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Regulation of Development and Function of Different T Cell Subtypes by Rel/NF- κ B Family Members

S. Vallabhapurapu

Institut für Toxikologie und Genetik

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Institut für Toxikologie und Genetik

von der Fakultät für Chemie und Biowissenschaften der

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Regulation of development and function of different T cell subtypes by Rel/NF- κ B family members

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SUMMARY

The Rel/NF- κ B family of transcription factors plays an important role in lymphocyte development and function and in the regulation of innate and adoptive immune responses. It has been shown that NF- κ B is dispensable for the development of mainstream T cells, but its role in the development of other sub-types, such as natural killer T (NKT) cells, has not been addressed. Similar to mainstream T cells, NKT cells also develop in the thymus although a few thymus-independent NKT cells may exist. Thymus-dependent NKT cells are key regulators of innate and adoptive immune responses and are involved in diverse immune functions ranging from suppression of autoimmunity to tumor rejection. They express the NK1.1 marker, an invariant V α 14-J α 18 T cell receptor (TCR), and are positively selected by the major histocompatibility complex (MHC) class I-like molecule CD1d. However, the molecular events involved downstream of CD1d are poorly understood. This study reveals the requirement of distinct members of the Rel/NF- κ B family in both hematopoietic and non-hematopoietic cells for the development of thymic NKT cells. Activation of NF- κ B via the classical I κ B α -regulated pathway is required within the NKT precursors for their efficient maturation from NK1.1⁻ precursors to mature NK1.1⁺ NKT cells. The Rel/NF- κ B family member RelB, on the other hand, is required in thymic stromal cells for the generation of very early NK1.1⁻ precursors. NF- κ B-inducing kinase (NIK) has also been shown to be required in thymic stromal cells for NKT cell development and this study demonstrates that NIK specifically regulates both constitutive and signal-induced DNA binding of RelB, but not RelA. Moreover, NIK-induced DNA binding of RelB depends on the processing of inhibitory p100 to p52, revealing an alternate pathway of NF- κ B induction. Thus, Rel/NF- κ B complexes activated by the classical I κ B α -regulated pathway in NKT precursors and an alternate NIK/p100/RelB pathway in thymic stromal cells regulate different stages of NKT cell development.

In addition to its role in lymphocyte development, NF- κ B also regulates survival and proliferation of peripheral T and B cells. At the end of an immune response, activated peripheral T cells are eliminated by activation-induced cell death (AICD), which is predominantly mediated by the FasL/Fas pathway. Mice with a defective Fas pathway due to mutations in FasL and Fas have impaired AICD and develop generalized lymphoproliferative disorder (*gld*) and lymphoproliferation (*lpr*), respectively. These mice have a similar phenotype characterized by progressive accumulation of abnormal Thy1⁺B220⁺CD4⁻CD8⁻ T cells, splenomegaly, lymphadenopathy, and increased serum Ig, leading to an autoimmune syndrome and premature death. This study demonstrates that inhibition of NF- κ B by transgenic overexpression of a non-degradable mutant I κ B α within T cells of FasL mutant *gld* mice prevents the accumulation of abnormal Thy1⁺B220⁺CD4⁻CD8⁻ T cells, partially reduces serum Ig levels, and protects from developing splenomegaly and lymphadenopathy. Block of NF- κ B in T cells of *gld* mice compensates for the lack of FasL/Fas-mediