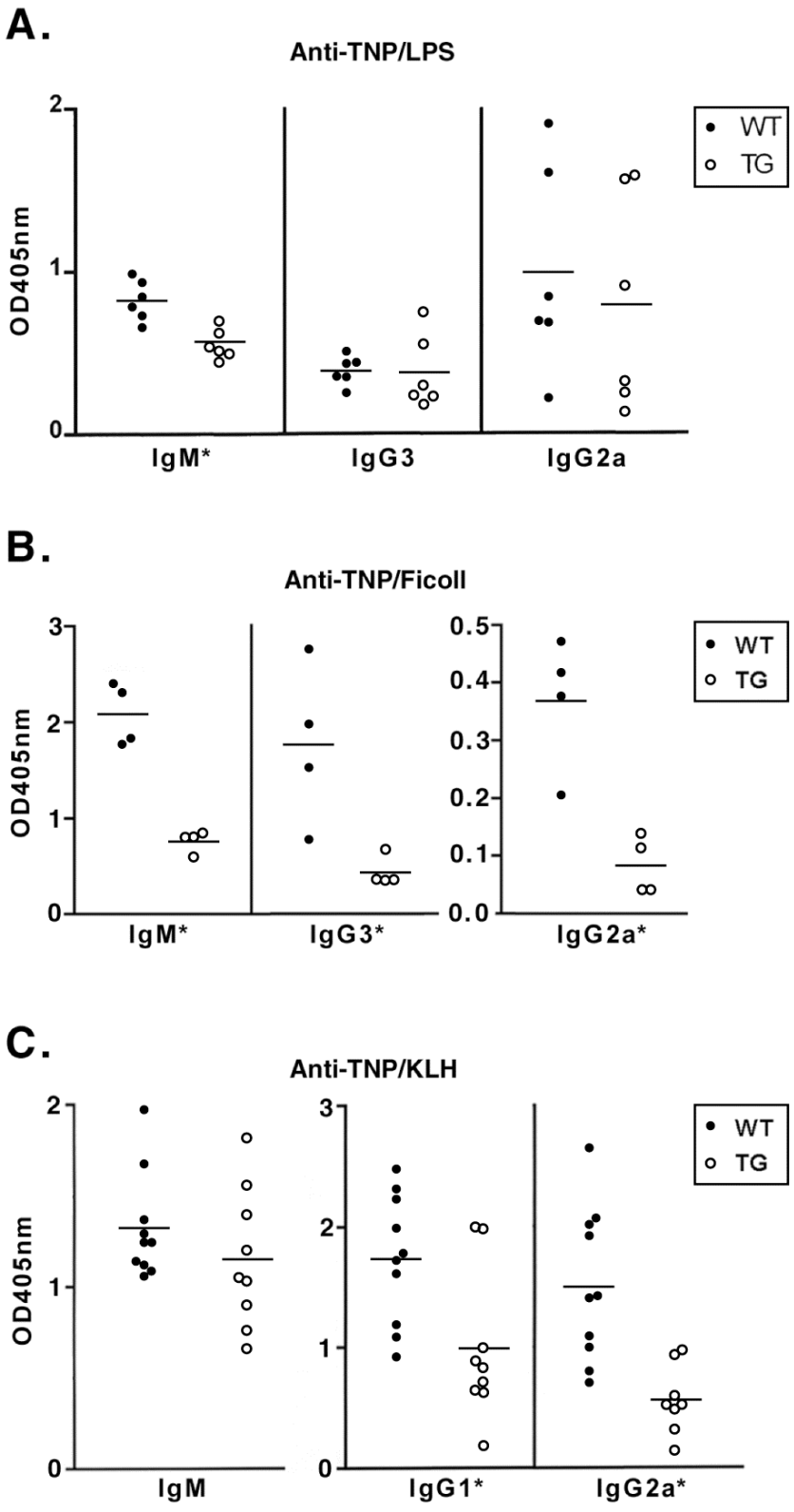

A.
Anti-TNP/LPS
OD405nm
2
1
0
WT
TG
IgM*
IgG3
IgG2a
B.
Anti-TNP/Ficoll
OD405nm
3
2
1
0
0.5
0.4
0.3
0.2
0.1
0.0
WT
TG
IgM*
IgG3*
IgG2a*
C.
Anti-TNP/KLH
OD405nm
2
1
0
3
2
1
0
WT
TG
IgM
IgG1*
IgG2a*

Figure 14. IgH/DNIKKβ transgenic mice display defects in specific antibody responses to T-dependent and T-independent antigens. Between 4-10 mice of either wild-type or transgenic littermates were immunized with (A) the type 1 T-independent antigen TNP-LPS, (B) the type 2 T-independent antigen TNP-Ficoll and (C) the T-dependent antigen TNP-KLH. The mice were injected intraperitoneally and 2 weeks after immunization, TNP-specific IgM, IgG2a,IgG3 and IgG1 were assayed in the sera of these mice as indicated. Statistical analysis was performed using the Student t Test to compare the immunoglobulin values for the transgenic mice and their normal littermates. "*" indicates that the values of the two groups are significantly different.

the levels of IgG2a, IgG3, and IgG1 in the serum of naive wild-type and transgenic mice were similar (Figure 13). These results indicate that blocking the activation of the NF-κB pathway by introduction of the DNIKKβ protein into B cells does not substantially affect basal immunoglobulin production.

Next we investigated whether blocking IKKβ function altered the responsiveness of mature B cells to specific antigenic challenge. DNIKKβ transgenic mice and their wild-type littermates were immunized with a type 1 T-independent antigen (TNP-LPS) and a type 2 T-independent antigen (TNP-Ficoll) as well as a T-dependent antigen (TNP-KLH). The levels of specific antibodies against TNP were tested by ELISA and the results are summarized in Figure 14. TNP-specific IgM was only slightly lower in the transgenic mice as compared to wild-type littermates in mice immunized with TNP-LPS, whereas TNP-specific IgG2a and IgG3 levels were similar (Figure 14A). These results indicate that *in vivo* class switching of TNP-specific antibodies to IgG2a and IgG3, which depends on the mitogenic properties of LPS, is not affected following immunization with a type 1 T-independent antigen.

In contrast, the response to the type 2 T-independent antigen TNP-Ficoll was significantly impaired in the transgenic mice. B cell activation by type 2 T-independent antigens depends on repetitive determinants that cause a high degree of B cell receptor crosslinking. TNP-specific IgM, IgG3 and IgG2a levels were reduced by 64%, 74%, and 77% of the levels seen in wild-type mice respectively (Figure 14B). These results indicate that the response to the type 2 T-independent antigen TNP-Ficoll requires NF-κB activity.

Surprisingly, the TNP-specific IgG1 and IgG2a, but not the IgM levels, were significantly lower in the transgenic mice immunized with the T-dependent antigen TNP-

KLH as compared to the wild-type mice (Figure 14C). This result indicates that class switching to γ1 and γ2a in response to *in vivo* immunization with a T-dependent antigen is altered in the DNIKKβ transgenic mice. Taken together, these results indicate that introduction of a dominant negative IKKβ mutant does not change the basal antibody production, but does play a role in the humoral response to a type 2 T-independent antigen. In addition, switching to downstream isotypes in response to both type 2 T-independent and T-dependent antigens may be affected.

D. Discussion

The NF-κB pathway is critical for the regulation of immune function (14, 19, 20). Since disruption of the IKKα and IKKβ genes results in early lethality of mice, we studied the role of a dominant negative IKKβ mutant expressed exclusively in B cells while maintaining intact T cell function. The use of a dominant negative IKKβ mutant does not totally inhibit IKKβ activity, but instead results in competition with the wild-type IKKβ protein. However, it allowed us to address the role of IKKβ on B cell development and function under conditions where the function of the wild-type protein is partially blocked.

The results of our study indicate that complete activation of the NF-κB pathway by IKKβ is not essential for the development of B cell subsets. Since there is likely residual IKKβ function in these transgenic mice, the complete loss of this kinase may result in more profound effects on B cell development. In addition, we did not detect abnormalities in lymph node or splenic architecture as has been noted with disruption of specific NF-κB

subunits (data not shown). Consistent with these findings, basal immunoglobulin levels were also not altered. Although previous data suggests that blocking specific components of the NF-κB pathway can result in enhanced apoptosis in B cells (140, 304, 306), we did not detect increased apoptosis of B cells when cells were either unstimulated or treated with LPS, anti-CD40 or anti-IgM. The failure to detect increased apoptosis when IKKβ function is altered in B cells may be due to residual NF-κB function or the fact that B cell apoptosis is less dependent on NF-κB than apoptosis in hepatocytes or T cells both of which undergo enhanced apoptosis in IKKβ knock-out animals (78, 93, 97).

However, our results indicate that IKKβ is critical for B cell proliferation in response to various mitogens and *in vivo* immunoglobulin production in response to both T-dependent and T-independent antigens. B cells isolated from the transgenic mice expressing the DNIKKβ exhibited proliferative defects in response to treatment with LPS, anti-IgM or CD40 ligand. The dominant negative IKKβ protein interfered with the proliferation of B cells induced by LPS more than that induced by anti-IgM and anti-CD40. This result is consistent with those obtained from p50 knockout mice which demonstrate that proliferation of B cells from these mice could be induced by antigen receptor engagement more efficiently than following treatment with LPS (252). Since NF-κB activation is not completely inhibited by the dominant negative IKKβ, it is possible that the remaining NF-κB activity induced by anti-IgM and anti-CD40 but not LPS is sufficient to transactivate genes involved in B cell proliferation. It is also possible that B cell proliferation induced by anti-IgM and anti-CD40 can partially bypass the NF-κB pathway.

The defects in B cell proliferation in response to LPS, anti-IgM and anti-CD40 are associated with reduced cell cycle progression from the G1 to S phases of the cell cycle as determined by BrdU labeling. Similar defects have been noted in B cells in which other NF-κB subunits such as p50 were disrupted (303). These results indicate that following mitogenic stimulation of B lymphocytes the NF-κB pathway is involved in the expression of genes that are critical for cell cycle progression. For example, NF-κB has been shown to be involved in the expression of cyclin D1, which in combination with CDK4, is critical for the phosphorylation of the retinoblastoma protein leading to the progression of cells from the G1 to the S phase (307). Additional studies are underway to identify factors regulated by NF-κB which are involved in the cell cycle progression of B cells.

Activation of the NF-κB pathway in B lymphocytes can be induced by engagement of either the BCR, CD40 or stimulation with LPS. The engagement of the BCR initiates signaling pathways mediated through non-receptor protein tyrosine kinases including Fyn, Lyn, Syk and BTK (308). Both BCR dependent and independent pathways can lead to NF-κB activation and there is likely cross-talk between these pathways. For example, LPS stimulation of B cell proliferation is mediated by the Toll-like receptor 4 (TLR4) in addition to other related receptors (309). Mice with mutations in these receptors exhibit defects in LPS-induced cell cycle progression and proliferation which are likely due at least in part to decreased activation of BTK and other downstream kinases (310, 311). Although the *in vitro* proliferation of transgenic B cells following LPS treatment is defective, the *in vivo* response of transgenic mice following immunization with the type 1 T-independent antigen TNP-LPS

is intact. Thus TNP-LPS may induce signals through both TLR-4 and the BCR that are sufficient to override the inhibitory effects of the dominant negative IKKβ mutant.

In contrast to the results with TNP-LPS, the transgenic mice exhibited marked defects in the B cell response to the type 2 T-independent antigen TNP-Ficoll. B cell responses to TNP-Ficoll are likely due to signaling through the BCR to activate BTK with subsequent activation of the NF-κB pathway (312). Marginal zone B cells have been shown to be critical for both the proliferative response to LPS and the humoral response to type 2 T-independent antigens (301, 302, 313). Mice deficient in B cell surface receptors such as TACI (314) or downstream signaling molecules such as Pyk-2 (302), BTK (315) and phospholipase Cγ2 (316) are also defective in development of marginal zone B cell as well as their response to type 2 T-independent antigens. We did not find changes in the development of these B cells in the transgenic mice despite the dramatic defects in response to type 2 T-independent antigens. Thus inhibiting IKKβ function likely prevents efficient signaling induced by the BCR that normally activates the NF-κB pathway in the marginal zone B cells.

The transgenic mice also exhibited a defective humoral response to the T-dependent antigen TNP-KLH which is likely due to effects on class switching. Since B cell activation in response to TNP-Ficoll is defective in the DNIKKβ mice, as indicated by the low IgM levels, it is not possible to determine if switching to downstream isotypes is compromised in response to this antigen. In contrast, IgM production in response to TNP-KLH is not affected in the transgenic mice, indicating that the initial stimulation of B cells following immunization with this T-dependent antigen does not require high levels of NF-κB. In contrast, switching to IgG1 and IgG2a is markedly reduced. It is interesting to note that

activation by anti-CD40 and isotype switching in response to cytokines *in vitro* are not affected in the transgenic B cells. Therefore, there must be significant differences in the level of NF-κB that are required for initial activation of B cells in response to CD40 and/or other T cell costimulatory signals in the context of BCR signaling versus the level of NF-κB required for the induction of class switch recombination.

Recent studies utilizing bone marrow chimeras has investigated the role of IKKα on B cell development (139, 140). Control and IKKα deficient fetal liver cells were transferred into lethally irradiated (139) or RAG2 deficient irradiated mice (140). These mice exhibited reduced numbers of mature B cells with decreased formation of secondary lymphoid organs and impaired antigen specific immune responses. The B cells also showed decreased survival and reduced proliferation to mitogens indicating a role for IKKα in preventing apoptosis and in facilitating the functional development of mature B cells. The major defect in these mice was found to be due to a lack of IKKα-mediated phosphorylation of NF-κB2, which is required for the processing of NF-κB2 and production of p52. Lack of p52 caused impared B cell maturation and the development of secondary lymphoid organs in these chimeras (139). Thus IKKα like IKKβ is also critical in regulating B cell function.

A similar type of analysis suggests that IKKβ deficient fetal liver cells were able to completely reconstitute T cell development. However, defects in these T cells were noted due to their increased sensitivity to TNFα induced apoptosis (138). This study and our analysis suggest that IKKβ is critical for both B and T cell function. The *in viv*o and *in vitro* analysis presented have allowed us to gain additional insights into the mechanism of B cell activation

that may not have been readily apparent in mice with specific disruption of NF-κB genes that affect additional cell types. Furthermore, the partial disruption of the NF-κB pathway in the transgenic B cells has permitted us to better define the signals transmitted via various B cell response elements that lead to activation of the NF-κB pathway and to establish a role for IKKβ in modulating B cell function.

CHAPTER III. GENERATION AND PHENOTYPIC STUDIES OF TRANSGENIC MICE EXPRESSING DNIKKα OR/AND DNIKKβ IN T CELLS

A. Introduction

The transcription factor NF-κB is a critical regulator of immune and inflammatory responses (14, 19, 55). NF-κB regulates the expression of a variety of genes encoding cytokines, cytokine receptors, chemokines, cell adhesion molecules and cell surface receptors that are critical for T and B-lymphocyte function (5). Members of the NF-κB/Rel family, which includes NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA/p65, RelB and c-Rel, are present predominantly in the cytoplasm of resting cells where they are bound to a group of inhibitory proteins known as IκB (10, 14, 19, 20). In response to a variety of stimuli, including the cytokines TNF-α and IL-1, the IκB proteins are specifically phosphorylated, leading to their ubiquitination and degradation by the 26S proteosome (10). This process results in nuclear translocation of NF-κB and the activation NF-κB target genes.

Phosphorylation of the IκB proteins by the IκB kinases (IKK) is a critical step involved in the control of the NF-κB pathway (55, 77, 78, 90, 93, 97). The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, in addition to a regulatory subunit known as IKKγ/NEMO. Both IKKα and IKKβ are able to phosphorylate IκB (77, 78, 90, 93, 97) while IKKγ/NEMO is a scaffold protein that is critical in regulating IKKα and IKKβ kinase activity (94-96, 283). IKKα and IKKβ have a high degree of amino acid homology and a similar domain organization that includes an N-terminal kinase domain, a leucine

zipper that facilitates their heterodimerization and homodimerization and a C-terminal helix-loop-helix domain (77, 78, 90, 93, 97, 107). IKKβ is a much more potent kinase for IκB than is IKKα, suggesting that IKKβ is the dominant kinase involved in cytokine mediated activation of the NF-κB pathway (78, 93, 99). However, IKKα and IKKβ have distinct functions *in vivo*. IKKα$^{-/-}$ mice die of severe skin and skeletal abnormalities shortly after birth (124, 125), whereas IKKβ$^{-/-}$ embryos die of severe liver degeneration due to massive hepatocyte apoptosis (103, 107, 129). These studies indicated that IKKβ is the critical kinase that phosphorylates IκB in response to proinflammatory cytokines and in addition is important in activating genes that prevent apoptosis (103, 138). In contrast, IKKα has a more important role in the development of the epidermis and skeletal system (124, 125).

As a key regulator of the NF-κB pathway, IKK must have essential role in regulating lymphocyte development and function. In chapter II, the role of IKKβ in B cell development and function is investigated. This chapter will focus on the role of IKKα and IKKβ in T cell development and function.

Previous studies using transgenic mice overexpressing IκBα (317) or expressing dominant negative forms of IκBα in T cells (279, 282) indicated that the NF-κB pathway is critical in regulating T cell proliferation and cytokine production (279, 282). The NF-κB pathway is also important for the development of CD8 positive T cells (279, 317) and for the regulation of T cell survival (279, 282). Using similar transgenic mice expressing dominant negative IκBα specifically in T cells, Voll *et al.* found that blocking the NF-κB pathway resulted in decreased population of thymocytes expressing pre-TCR (166). In contrast,

constitutive NF-κB activity leads to increased number of this population. These results indicated that activation of NF-κB is a survival signal in thymocytes expressing pre-TCR, thereby determining the fate of developing T cells (166). Recently, several groups utilized IKKα$^{-/-}$ and IKKβ$^{-/-}$ radiation chimeras to investigate the roles of these kinases in the development and function of the immune system (138-140). These studies demonstrated that IKKα is important in B cell maturation and the formation of secondary lymphoid organs through its ability to phosphorylate and induce processing of p100, whereas IKKβ is critical in preventing TNF-α induced apoptosis in developing lymphocytes. However, NF-κB could promote apoptosis in T cells under some circumstances (282, 318). Although it was shown that mature B cells lacking IKKα have an increased turnover rate and increased spontaneous apoptosis *in vitro* (139, 140), the role of IKKα in regulating apoptosis in T cells is unclear, as is the role of IKKα and IKKβ in regulating T cell proliferation and cytokine production.

A direct comparison of the role of IKKα and IKKβ on the development and function of T lymphocytes has not previously been reported. Here we utilize transgenic mice expressing dominant negative IKKα (DNIKKα) and dominant negative IKKβ (DNIKKβ) either individually or in combination specifically in T cells. We demonstrate that IKKα and IKKβ have distinct roles in T cell function. Both DNIKKα and DNIKKβ inhibited NF-κB activation in T cells following treatment with PMA and ionomycin. DNIKKβ was a stronger inhibitor of anti-CD3 induced NF-κB activation than was DNIKKα. Accordingly, DNIKKβ but not DNIKKα, markedly reduced the proliferative response of T cells following TCR cross-linking by inhibiting cell cycle progression. In addition, thymocytes from mice

expressing both DNIKKα and DNIKKβ exhibited severe defects in cytokine production. Finally, we assayed the effect of DNIKKα and DNIKKβ on the apoptosis of thymocytes. Surprisingly, following the *in vivo* administration of anti-CD3, DNIKKα mice exhibited increased apoptosis of double positive thymocytes, while DNIKKβ mice exhibited decreased apoptosis. These results provide the first direct comparison of the roles of IKKα and IKKβ on the development and function of murine T lymphocytes.

B. Materials and Methods

Generation of CD2/DNIKKα and CD2/DNIKKβ transgenic mice

An influenza hemagglutinin (HA)-tagged human IKKα cDNA containing substitution of serine residue 176 with alanine (HA-IKKαA176) or a Flag-tagged human IKKβ cDNA containing substitutions of serine residues 177 and 181 with alanine (FL-IKKβA177/181) were inserted into the VA hCD2 cassette (319) in order to obtain T cell specific expression of these genes. The linearized transgenes CD2/DNIKKα and CD2/DNIKKβ were microinjected into the pronuclei of F1 zygotes of the C57BL/6 and DBA2 strains. Transgenic founders were bred and maintained in a specific pathogen free (SPF) colony. PCR reactions using primer pairs hybridizing to the 5'and 3' portions of the HA-IKKαA176 or FL-IKKβA177/181 cDNA were used to genotype transgenic progeny and their littermates in the colony. Southern blot analysis was used to confirm the presence of the transgenes in the founders. Progeny of different founders were tested for transgene protein expression and

those with highest levels of expression were crossed to generate transgenic mice that exhibited T cell specific expression of both DNIKKα and DNIKKβ.

Flow cytometry analysis

Thymocytes and splenocytes from either wild-type or transgenic littermates were prepared in RPMI media and stained with fluorescent antibodies against cell surface markers. The antibodies and reagents used for cell surface staining were: FITC-conjugated anti-CD4, PE-conjugated anti-CD3, PerCP-conjugated anti-CD8 (Pharmingen). Fluorescence analysis was performed using a FACSCALIBUR flow cytometer (Becton Dickinson).

Immunoprecipitation and Western blot analysis

Thymocytes were lysed in TNE buffer (1%Triton X-100, 10mM Tris-HCl pH8.0, 150mM NaCl, 1mM EDTA) containing a cocktail of protease inhibitors (Roche). Cell lysates were then incubated overnight with an M2 monoclonal antibody against the Flag epitope (Sigma) or a polyclonal antibody against the HA epitope (Santa Cruz) followed by incubation with protein G sepharose beads for 1 hour. The immunoprecipitates were then subjected to Western blot analysis using either IKKα or IKKβ antibodies (Santa Cruz).

RT-PCR analysis of IKK mRNA isolated from thymocytes of wild-type and transgenic mice

Total RNA was extracted from thymocytes using RNeasy mini-columns (Qiagen) and subjected to RT-PCR analysis. The oligonuleotide primers used to amplify GAPDH have been described (289). Primers used to amplify a 411bp fragment of both the mouse and

human IKKα cDNA included the 5' primer: 5'-ctgaggttggtgtcattgg-3' and the 3' primer: 5'-cagaactctgtgtacaggc-3'. Primers used to amplify a 341bp fragment of both mouse and human IKKβ were the 5' primer 5'-gtgtcagctgtatccttc-3' and the 3' primer: 5'-gctccacagcctgctcc-3'. The sense primers were end-labeled with [γ-^{32}P]-ATP. The PCR products were analyzed by digestion with BstEII or EcoRI for IKKα and IKKβ, respectively. BstEII cuts the cDNA fragment amplified from endogenous mouse IKKα but not DNIKKα (human) to generate two fragments of 247 and 164 bp, whereas EcoRI cuts the cDNA fragment amplified from DNIKKβ (human) but not the mouse IKKβ to generate fragment of 176 and 165 bp. Following gel electrophoresis and autoradiography, the intensity of the radioactive species was measured by PhosphorImager analysis (Cyclone, Packard).

Stimulation of thymocytes and electrophoretic mobility shift assay

Thymocytes from wild-type and transgenic mice were either incubated in complete RPMI alone or with PMA (50ng/ml) and ionomycin (200ng/ml) for 15 minutes or with immobilized anti-CD3 (10μg/ml) for 4 hours at 37^{o}C. Nuclear extract were then prepared from the cells (290, 291). To test NF-κB binding, a ^{32}P-labeled oligonucleotide probe containing the MHC class I κB site (292) or the NF-Y binding site was incubated with the nuclear extracts at room temperature for 30 minutes and subjected to electrophoresis on a 5% polyacrylamide gel in 0.5x TBE buffer. The binding reaction contained 60,000 cpm of the radio-labeled probe, 4 μg of nuclear protein, 500 ng of poly (dI-dC) (Pharmacia), 10 μg BSA, 20 mM HEPES pH 7.9, 1 mM EDTA, 1% NP-40, 5% glycerol, 5 mM DTT in a final

volume of 20 μl. For super-shift assays, 5 μg of goat polyclonal antibody directed against p65 or normal goat sera was added to the binding reactions and incubated for 30 minutes on ice before the samples were subjected to gel electrophoresis. The gels were dried and exposed to X-ray film and quantified by PhosphorImager analysis.

Cell culture and proliferation assay

Freshly isolated thymocytes and splenocytes were cultured in RPMI containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 25 mM HEPES, and 50 mM 2-mercaptoethanol in 96 well plates at 37^{o}C, 5% CO_2. Anti-CD3 antibody was purified from the culture supernatant of 145-2C11 hybridoma using HiTrap rProtein A column (Amersham) and used at 10 μg/ml to coat the plates overnight at 4^{o}C. Mouse recombinant IL-2 (Endogen) was added to the cultures at a concentration of 1 to 10 ng/ml. The cells were cultured for 48 hours before they were pulsed with [^{3}H]-thymidine for 14-18 hours and harvested onto glass fiber filter paper. The amount of [^{3}H]-thymidine incorporated was quantified by a β scintillation counter.

Cell cycle analysis

Cell cycle analysis of non-stimulated and stimulated thymocytes was performed using a BrdU Flow Kit (Pharmingen). Thymocytes were cultured in RPMI in 24 well plates with or without anti-CD3 (10μg/ml) and IL-2 (2ng/ml) for 60 hours before they were pulsed with BrdU for 30 minutes. According to the manufacturer's instructions, the cells were then processed and stained with FITC-conjugated anti-BrdU and 7-AAD to determine the amount

of BrdU incorporated and the total DNA content, respectively. Flow cytometry analysis was performed using FACSCALIBUR (Becton Dickinson).

Ribonuclease protection assay (RPA)

To measure the mRNA levels of multiple cytokines in thymocytes, freshly isolated thymocytes were stimulated with immobilized anti-CD3 (10μg/ml) and anti-CD28 antibodies (Southern Biotechnology Associates) (16 μg/ml) for 4-6 hours. Total RNA was prepared from $2x10^7$ stimulated cells using Trizol (Gibco BRL) in combination with the RNeasy Kit (Qiagen). Briefly, the stimulated cells were immediately homogenized with Trizol Reagent and extracted with chloroform. Total RNA in the cell extract was then bound to silica-gel-based mini-column and eluted with DEPC water. Approximately 2 μg of the RNA samples were subjected to electrophoresis on a 1.2% denaturing formaldehyde agarose gel in MOPS buffer to confirm the integrity of the RNA. Following DNaseI treatment, 4 μg RNA from each sample was used to hybridize with labeled mCK-1b probe that contains fragments of multiple cytokine mRNA using the Riboquant RPA Kit (Pharmingen). The hybridized samples were treated with RNase followed by proteinase K and fractionated on a 5% denaturing polyacrylamide gel. The radioactive species on the gel were identified according to their mobility after autoradiography.

In vivo apoptosis analysis

Wild-type and transgenic littermates that were 5-7 weeks old were injected intraperitoneally with 100 μl of PBS or PBS containing 25 μg or 50 μg anti-CD3 (145-2C11)

(282). At 48 hours after anti-CD3 administration, the thymocytes of treated and control mice were counted and analyzed by flow cytometry for the surface expression of CD4 and CD8. The absolute numbers of thymocyte subsets were calculated.

C. Results

Generation of transgenic mice expressing dominant negative IKKα (DNIKKα) and dominant negative IKKβ (DNIKKβ) mutants in T lymphocytes

In an attempt to inhibit the function of endogenous IKKα and IKKβ specifically in T cells, we generated transgenic mice that expressed dominant negative forms of human IKKα (A176) (108) or human IKKβ (A177/181) (78, 119) by inserting these dominant negative genes into a modified human CD2 promoter cassette. This cassette confers position independent and transgene copy number dependent expression of these genes in the T cell lineage (319). The DNIKKα and DNIKKβ cDNAs contained amino terminal HA and Flag epitopes, respectively, to facilitate their detection in murine T cells. The IKKβ protein in which serine residues 177 and 181 in the MAP3 kinase activation loop were substituted with alanine has a dominant negative phenotype that inhibits NF-κB activation in response to treatment with proinflammatory cytokines such as TNFα and IL-1 (78, 90, 97, 119). The IKKα protein in which serine residue 176 was substituted with alanine could not be phosphorylated or activated by the upstream kinase NIK and inhibited endogenous IKKα

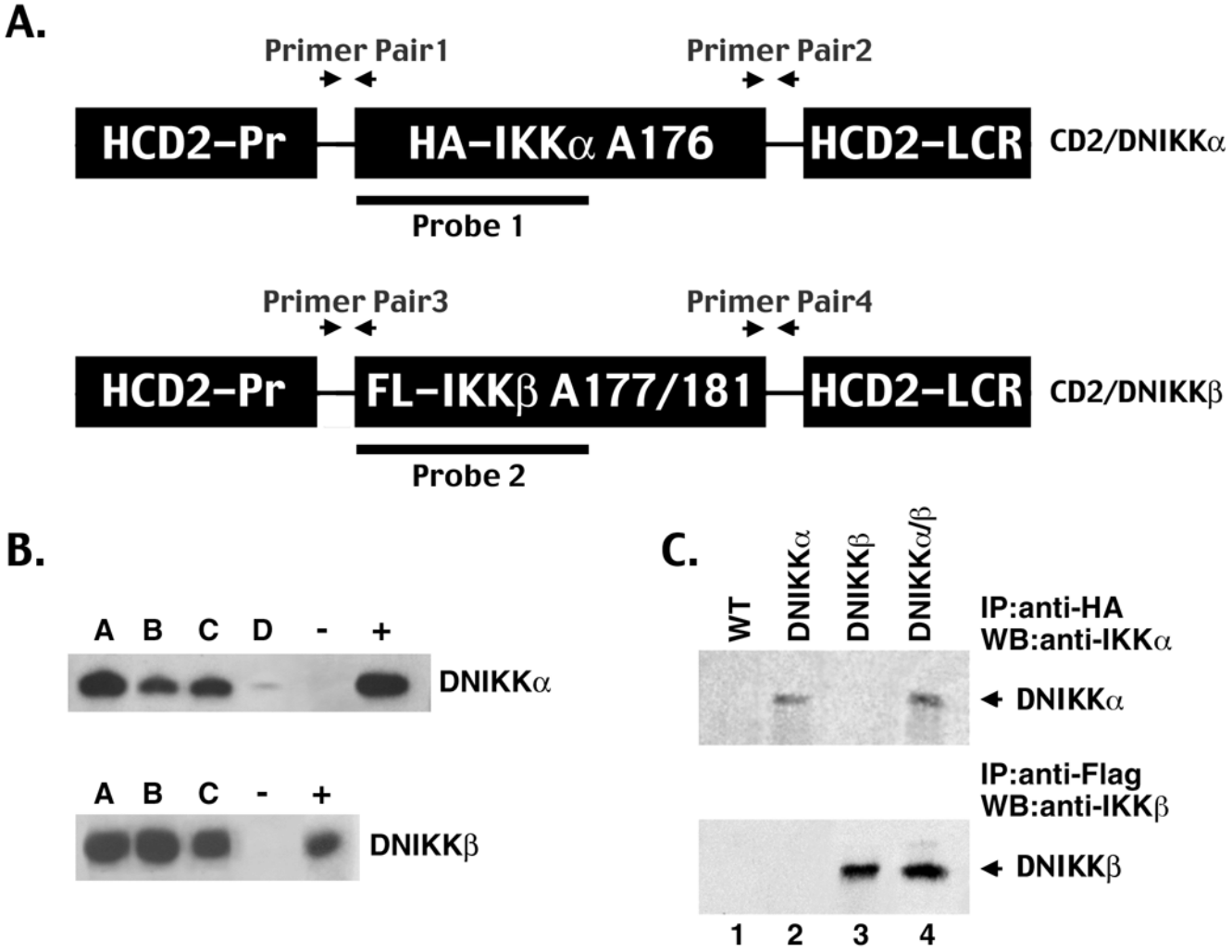

A.
Primer Pair1
Primer Pair2
HCD2-Pr
HA-IKKα A176
HCD2-LCR
CD2/DNIKKα
Probe 1
Primer Pair3
Primer Pair4
HCD2-Pr
FL-IKKβ A177/181
HCD2-LCR
CD2/DNIKKβ
Probe 2
B.
A B C D - +
DNIKKα
A B C - +
DNIKKβ
C.
WT
DNIKKα
DNIKKβ
DNIKKα/β
IP:anti-HA
WB:anti-IKKα
DNIKKα
IP:anti-Flag
WB:anti-IKKβ
DNIKKβ
1 2 3 4

Figure 15. Generation of transgenic mice expressing DNIKKα and/or DNIKKβ proteins in T lymphocytes. (A) A schematic of the HA-tagged DNIKKα (HA-IKKαA176) or Flag-tagged DNIKKβ (FL-IKKβA176/181) inserted between the human CD2 promoter region (hCD2-Pr) and human CD2 locus control region (hCD2-LCR) is shown. Primer pairs (1, 2, 3, and 4) that hybridize to the 5' and 3' portions of the epitope tagged DNIKKα or DNIKKβ were used for PCR genotyping. Probes 1 and 2 were used in southern blot analysis of genomic DNA of DNIKKα and DNIKKβ mice, respectively. (B) Southern blot analysis of genomic DNA from four DNIKKα founders (A, B, C, and D) and three DNIKKβ founders (A, B, and C) and control DNA (-/ +) was performed using the ^{32}P-labled probes 1 and 2, respectively. (C) Cell lysates from wild type (WT), DNIKKα, DNIKKβ and DNIKKα/β thymocytes (lanes 1, 2, 3, and 4) were immunoprecipitated (IP) with antibodies directed against HA (top panel) or Flag (bottom panel) and Western-blotted (WB) with antibodies directed against IKKα (top panel) or IKKβ (bottom panel). The positions of the DNIKKα and DNIKKβ on SDS gels are shown.

function (108). Since mouse and human IKKα or IKKβ have greater than 90% amino acid identity, we expected that these dominant negative forms of IKKα and IKKβ would inhibit the function of endogenous mouse IKKα and IKKβ and thus alter NF−κB activation in T lymphocytes.

Constructs containing either the dominant negative IKKα or IKKβ cDNAs were microinjected into the pronuclei of C57BL/6XDBA/2 zygotes (Fig. 15). Southern blot analysis indicated that four founders designated A, B, C, and D for the CD2/DNIKKα construct and three founders designated A, B, and C for the CD2/DNIKKβ construct contained integrated transgenes (Fig. 15B). As expected, progeny from all of these founders were able to express the dominant negative IKK proteins in T cells in a copy number-dependent manner (data not shown). Progeny of the B founders of DNIKKα and DNIKKβ that expressed high levels of DNIKKα and DNIKKβ respectively were crossed to generate DNIKKα/β mice that expressed both DNIKKα and DNIKKβ. As shown by immunoprecipitation and Western blot assays of protein extracts prepared with thymocytes from wild-type and DNIKK mice (Fig. 15C), DNIKKα protein was expressed at similar levels in the thymocytes of DNIKKα and DNIKKα/β mice and DNIKKβ protein was expressed at similar levels in the thymocytes from DNIKKβ and DNIKKα/β mice.

Since substantial levels of dominant negative proteins are frequently required to inhibit endogenous protein function, we utilized RT-PCR analysis of mRNA isolated from transgenic T cells to compare DNIKKα and DNIKKβ mRNA levels with that of the endogenous IKKα and IKKβ mRNA (Fig. 16). To distinguish the expression of the mouse

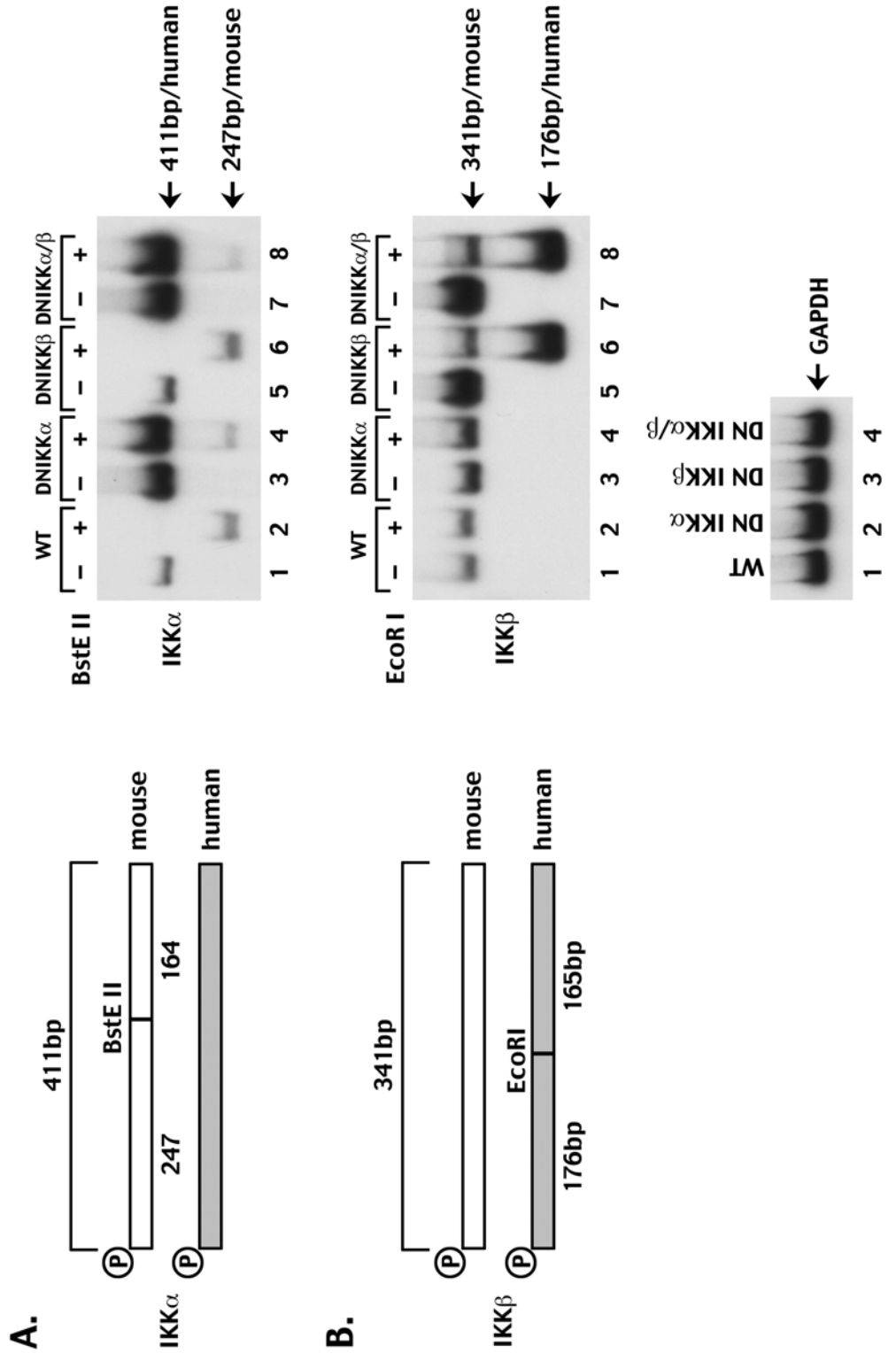

A.
411bp
BstE II
mouse
247
164
human
IKKα
B.
341bp
mouse
EcoRI
human
176bp
165bp
IKKβ
WT
DNIKKα
DNIKKβ
DNIKKα/β
BstE II
IKKα
411bp/human
247bp/mouse
1 2 3 4 5 6 7 8
EcoR I
IKKβ
341bp/mouse
176bp/human
1 2 3 4 5 6 7 8
WT
DN IKKα
DN IKKβ
DN IKKα/β
GAPDH
1 2 3 4

Figure 16. Comparison of DNIKKα and DNIKKβ (human) and endogenous IKKα or IKKβ (mouse) expression levels. RT-PCR analysis of endogenous (murine) and exogenous (human) IKKα and IKKβ mRNA was performed. Oligonucleotide primers with one primer end-labeled with ^{32}P were used to amplify (A) a 411 bp fragment of IKKα or (B) a 341 bp fragment of IKKβ. The PCR products were digested with either (A) BstEII that cuts only the mouse IKKα fragment or (B) EcoRI that cuts only the human IKKβ fragment. Digested samples (lanes 2, 4, 6, and 8) and non-digested samples (lanes 1, 3, 5, and 7) were subjected to electrophoresis on a 5% native polyacrylamide gel. PhosphorImager analysis indicated that (A) the 411 bp fragment (human IKKα) in lanes 4 and 8 was 20-fold stronger than the 247 bp fragment (mouse IKKα), whereas (B) the 176 bp fragment (human IKKβ) in lanes 6 and 8 was 10-fold stronger than the 341 bp fragment (mouse IKKβ). (C) RT-PCR of GAPDH indicated that equivalent amount of template cDNA was used in the PCR reactions.

and human IKK RNAs, we chose to amplify either a 411bp fragment with a BstEII site in the mouse, but not the human IKKα (Fig. 16A), or a 341bp fragment with an EcoRI site in the human, but not the mouse IKKβ (Fig. 16B). The level of DNIKKα mRNA, represented by the 411bp fragment, was more than 20-fold higher than that of the endogenous mouse IKKα mRNA represented by the 247bp fragment in the DNIKKα and DNIKKα/β mice (Fig. 16A). The level of DNIKKβ mRNA, represented by the 176bp fragment, was 10-fold higher than that of the endogenous mouse IKKβ represented by the 341bp fragment in the DNIKKβ and DNIKKα/β mice (Fig. 16B). These results suggest that both DNIKKα and DNIKKβ were expressed at significantly higher levels than the endogenous kinases.

Reduced NF-κB DNA binding in the thymocytes from the DNIKK transgenic mice

Next we addressed whether DNIKK expression altered NF-κB activation in T cells. To analyze the effects of DNIKKα and DNIKKβ expression on NF-κB DNA binding, we performed electrophoretic mobility shift assays (EMSA) using nuclear extracts prepared from both non-stimulated and PMA/ionomycin or anti-CD3 stimulated thymocytes isolated from wild-type and the different DNIKK transgenic mice. NF-κB DNA binding activity was strongly induced by both PMA/ionomycin and anti-CD3 stimulation in thymocytes isolated from wild-type mice (Fig. 17A & B). PMA/ionomycin induced NF-κB DNA binding activity was reduced in thymocytes isolated from DNIKKα and DNIKKβ mice, and further reduced in the thymocytes from DNIKKα/β mice (Fig. 17A). There was only a moderate decrease in anti-CD3 induced NF-κB DNA binding activity in DNIKKα thymocytes as compared to the

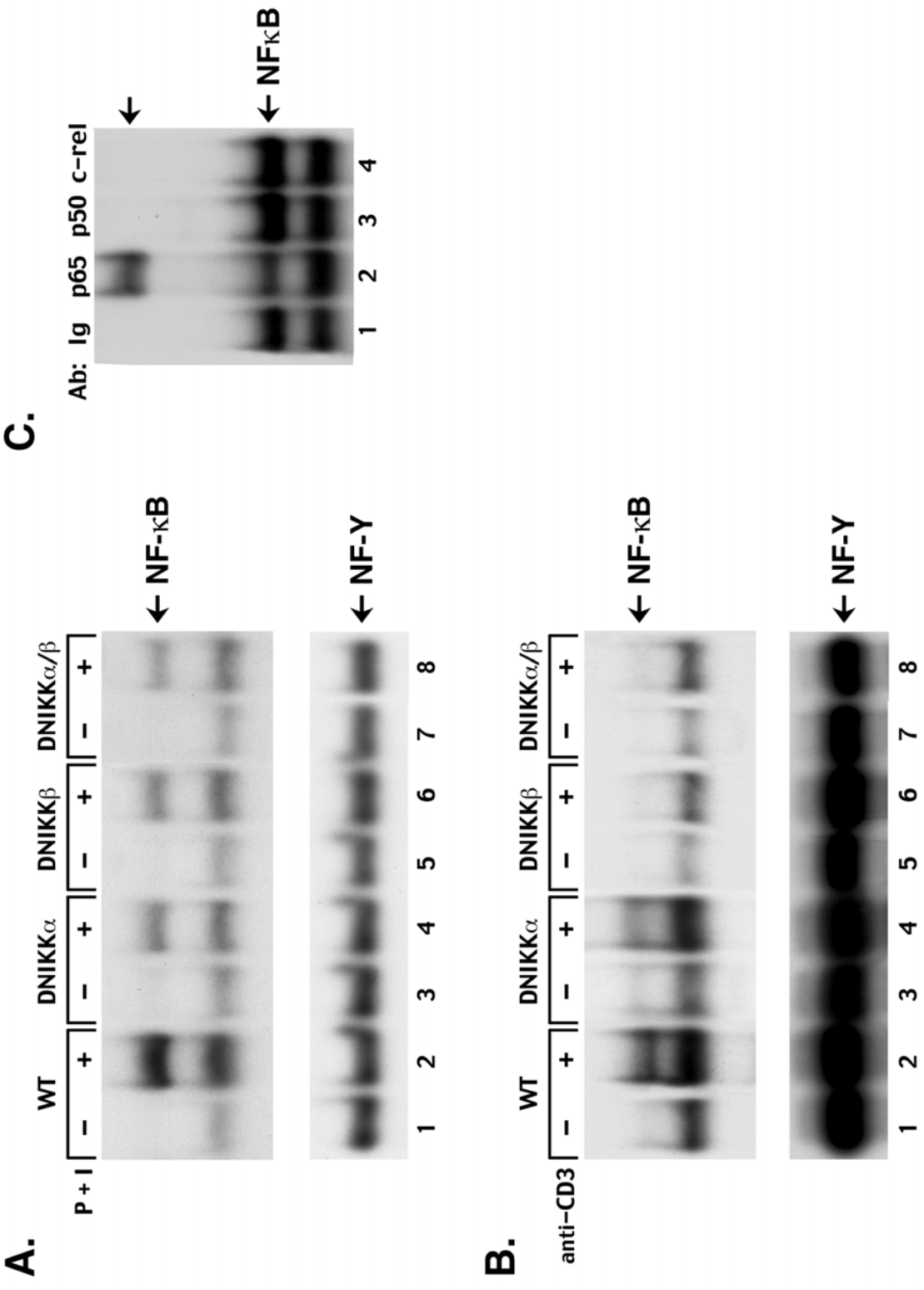

A.
WT
DNIKKα
DNIKKβ
DNIKKα/β
P + I
– + – + – + – +
NF-κB
NF-Y
1 2 3 4 5 6 7 8
B.
WT
DNIKKα
DNIKKβ
DNIKKα/β
anti-CD3
– + – + – + – +
NF-κB
NF-Y
1 2 3 4 5 6 7 8
C.
Ab: Ig p65 p50 c-rel
NFκB
1 2 3 4

Figure 17. Inhibition of NF-κB DNA binding activity in T cells isolated from DNIKK transgenic mice. (A) Thymocytes from wild-type (WT), DNIKKα, DNIKKβ, or DNIKKα/β mice were either left untreated (A & B, lanes 1, 3, 5, 7) or stimulated with (A) PMA (50ng/ml) and ionomycin (200 ng/ml) (lanes 2, 4, 6, 8) for 15 minutes or (B) anti-CD3 (10μg/ml) (lanes 2, 4, 6, 8) for 4 hours. Nuclear extracts from these cells were subjected to EMSA using a radio-labeled NF-κB probe (top panel) or an NF-Y probe (bottom panel) as a control. (C) The nuclear extract of stimulated thymocytes from wild-type mice was subjected to a super-shift assay with 5μg of either normal goat IgG (lane 1) or antibodies against p65 (lane 2), p50 (lane 3) or c-rel (lane 4). The positions of NF-κB binding complex and the super-shifted complex are indicated.

wild-type thymocytes, whereas this activity was markedly reduced in DNIKKβ and DNIKKα/β thymocytes (Fig. 17B). In these extracts, there was comparable DNA binding activity to NF-Y, a constitutively active transcription factor (Fig. 17A & B). A supershift assay using p65 antibody indicated that the NF-κB DNA binding complex induced by PMA and ionomycin contained the p65 NF-κB subunit (Fig. 17C). These results indicated that PMA/ionomycin induced NF-κB activation was inhibited in thymocytes from all three lines of transgenic mice, whereas anti-CD3 induced NF-κB activation was blocked predominantly in thymocytes isolated from DNIKKβ and DNIKKα/β transgenic mice but only to a lesser degree in thymocytes from DNIKKα mice, suggesting that IKKβ plays a more important role in anti-CD3 mediated NF-κB activation.

Normal T cell development in DNIKK transgenic mice

Since both dominant negative forms of IKKα and IKKβ inhibited NF-κB activation in the thymocytes isolated from the transgenic mice, we next asked whether the expression of these dominant negative kinases altered T cell development. The thymus and spleen isolated from the DNIKKα, DNIKKβ, and DNIKKα/β mice were of normal size and structure as determined by pathological examination with hemotoxylin and eosin staining (data not shown). Thymocytes and splenocytes isolated from these transgenic mice were analyzed by flow cytometry analysis of the surface expression of CD4 and CD8. There were normal percentages of single positive as well as double positive populations in thymocytes isolated from all of these mice (Fig. 18). The percentage of single positive T cells in the spleens of

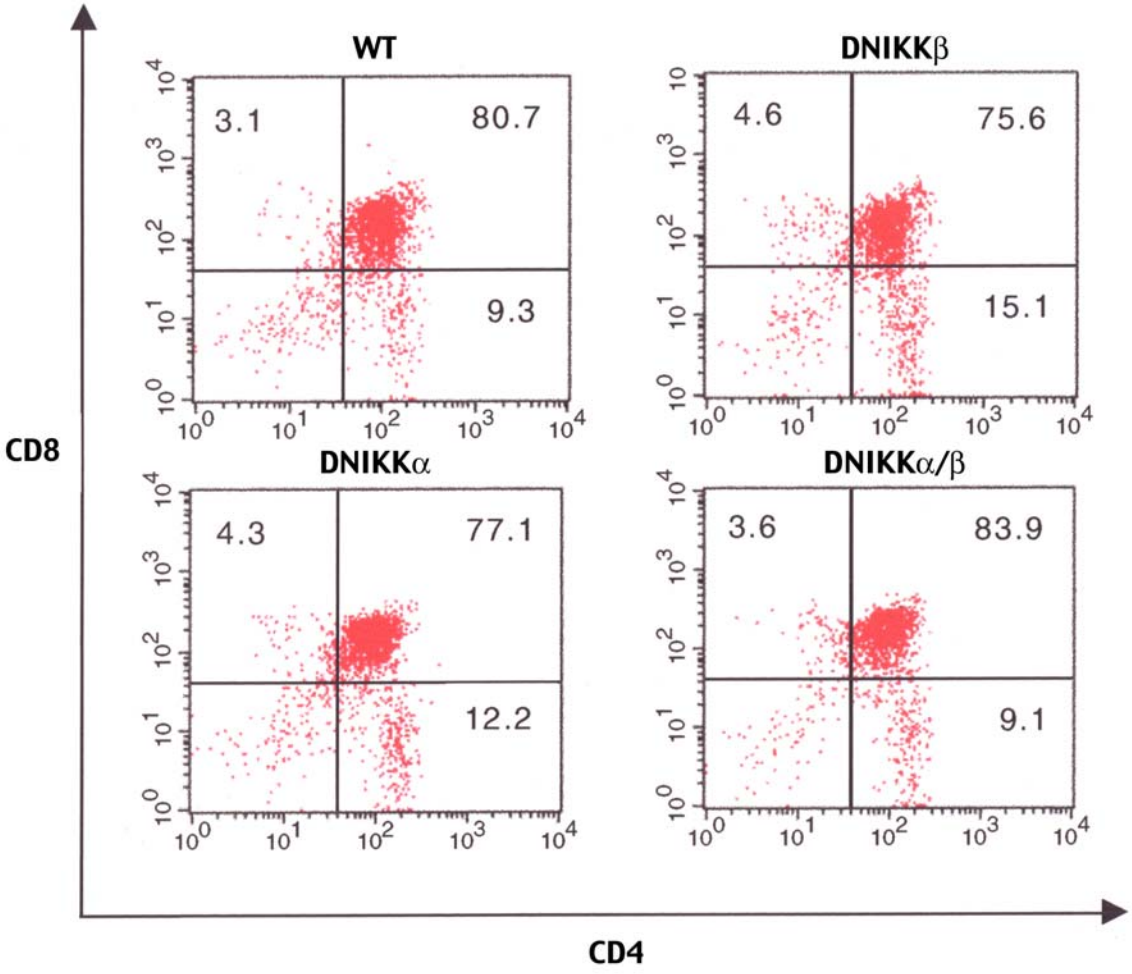

WT
3.1
80.7
9.3
DNIKKβ
4.6
75.6
15.1
DNIKKα
4.3
77.1
12.2
DNIKKα/β
3.6
83.9
9.1
CD8
CD4

Figure 18. Normal T cell development in DNIKK transgenic mice. Thymocytes from wild-type (WT), DNIKKα, DNIKKβ, and DNIKKα/β mice were stained with FITC-conjugated anti-CD4 and PerCP-conjugated anti-CD8 and analyzed by flow cytometry. The percentages of double positive cells (upper right quadrant), CD4 single positive cells (lower right quadrant), and CD8 single positive cells (upper left quadrant) are shown.

these transgenic mice was also similar (data not shown). These results suggest that the overall T cell development in the DNIKK transgenic mice is not altered by the expression of DNIKKα or DNIKKβ.

IKKβ is the dominant kinase regulating the proliferation of T cells in response to anti-CD3 treatment

NF-κB is important in mediating T cell receptor (TCR) signaling (320, 321). Activation of T cells by TCR engagement results in their proliferation and cytokine production. First, we addressed whether the expression of DNIKKα and DNIKKβ altered the proliferation of T cells in response to anti-CD3 stimulation. Thymocytes and splenocytes from wild-type and transgenic mice were stimulated with either immobilized anti-CD3 antibody alone or in combination with IL-2. The incorporation of [^{3}H]-thymidine was then measured. The proliferative response of thymoctyes from DNIKKβ and DNIKKα/β mice was significantly reduced compared to that seen with thymocytes isolated from DNIKKα and wild-type mice (Fig. 19A). The proliferative defects seen in the thymocytes from DNIKKβ and DNIKKα/β mice could be substantially rescued by the addition of IL-2 to the culture media (Fig. 19A). These results indicated that IKKβ was more important than was IKKα in regulating the T cell proliferative response induced by TCR crosslinking and that IL-2 could overcome this defect. In contrast, anti-CD3 induced proliferation of the splenic T cells from DNIKKβ mice was comparable to that found in the splenic T cells isolated from wild-type and DNIKKα mice (Fig. 19B). However, anti-CD3-mediated proliferation of the splenic T

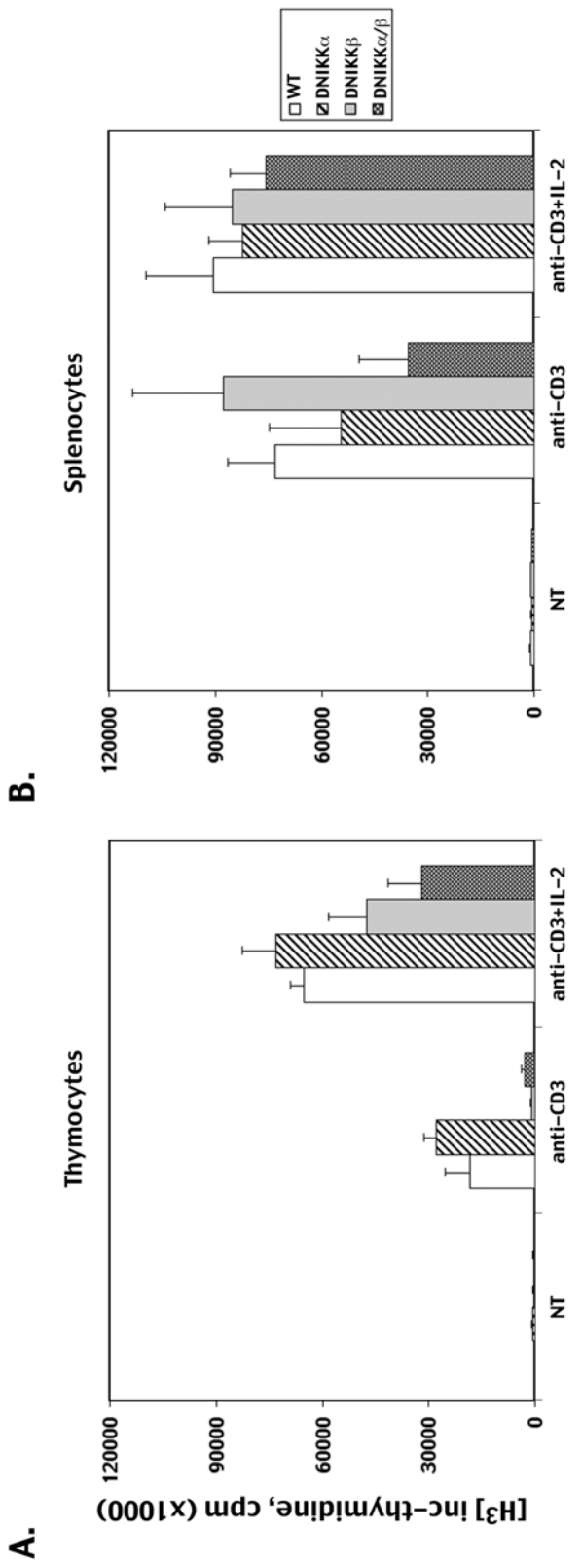

A.
Thymocytes
[H3] inc-thymidine, cpm (x1000)
120000
90000
60000
30000
0
NT
anti-CD3
anti-CD3+IL-2
B.
Splenocytes
WT
DNIKKα
DNIKKβ
DNIKKα/β
120000
90000
60000
30000
0
NT
anti-CD3
anti-CD3+IL-2

Figure 19. Thymic and splenic T cells from DNIKKβ and DNIKKα/β mice exhibit proliferative defects. Thymocytes (A) and splenocytes (B) from wild type (WT), DNIKKα, DNIKKβ, and DNIKKα/β mice were cultured in RPMI alone (NT) or in the presence of plate-coated anti-CD3 (10μg/ml), plated-coated anti-CD3 and mouse recombinant IL-2 (2ng/ml) for a period of 60 hours with the last 16 hours pulsed with [^{3}H]-thymidine. The mean of the incorporated [^{3}H]-thymidine with standard deviation in four wells is shown and the results are representative of at least four independent experiments.

cells from DNIKKα/β mice was reduced 50% as compared to wild-type splenocytes. These results indicated that while inhibition of IKKβ alone was sufficient to reduce thymocyte proliferation in response to anti-CD3, inhibition of both IKKα and IKKβ activity was required to reduce TCR-induced proliferation of peripheral T cells.

Impaired cell cycle progression in DNIKKβ thymocytes

To investigate the mechanisms involved in the proliferative defects seen in T cells isolated from transgenic mice containing DNIKKβ, the cell cycle progression of thymocytes stimulated with anti-CD3 was analyzed by BrdU labeling followed by staining with anti-BrdU and 7-AAD. Flow cytometry analysis was then performed to determine the incorporation of BrdU and the total DNA content (Fig. 20). Only cells in the S phase incorporate significant amounts of BrdU during the 30 minute labeling period, while cells in the G0/G1 and G2/M phase can be distinguished by differences in their DNA content.

As shown in Figure 20, approximately 20% of the thymocytes from wild-type and DNIKKα mice were in the S phase after anti-CD3 treatment. However, only 5-10% thymocytes isolated from DNIKKβ and DNIKKα/β mice were in S phase. The decrease in the percentage of the S phase cells of DNIKKβ and DNIKKα/β thymocytes was associated with a corresponding increase in the percentage of cells in the G0/G1 phase of the cell cycle. These results indicated that the progression from the G0/G1 phase to the S phase in the thymocytes from DNIKKβ and DNIKKα/β mice was reduced. In contrast, the expression of DNIKKα did not significantly alter thymocytes cell cycle progression. The defect in cell

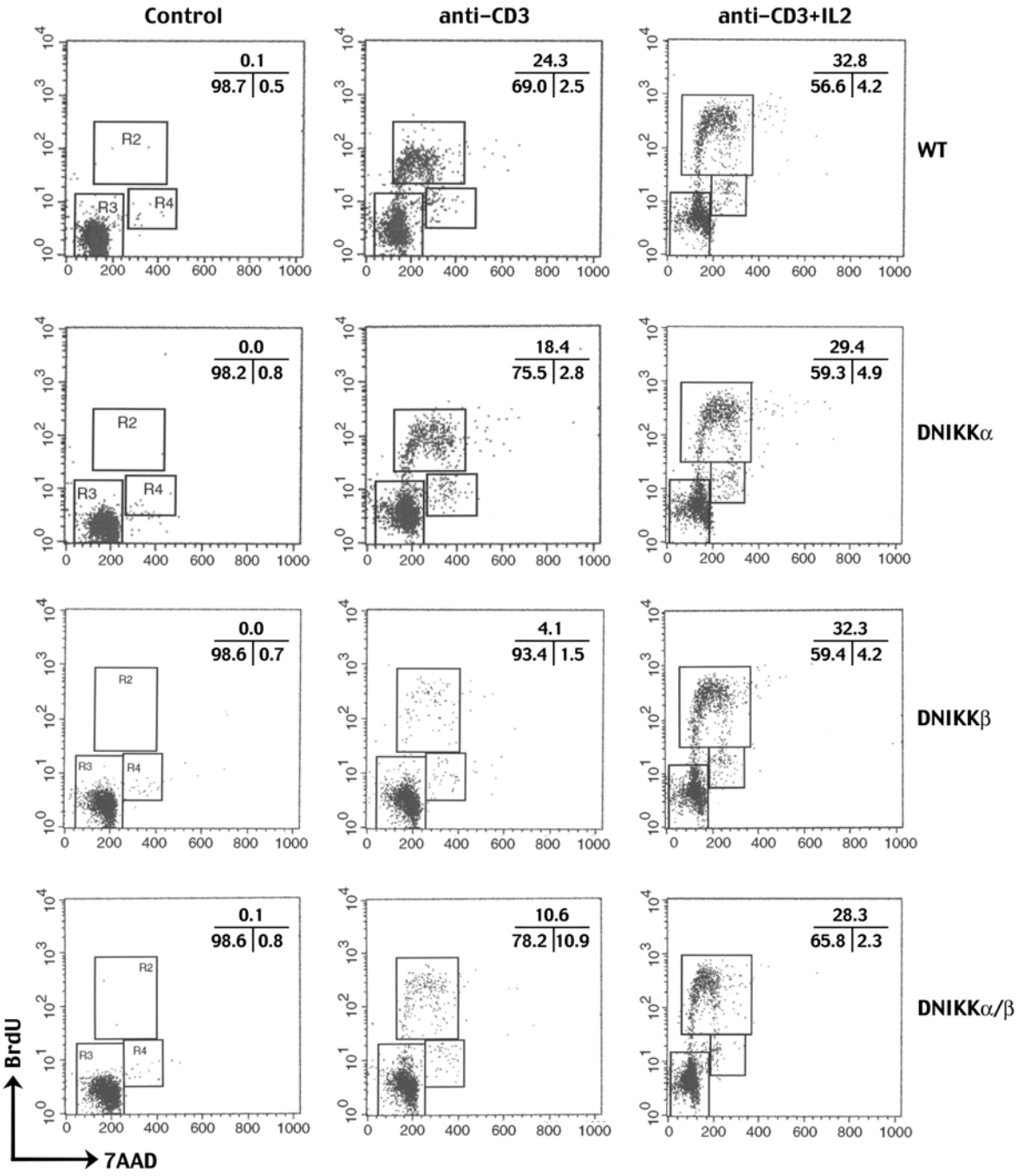

Control
anti-CD3
anti-CD3+IL2
0.1
98.7 | 0.5
24.3
69.0 | 2.5
32.8
56.6 | 4.2
WT
0.0
98.2 | 0.8
18.4
75.5 | 2.8
29.4
59.3 | 4.9
DNIKKα
0.0
98.6 | 0.7
4.1
93.4 | 1.5
32.3
59.4 | 4.2
DNIKKβ
0.1
98.6 | 0.8
10.6
78.2 | 10.9
28.3
65.8 | 2.3
DNIKKα/β
R2
R3
R4
BrdU
7AAD

Figure 20. Impaired cell cycle progression in T cells isolated from transgenic mice expressing DNIKKβ. The cell cycle profile of thymic T cells that were stimulated for 60 hours in RPMI with or without immobilized anti-CD3 (10μg/ml) or immobilized anti-CD3 and IL-2 (2ng/ml) was obtained using a BrdU Flow kit. Cells were pulsed with BrdU for 30 minutes and processed prior to staining with anti-BrdU to identify cells synthesizing DNA and 7-AAD to determine the total DNA content in the cells. The percentage of cells in the S phase (R2), G0/G1 phase (R3) and G2/M phase (R4) are shown in the upper right corner of each panel in the corresponding positions.

cycle progression seen with the DNIKKβ and DNIKKα/β thymocytes could be rescued by treatment with IL-2, as reflected in the similar percentages of S phase cells seen in wild-type and transgenic thymocytes. These results are consistent with the reduced NF-κB DNA binding activity and the reduced [^{3}H]-thymidine incorporation in thymocytes isolated from mice expressing DNIKKβ following stimulation with anti-CD3.

Differential cytokine expression in thymocytes isolated from DNIKK transgenic mice

To determine whether the cytokine production by T cells is affected by inhibition of IKKα or IKKβ, we analyzed the cytokine profile of both untreated and anti-CD3/anti-CD28 stimulated thymocytes isolated from wild-type and the DNIKK transgenic mice (Fig. 21). The mRNA levels of multiple cytokines were analyzed using RNase protection assays performed with a mouse cytokine multi-probe template set. Similar amounts of RNA were analyzed from each of these mice as demonstrated by the detection of similar levels of the house-keeping genes L32 and GAPDH (Fig. 21). Thymocytes isolated from DNIKKα and DNIKKβ transgenic mice produced relatively comparable levels of multiple cytokines including IL-4, IL-5, IL-10, IL-13, IL-2, IL-3 and IFNγ when compared with thymocytes isolated from wild-type mice. However, there were some differences in IL-4, IL-5, IL-13 and IFN-γ noted in the DNIKKα and DNIKKβ transgenic mice. In contrast, thymocytes isolated from DNIKKα/β transgenic mice produced markedly reduced levels of multiple cytokines (Fig. 21), indicating that inhibition of the activity of both IKKα and IKKβ can block the production of multiple cytokines in thymocytes.

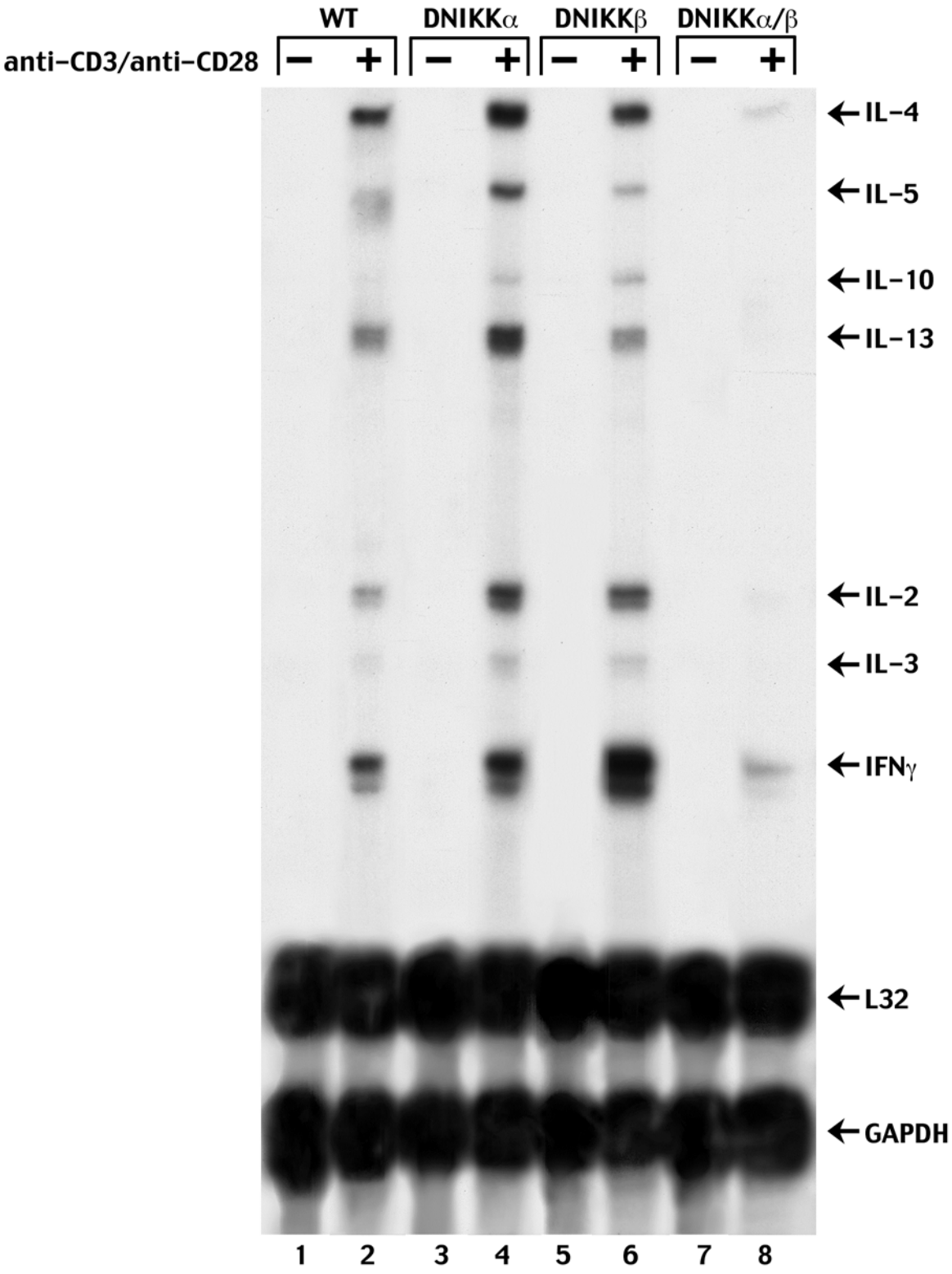
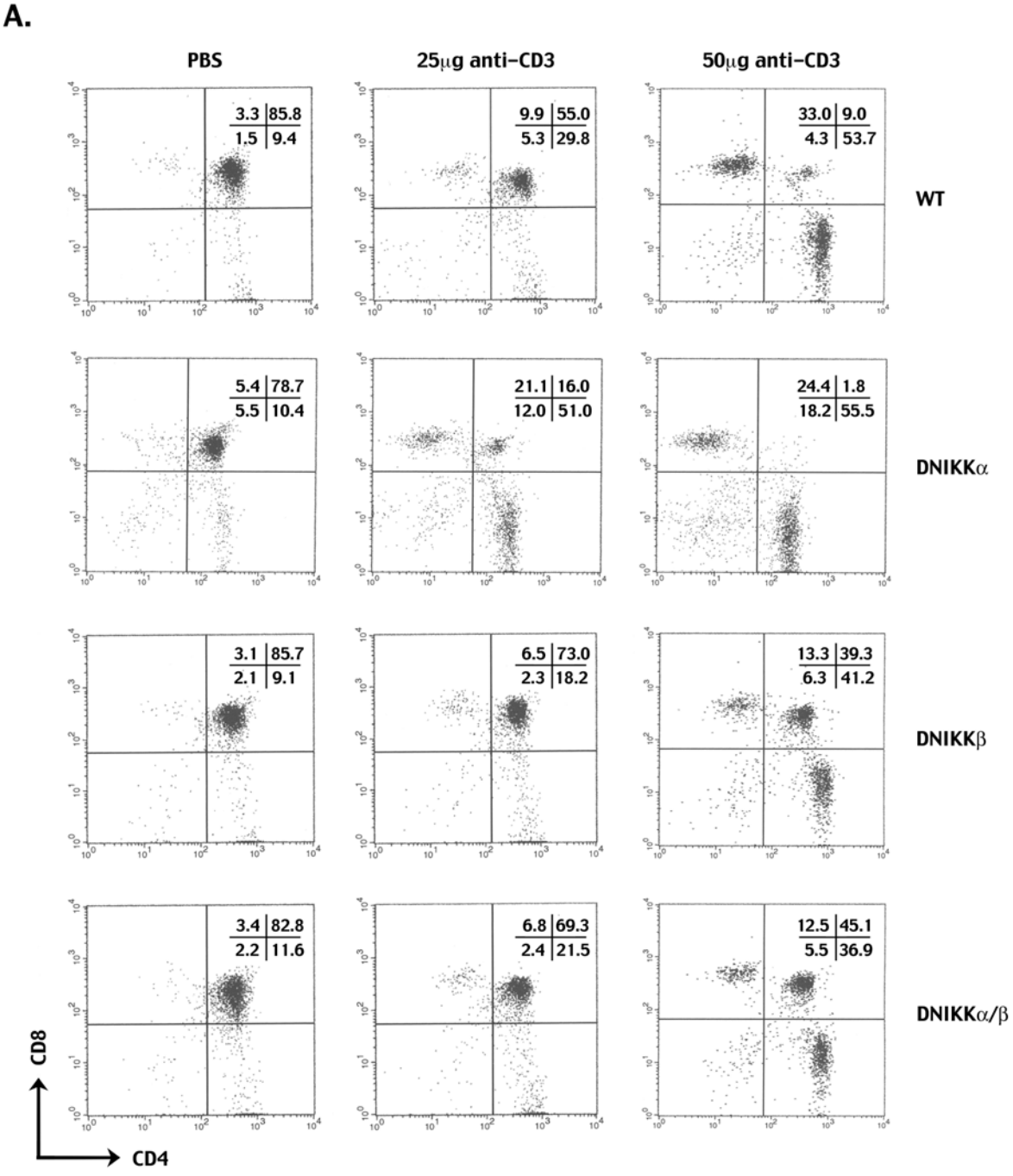

WT
DNIKKα
DNIKKβ
DNIKKα/β
anti-CD3/anti-CD28
− + − + − + − +
IL-4
IL-5
IL-10
IL-13
IL-2
IL-3
IFNγ
L32
GAPDH
1 2 3 4 5 6 7 8

Figure 21. Differential cytokine production in wild-type and transgenic thymocytes. Thymoctyes from wild-type and transgenic mice were cultured for 4-6 hours in complete RPMI media alone (lanes 1, 3, 5, 7) or with plated-coated anti-CD3 (10μg/ml) and anti-CD28 (16μg/ml) (lanes 2, 4, 6, 8). Total RNA was isolated and subjected to an RNase protection assay using a mouse cytokine multi-probe template set, mCK-1b. The positions of protected probes of IL-4, IL-5, IL-10, IL-13, IL-2, IL-3, IFNγ and house keeping genes L32 and GAPDH are shown.

Differential effects of DNIKKα and DNIKKβ on anti-CD3 induced apoptosis in double positive T cells

$CD4^+CD8^+$ thymocytes undergo apoptotic cell death when activated through the T cell receptor complex by systemic administration of anti-CD3 antibody, a phenomenon that is probably related to auto-antigen induced negative selection during T cell maturation (322-327). Previously, it was noted that the $CD4^+CD8^+$ thymocytes isolated from the transgenic mice that expressed a dominant negative IκBα protein in T cells were protected from anti-CD3 induced apoptosis (282). Since the dominant negative IκBα protein inhibited nuclear translocation of NF-κB proteins, these results suggested that activation of the NF-κB pathway leads to pro-apoptotic effects in immature thymocytes stimulated with anti-CD3.

Thus we addressed whether inhibition of IKKα or/and IKKβ altered the survival of $CD4^+CD8^+$ T cells following the *in vivo* administration of anti-CD3 (Fig. 22). In both wild-type and transgenic mice, 50 μg of anti-CD3 depleted more thymocytes than 25 μg of anti-CD3, indicating a dose-dependent effect of anti-CD3 antibody on thymocyte depletion (Fig. 22A&B). A decrease in the size of the thymus and fewer numbers of total thymocytes were noted in mice following anti-CD3 treatment (Fig. 22B). This depletion of thymocytes was associated with a selective decrease in the number of the double positive but not the single positive or the double negative thymocytes (Fig. 22B). Flow cytometry analysis indicated that the double positive thymocytes from DNIKKβ transgenic mice were significantly protected against anti-CD3 induced apoptosis (Fig. 22A). This result is similar to that obtained with DNIκBα transgenic mice (282). In contrast, thymocytes isolated from the DNIKKα transgenic mice had the opposite phenotype with increased susceptibility to


A.
PBS
25μg anti-CD3
50μg anti-CD3
3.3 85.8
1.5 9.4
9.9 55.0
5.3 29.8
33.0 9.0
4.3 53.7
WT
5.4 78.7
5.5 10.4
21.1 16.0
12.0 51.0
24.4 1.8
18.2 55.5
DNIKKα
3.1 85.7
2.1 9.1
6.5 73.0
2.3 18.2
13.3 39.3
6.3 41.2
DNIKKβ
3.4 82.8
2.2 11.6
6.8 69.3
2.4 21.5
12.5 45.1
5.5 36.9
DNIKKα/β
CD8
CD4

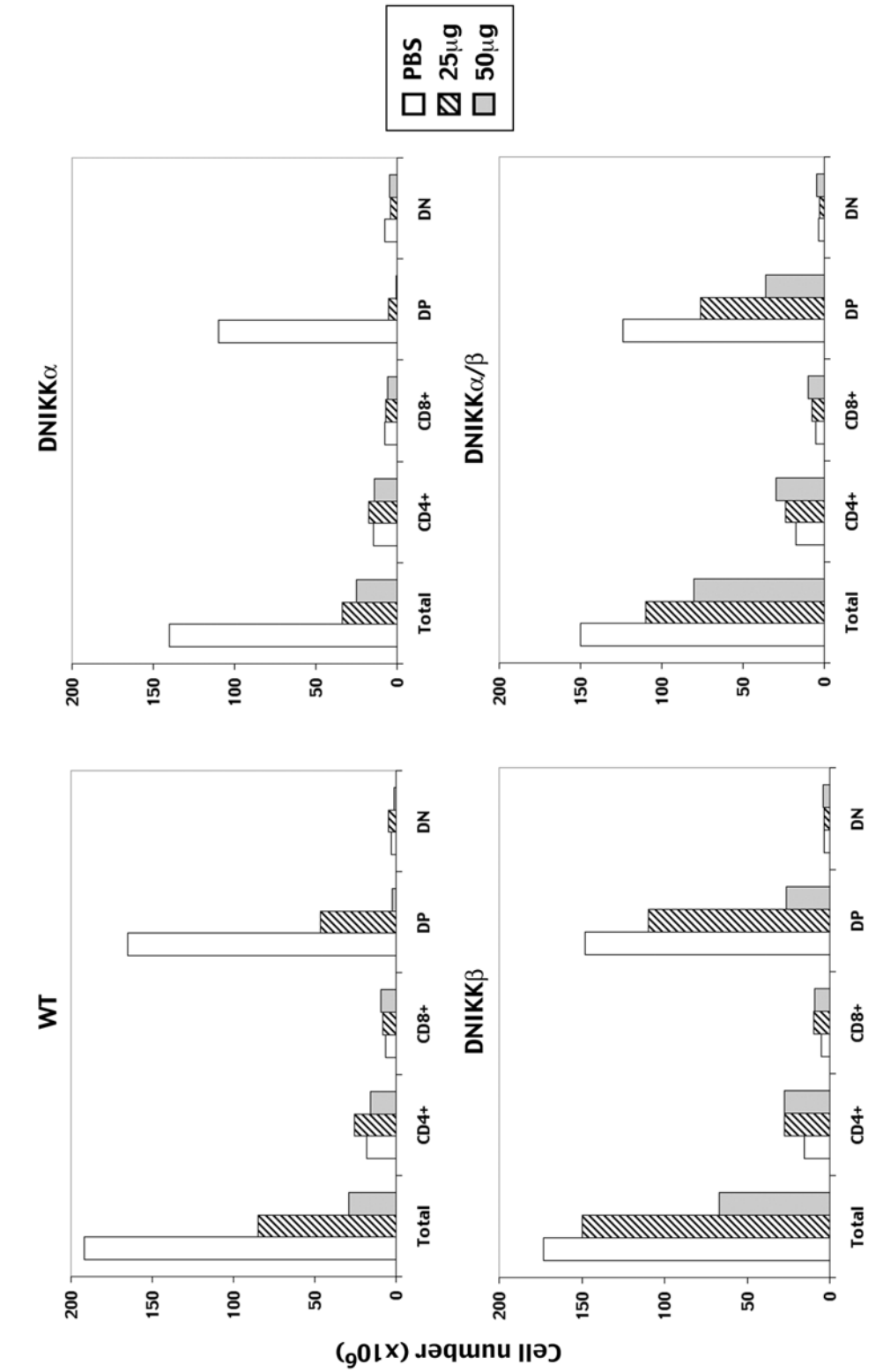

B.
Cell number (x10⁶)
WT
DNIKKβ
DNIKKα
DNIKKα/β
PBS
25μg
50μg
Total
CD4+
CD8+
DP
DN
0
50
100
150
200

Figure 22. DNIKKα and DNIKKβ result in different effects on α-CD3 induced apoptosis. Wild type (WT), DNIKKα, DNIKKβ, and DNIKKα/β mice were each injected intraperitoneally with 100μl of PBS alone or PBS containing 25μg or 50μg of anti-CD3. Thymocytes of these mice analyzed by (A) flow cytometry 48 hours after anti-CD3 administration with fluorescent anti-CD4 and anti-CD8. Percentages of each subsets were shown in the upper right corner of each panel in corresponding positions. (B) Absolute numbers of total and different subsets of thymocytes. Numbers of T cell subsets were calculated according to the numbers of total thymocytes and the percentages of subsets obtained from (A). Total, total thymocytes isolated from each thymus; $CD4^+$, $CD4^+$ $CD8^-$ single positive cells; $CD8^+$, $CD4^-CD8^+$ single positive cells; DP, $CD4^+CD8^+$ double positive cells; DN, $CD4^-CD8^-$ double negative cells.

apoptosis as compared to thymocytes isolated from either wild-type or DNIKKβ mice (Fig. 22A). Finally, there was increased protection against anti-CD3 induced apoptosis in thymocytes expressing both DNIKKα and DNIKKβ as compared to thymocytes obtained from wild-type mice (Fig. 22A). These results suggest that IKKα has anti-apoptotic effects on anti-CD3 activated double positive T cells while IKKβ has pro-apoptotic effects on these cells. In thymocytes containing both DNIKKα and DNIKKβ, the pro-apoptotic role of IKKβ was dominant over the anti-apoptotic effects of IKKα.

D. Discussion

There is little data available concerning the role of IKKα and IKKβ in the ontogeny and regulation of T cell function. Since disruption of IKKα (124, 125) and IKKβ (103, 107, 129) genes results in early lethality of mice, we studied the effects of dominant negative mutants of IKKα and IKKβ on both the development and function of T lymphocytes. Since the dominant negative mutants were expressed at higher levels than the endogenous kinases, the endogenous kinases were likely substantially inhibited and the function of these kinases in T cells could be addressed.

NF-κB DNA binding activity was substantially inhibited in thymocytes from both DNIKKα and DNIKKβ mice following treatment with PMA and ionomycin. There was even greater inhibition noted in thymocytes from DNIKKα/β double mutant mice. Although IKKα is not as critical as IKKβ in the cytokine-mediated activation of NF-κB pathway (107, 124, 125), there is significant evidence demonstrating that IKKα contributes to the activation

of the conventional NF-κB pathway (108-110, 328). Our results suggest that IKKα is involved in the activation of NF-κB pathway in T cells following treatment with PMA and ionomycin. However, IKKα appeared to be dispensable for the NF-κB activation induced by anti-CD3 treatment in thymocytes. Consistent with previous results demonstrating that PKC-θ mediates NF-κB activation upon TCR engagement (85, 87) by activating IKKβ (88), our data suggest that IKKβ is the dominant kinase mediating TCR induced NF-κB activation.

The impaired proliferative response induced by anti-CD3 mediated TCR-crosslinking in thymocytes isolated from DNIKKβ and DNIKKα/β transgenic mice suggests that IKKβ is the dominant kinase involved in this process. This defect was associated with marked alterations in cell cycle progression. The relatively normal cell cycle progression and proliferative response induced by anti-CD3 in thymocytes from DNIKKα mice may be attributed to their relatively normal NF-κB activation. It is interesting to note that splenocytes from DNIKKβ mice exhibited a relatively normal proliferative response to anti-CD3 stimulation. This may reflect the state of maturation of the splenic T cells and additional growth signals that are provided by other types of cells in the spleen. Unlike the data from mice expressing a dominant negative IκBα molecule (279), IL-2 was able to largely correct the proliferative defects of thymocytes from DNIKKβ and DNIKKα/β transgenic mice in response to anti-CD3 treatment. Furthermore, these results suggest that the IL-2 signaling pathway in these cells is intact.

IKKα and IKKβ are critical for the production of cytokines in thymocytes. Inhibition of both IKKα and IKKβ in thymocytes from DNIKKα/β transgenic mice reduced the

expression of both Th1 and Th2 cytokines following stimulation with anti-CD3 and anti-CD28. However, thymocytes isolated from DNIKKα or DNIKKβ transgenic mice expressed significant levels of these cytokines. These results suggest that IKKα and IKKβ likely provide redundant functions required for production of Th1 and Th2 cytokines. Interestingly, thymocytes from DNIKKβ mice expressed somewhat increased levels of mRNA encoding the Th1 cytokine IFN-γ, while DNIKKα thymocytes tended to express somewhat higher levels of IL-4, IL-5, and IL-13 as compared to thymocytes obtained from wild-type mice. Previously it was shown that $p50^{-/-}$ mice were unable to mount an airway eosinophilic inflammatory response (329, 330) due to their inability to activate the GATA-3 transcription factor that regulates the production of the Th2 cytokines IL-4, IL-5, and IL-13 (331). However, $CD4^+$ T cells from these mice showed unimpaired production of IFN-γ under Th1-differentiating conditions (329). In contrast, NF-κB2$^{-/-}$ mice failed to mount a cell-mediated immune response against the intracellular parasite *Toxoplasma gondii* (249, 332), and this correlated with the inability of their T cells to produce the Th1 cytokine IFN-γ (332). These results suggest that differential regulation of the NF-κB pathway can alter the production of Th1 and Th2 cytokines. Our data demonstrates that subtle changes in the cytokine profiles of DNIKKα and DNIKKβ thymocytes likely reflect distinct effects of IKKα and IKKβ on NF-κB induction of Th1 and Th2 cytokines.

NF-κB has been demonstrated to have both anti- (279) and pro-apoptotic (279, 282, 318) effects on the survival of lymphocytes. Hettmann *et al*. demonstrated that $CD4^+CD8^+$ thymocytes isolated from transgenic mice expressing a dominant negative IκBα mutant were

more resistant to anti-CD3 induced apoptosis than were wild-type cells (282). This protective effect of blocking the NF-κB pathway in double positive thymocytes correlated with the high levels of the anti-apoptotic protein bcl-xl (333). In agreement with these results, we found that there was reduced apoptosis in double positive thymocytes isolated from the DNIKKβ mice following systematic administration of anti-CD3. However, peripheral $CD4^+$ and $CD8^+$ single positive T cells isolated from transgenic mice expressing dominant negative IκBα exhibited increased apoptosis upon anti-TCR stimulation (279). Thus, NF-κB is likely to regulate the expression of distinct pro-apoptotic and anti-apoptotic genes in different developmental stages and in response to different stimuli.

While IKKβ mediated NF-κB activation may prevent apoptosis induced by TNF-α treatment during the physiological development of lymphocytes and hepatocytes (103, 129, 138), our results suggest that it may promote apoptosis induced by TCR activation in double positive thymocytes. In contrast to the results with IKKβ, IKKα has an anti-apoptotic role in double positive thymocytes following treatment with anti-CD3. IKKα has also been demonstrated to prevent apoptosis in B cells (139, 140). Although the mechanism by which IKKα prevents apoptosis remains to be elucidated, it is possible that it mediates these effects through p52, which has been demonstrated to be important in preventing apoptosis of T cells (332). Our results indicate that the pro-apoptotic effects of DNIKKβ are dominant over the anti-apoptotic effects of DNIKKα as reflected by the decreased amounts of apoptosis seen in double positive T cells isolated from DNIKKα/β transgenic mice.

In this study, transgenic mice in which IKKα and IKKβ function is partially inhibited have allowed us to gain additional insights into the distinct physiological roles of these kinases in the development and function of T cells. Normal levels of IKKα and IKKβ activity are not required for the development of T lymphocytes. However, IKKβ, but not IKKα, is critical in mediating the proliferative response of thymic T cells upon TCR-stimulation while both of these kinases are critical in regulating cytokine production. Finally, IKKα and IKKβ have important and distinct roles in regulating apoptosis of immature thymocytes. These studies will help to address the distinct roles of IKKα and IKKβ in regulating immune function.

CHAPTER IV. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

In order to evaluate the role of IKKα and IKKβ in lymphocyte development and function, dominant negative forms of IKKα and IKKβ were targeted into mouse lymphocytes using either B cell- or T cell-specific promoters. Phenotypic studies of IgH-DNIKKβ, CD2-DNIKKα, CD2-DNIKKβ and CD2-DNIKKα/β transgenic mice presented in this thesis have contributed to our overall understanding of the physiological role of IKKα and IKKβ in the development and function of lymphocytes. It is clear that partial inhibition of IKKβ did not affect B cell ontogeny while inhibition of either or both IKKα and IKKβ did not alter T cell ontogeny. It is also clear that inhibition of IKKβ specifically in B or T cells prevented NF-κB activation in response to potent B or T cell mitogens. Accordingly, their proliferative response was markedly reduced due to impaired cell cycle progression. In contrast, inhibition of IKKα in T cells did not significantly inhibit the NF-κB activation, cell cycle progression or proliferation of thymic T cells in response to TCR crosslinking. IKKβ was found to have a critical role in mediating the TI-2 response and isotype switching in TD response. In addition, IKKα and IKKβ may have redundant functions in mediating cytokine expression by thymocytes but distinct roles in regulating thymocyte apoptosis. Table 3 and 4 illustrates some of the major findings in this thesis and some recent results of the role of IKKα and IKKβ in the development and function of B and T lymphocytes.

Table 3. Role of IKKα and IKKβ in the development and function of B lymphocytes

Function	IKKα	IKKβ
Development	*required	normal level not required
Proliferation	?	required
Cell cycle progression	?	required
In vitro Ig secretion	?	normal level not required
In vivo basal Ig	?	normal level not required
TI-1 response	?	normal level not requried
TI-2 response	?	required
TD response	?	required

"*" indicates result not from the thesis

Table 4. Role of IKKα and IKKβ in the development and function of T lymphocytes

Function	IKKα	IKKβ
Development	normal level not required	normal level not required
TCR mediated proliferation of thymocytes	normal level not required	required
TCR mediated Cell cycle progression of thymocytes	normal level not required	required
Cytokine production of thymocytes	not required in the presence of IKKβ	not required in the presence of IKKα
Anti-CD3 induced apoptosis of DP cells	anti-apoptotic	pro-apoptotic

A. The role of IKKα and IKKβ in the development and function of B cells

The normal B cell development seen in IgH-DNIKKβ transgenic mice is reflected by the normal structure and cellularity in their spleen with normal numbers of $B220^{+}$ cells, mature B cells ($IgM^{low}IgD^{high}$) and marginal zone B cells ($CD21^{high}CD23^{low}$). B cells from these mice showed normal usage of the κ chain. These results indicated that normal levels of IKKβ in B lineage cells is not required for B cell development. However, when IKKβ activity is completely eliminated, as is the situation of recent studies when fetal liver cells from $IKK\beta^{-/-}$ mice were transplanted into lethally irradiated hosts, maturation of B cells did not occur (138, 139). The absence of B cell maturation in the $IKK\beta^{-/-}$ radiation chimeras was rescued by the co-transplantation of $IKK\beta^{-/-}$ fetal liver cells with wild-type bone marrow cells, which provide normal stromal cells in the hematopoietic microenvironment (138). This result is strikingly similar to that obtained with p50/p65 double knockout radiation chimeras (132) which suggested that the normal NF-κB activation in the surrounding cells rather than the developing cells is more important for the maturation of B cells. Therefore, the absence of B cells in $IKK\beta^{-/-}$ chimeras is most likely due to absence of stromal cells that have normal NF-κB activity. However, Senftleben *et al*. proposed that another reason for the failure of B cell development is the increased apoptosis of developing $IKK\beta^{-/-}$ cells induced by TNF-α (138). It is intriguing to determine which of these possibilities is the major reason for failure of B cell maturation in the absence of IKKβ. Generation of conditional knockout mice with IKKβ absent in B lymphocytes and their progenitors would give additional insights into the role of IKKβ in the development of B cells. If such mice exhibited normal development of B

cells and normal B cell sensitivity to TNF-α induced apoptosis, it would suggest a critical role for NF-κB activation in the stromal cells that is required for the development of B cells. However, if these mice did not have normal B cell development and the removal of TNF-α signaling could rescue the developmental defects, it would suggest that the NF-κB activity in the developing B cells is required to prevent TNF-α induced apoptosis, leading to the normal development of B lymphocytes.

In contrast to IKKβ, IKKα has a much more defined role in the maturation of B cells as recently revealed through studies of the IKKα$^{-/-}$ radiation chimeras (139, 140). IKKα mediates the processing of NF-κB2 by phosphorylating the C-terminal region of this molecule, leading to its subsequent proteolytic processing and generation of p52. The p52 subunit is important for the B cell maturation and formation of secondary lymphoid organs. IKKα$^{-/-}$ radiation chimeras exhibited a similar phenotype as that seen in the NF-κB2 deficient mice due to their inability to generate p52 in the developing B lineage cells. Both of these mutant mice exhibited severe developmental defects in their B cell compartment (248, 249). The generation of transgenic mice that exhibit B cell specific expression of a dominant negative IKKα mutant is ongoing. Analysis of B cell population as well as the lymphoid structure in these mice will help to elucidate whether the amount of IKKα activity is important for the production of sufficient p52 to support the development of B cells and formation of secondary lymphoid organs. Moreover, due to the B cell specific expression of the DNIKKα mutant, these mice can be used to address whether the presence of IKKα in

developing B cells or in the stromal cells is important for the maturation of B cells and the formation of secondary lymphoid organs.

The phenotypic analysis of IgH-DNIKKβ mice revealed a clear role of IKKβ in B cell function in terms of B cell proliferation, cell cycle progression, basal production of immunoglobulins and antibody response to specific antigens. *In vitro*, inhibition of IKKβ activity resulted in decreased proliferation and impaired cell cycle progression from G0/G1 to S phase in response to LPS, anti-IgM and anti-CD40. The inhibitory effect on the proliferation in response to LPS was greater than that in response to anti-IgM and anti-CD40. Consistent with previous findings on $p50^{-/-}$ B cells (252), these results suggest that the proliferation of B cells in response to LPS is more dependent on intact IKKβ and p50 mediated NF-κB activation than is their response to anti-IgM and anti-CD40. It is likely that TLR (Toll like receptor) mediated signaling initiated by LPS target on NF-κB activation to induce B cell growth, whereas signaling mediated by BCR and CD40 target on other pathways in addition to the NF-κB pathway to increase B cell proliferation. The reduced immunoglobulin secretion by B cells from DNIKKβ transgenic mice in response to these B cell mitogens was proportional to their proliferation, indicating that the ability of B cells from the DNIKKβ transgenic mice to secrete immunoglobulin was not impaired.

In vivo analysis of DNIKKβ transgenic mice revealed that DNIKKβ did not affect the overall basal levels of IgG1, IgG3 and IgG2a in the sera of naïve mice. It is possible that basal Ig production requires very low levels of NF-κB activity that is present in DNIKKβ transgenic mice. It is also possible that basal Ig production is mainly regulated by T cell function that is intact in these mice. Consistent with the latter possibility, the $p50^{-/-}$, c-$rel^{-/-}$

mice, with impaired NF-κB activation in their T cells, have reduced basal Ig levels (251, 252). Since a substantial number of splenic B1 B cells are IgM-secreting plasma cells and their antibody dominates serum IgM (334, 335), the slightly lower basal IgM levels in the DNIKKβ suggest a possible role of IKKβ in mediating IgM secretion of B1 B cells.

The impaired humoral response to the type 2 T-independent antigen (TI-2) TNP-Ficoll and the normal response to the type 1 T-independent antigen (TI-1) TNP-LPS in the DNIKKβ transgenic mice indicated that these mice have a similar phenotype to that exhibited by the *xid* (X-linked immunodeficiency) mice, which have a mutant Bruton's tyrosine kinase (Btk) gene (336, 337). These defects are probably due to the fact that IKKβ is a downstream kinase that is regulated by Btk mediated signaling (84, 336, 338). However, DNIKKβ mice exhibited additional defects in humoral response in that administration of a T-dependent antigen TNP-KLH in these mice elicited reduced levels of TNP-specific IgG1 and IgG2a, which were normal in *xid* mice (339). Since anti-CD40 stimulation resulted in normal IgG1 production *in vitro*, there are probably additional signals, which are interrupted by DNIKKβ, that are required for isotype switching *in vivo*. These signals may be provided by cytokines such as IL-6 (340, 341) and cognate interaction of B and T cells mediated by CD40-CD40 ligand as well as adhesion molecules such as LFA-1-ICAM-1 and LFA-3-CD2.

Phenotypic analysis of DNIKKβ mice indicated that IKKβ activity in B cells is not required for the development of B cells, but is critical for their proliferative response following mitogenic stimulation and their antibody response to TI-2 and TD antigens. Once again, the generation of conditional knock out mice with a B cell specific loss of IKKβ would provide additional information about the role of IKKβ in regulating B cell function.

For expample, if B cell proliferation in response to BCR crosslinking and anti-CD40 was eliminated in the complete absence of IKKβ, it would suggest this B cell function only requires only low levels of IKKβ activity and therefore NF-κB activation. However, if B cells from such mice could not proliferate in response to either mitogenic stimulation or *in vivo* specific antigen priming, these cells could not be untilized to investigate the role of IKKβ in regulating the humoral response to both TD and TI antigens. Therefore, the transgenic approach used in this research might have advantages over the conditional knockout strategy.

B. The role of IKKα and IKKβ in the development and function of T cells

Generation of transgenic mice with the T cell specific expression of dominant negative IKKα (DNIKKα) or/and dominant negative IKKβ (DNIKKβ) provides a powerful tool to study the role of these two catalytic subunits of IKK complex in the development and function of T cells.

Our data suggested that partial inhibition of either IKKα or IKKβ or both kinases in T lineage cells does not alter T cell development in a wild type background. Previous data indicated that the homozygous deletion of IKKα did not alter the establishment of normal T cell population. Fetal liver cells from IKKα$^{-/-}$ mice were able to reconstitute normal numbers of thymic and peripheral T cells in lethally irradiated hosts (139, 140), indicating that IKKα is not required either directly or indirectly for the development of T lymphocytes.

The role of IKKβ in T cell development was more intriguing. IKKβ$^{-/-}$ fetal liver cells were unable to reconstitute mature T cells in transfer experiments (138). This failure of T cell

development was rescued by either co-transplantation of wild type bone marrow cells with the IKKβ$^{-/-}$ fetal liver cells or transplantation of IKKβ$^{-/-}$TNFR$^{-/-}$ double mutant fetal liver cells into the irradiated hosts. This suggested two possible mechanisms for the failure of IKKβ$^{-/-}$ fetal liver cells to reconstitute T cells. First, T cells lacking IKKβ may develop normally as long as there are surrounding stromal cells with normal NF-κB activation. Secondly, the developing T cells may be able to give rise to normal T cell subsets as long as they are protected from TNF-α induced apoptosis. However, since the immature IKKβ$^{-/-}$ thymocytes from the chimeras are more sensitive to TNF-α mediated apoptosis and the level of circulating TNF-α in these chimeras is higher than that seen in the wild type chimeras, Senftelben *et al.* proposed that the TNF-α induced apoptosis is the predominant reason for the absence of T lymphocytes in IKKβ$^{-/-}$ chimeras. The altered hematopoietic microenvironment in the IKKβ$^{-/-}$ chimeras had higher levels of TNF-α than that seen in wild type chimeras, probably due to the increased number of myeloid cells. Therefore, TNF-α induced apoptosis is likely to be a major reason for the failure of T cell ontogeny in the IKKβ$^{-/-}$ chimeras. The normal development of T cells in DNIKKβ transgenic mice indicated that normal levels of IKKβ activity in developing T cells is not critical in order to prevent these cells from undergoing apoptosis. However, to examine the role of TNF-α induced apoptosis and the hematopoietic microenvironment in regulating T cell development, it is necessary to generate conditional knockout mice with homozygous deletion of IKKβ specifically in T lineage cells. The success or failure of T cell development in these mice would one to assess the importance of IKKβ activity in the stromal cells and in the

developing T cells, respectively. The normal T cell development in the DNIKKα/β double mutant mice suggest that there is no functional redundancy between IKKα and IKKβ in terms of their role in mediating T cell development.

IKKα and IKKβ have distinct roles in T cell functions in terms of proliferation, cytokine production and survival. The research presented in this dissertation was focused on TCR mediated function of thymic T cells.

T cell proliferative responses in response to TCR cross-linking are mediated by cellular signaling pathways that activate NF-κB (320, 321) and other transcription factors such as AP-1 (342) and NF-AT (343-345). Multiple NF-κB subunits, such as p50, c-rel, RelA, are involved in the T cell proliferative response as indicated by the impaired proliferation of T cells from mice that are deficient in each of these subunits (250-252). The decreased proliferation of T cells from DNIKKβ, but not DNIKKα, transgenic mice suggested that IKKβ is more important than IKKα in mediating T cell proliferation. Several groups demonstrated that TCR stimulation activates NF-κB through stimulating IKK activity (85-88). Therefore, it is likely that IKKβ- but not IKKα- mediated activation of the NF-κB pathway leads to the nuclear translocation of NF-κB dimers containing p50, c-rel and RelA, which activate genes that are involved in T cell proliferation. Proliferation of DNIKKα/β thymocytes in response to TCR crosslinking was reduced to a similar extent to that seen in DNIKKβ thymocytes, indicating that the inhibitory function of DNIKKβ is not altered by the presence of DNIKKα. Interestingly, the proliferative response of splenic T cells from DNIKKβ mice appeared normal, indicating that normal levels of IKKβ activity are not

required for the proliferation of mature T cells. The differential proliferative responses of thymic and splenic T cells was also observed in the $fyn^{-/-}$ mice (346, 347), which lack the $p59^{fyn}$, a non-receptor tyrosine kinase that is involved in the TCR-induced lymphocyte activation. There are two possible reasons for the differential dependence on IKKβ seen with thymic versus splenic T cells in response to TCR derived signals. First, the degree of maturity of the single positive thymocytes as opposed to the peripheral T cells may render the dependence on IKKβ of these cells for TCR-crosslinking induced proliferation (348). Second, IKKα and IKKβ may have redundant functions in regulating the proliferation of the peripheral but not the thymic T cells. In fact, splenic T cells from DNIKKα/β transgenic mice exhibited reduced proliferation in response to anti-CD3, suggesting that IKKα may compensate for IKKβ in mediating TCR-triggered proliferative response of peripheral T cells. The selective proliferative defects in thymic but not splenic T cells may also be due to the fact that the latter were co-cultured with other splenic cells that provide growth signals through either cell-cell contact or cytokine production. Proliferation assays using purified splenic T cells will be needed to test this hypothesis.

The DNIKK transgenic mice exhibited interesting phenotypes in terms of the cytokine production of their thymocytes. While thymocytes isolated from DNIKKα and DNIKKβ mice were able to produce multiple cytokines including IL-4, IL-5, IL-10, IL-13, IL-2, IL-3, and IFN-γ to a comparable level as thymocytes from normal mice, expression of all these cytokines was severely inhibited in thymocytes from DNIKKα/β transgenic mice. These results indicated that IKKα and IKKβ may have redundant function in regulating

cytokine production. DNIKK transgenic mice could be used as powerful tools to study the role of IKK in regulating Th1 and Th2 responses. Previously, it was shown that T cells from $p50^{-/-}$ mice were not able to produce Th-2 cytokines IL-4, IL-5, IL-13 when the animals were exposed to antigens that can cause airway inflammation in normal mice (329-331). In contrast, NF-$\kappa B2^{-/-}$ mice could not produce IFN-γ that is critical to mediate the cellular immunity to clear the infection of *T. gondii* (248, 249, 332). It would be interesting to see how DNIKK transgenic mice respond to these antigenic challenges. If DNIKKα transgenic mice exhibited a similar phenotype as did NF-$\kappa B2^{-/-}$ mice when challenged with *T. gondii*, it would suggest that DNIKKα inhibited the Th1 response by inhibiting the processing of NF-κB2 and the generation of p52, which seem to be important for the production of IFN-γ and the clearance of the pathogen. Western blot analysis to detect the levels of p100 and p52 in T cells from DNIKKα mice could be used to further examine this possibility. In addition, if DNIKKβ transgenic mice were not able to mount airway inflammation as seen in $p50^{-/-}$ mice, it would suggest that inhibition of IKKβ blocked NF-κB activation that normally up-regulates Th2 cytokine expression. These studies would provide novel information about the role of IKKα and IKKβ in regulating cytokine production in mature T cells and Th1 and Th2 responses.

The use of anti-CD3 to induce apoptosis of $CD4^+CD8^+$ double positive thymocytes revealed another distinct role of IKKα and IKKβ in regulating T cell function. The role of NF-κB in regulating lymphocyte apoptosis has been intriguing due to its opposite effects under different condition (138, 140, 279, 282). The results obtained from our studies using

DNIKK transgenic mice suggested that IKKα has anti-apoptotic effect on double positive thymocytes in mice treated with anti-CD3, whereas IKKβ has pro-apoptotic effect. In addition, the pro-apoptotic effects of IKKβ are dominant over the anti-apoptotic effect of IKKα. Double positive thymocytes in transgenic mice expressing DNIκBα exhibited reduced apoptosis following administration of anti-CD3 (282). These results suggest that IKKβ mediated-NF-κB activation elicits cell death signals in double positive thymocytes stimulated with anti-CD3 *in vivo*, whereas signaling mediated by IKKα elicits cell survival signals. Inhibition of both the death signal and the survival signal resulted in cell survival in the double positive T cells from DNIKKα/β mice, indicating that the survival signal mediated by IKKα is not necessary for the cells to survive if the death signal is blocked by DNIKKβ.

Since anti-CD3 induced apoptosis of double positive T cells is a phenomenon that is related to autoantigen induced negative selection during T cell maturation (322-327), the distinct effects of DNIKKα and DNIKKβ on anti-CD3 induced apoptosis of double positive thymocytes suggested that the DNIKK transgenic mice can be used to study the role of IKKα and IKKβ in T cell negative selection. In order to do so, we crossed DNIKKα and DNIKKβ mice to the HY male antigen TCR transgenic mice. HY-TCR transgenic male mice do not have single positive T cells due to depletion of virtually all T cells during negative selection. HY-TCR transgenic female mice have enlarged proportion of CD8+ T cells that are positively selected by the MHC class I H-2D^{b} during positive selection (349, 350). If IKKβ provides a cell death signal as suggested by the decreased apoptosis of DNIKKβ double

positive thymocytes, expression of DNIKKβ would inhibit negative selection of T cells in HY-TCR transgenic mice. Therefore, there would be some single positive T cells in the HY-TCR/DNIKKβ transgenic mice. The analysis of the thymocyte population of HY-TCR/DNIKKα and HY-TCR/DNIKKβ transgenic mice is currently being performed.

IKKβ$^{-/-}$ fetal liver cells were unable to reconstitute lymphocytes in irradiated chimeras due to apoptosis induced by TNF-α (138). Combined with the observations in the IKKβ$^{-/-}$, IKKγ$^{-/-}$, and RelA$^{-/-}$ mice (103, 129, 131, 134), which die of liver degeneration due to massive apoptosis of hepatocytes, these results suggest that IKKβ mediated NF-κB activation is critical in preventing developing cells from undergoing TNF-α mediated apoptosis. Taken together, IKKβ appeared to have an anti-apoptotic role in lymphocytes during their physiological development, but a pro-apoptotic role when developing cells encounter exogenous stimulation such as cross-linking of TCR. However, blocking the NF-κB pathway by overexpression of IκBα was shown to prevent apoptosis of mature T cells caused by TCR stimulation (279). Investigation of both the turnover of mature IKKβ$^{-/-}$ T cells and the rate of spontaneous and activation induced death of IKKβ$^{-/-}$ T cells would give additional insight into the role of IKKβ in regulating T cell survival.

In contrast, IKKα seemed to consistently have anti-apoptotic effect. IKKα$^{-/-}$ B cells from IKKα$^{-/-}$ cell reconstituted radiation chimeras have a higher turn over rate as well as a higher rate of spontaneous and activation induced death (138, 140), suggesting that IKKα prevents apoptosis in mature B cells. Our results demonstrated that IKKα is anti-apoptotic in developing T cells when they are stimulated with anti-CD3. Analysis of the

apoptosis in both thymic and peripheral T cells *in vivo* and *in vitro* in DNIKKα mice and IKKα$^{-/-}$ radiation chimeras will be helpful in further determining the role of IKKα in regulating apoptosis in T lymphocytes.

Taken together, the research presented in this thesis provides a characterization of the role of IKKα and IKKβ in the development and function of both B and T lymphocytes. The generation of IgH-DNIKKβ, CD2-DNIKKα, CD2-DNIKKβ, and CD2-DNIKKα/β transgenic mice provides powerful tools to further investigate the role of IKKα and IKKβ in the function of the immune system and may yield useful insights for better understanding the immune response to various pathogens, allergic and autoimmune response.

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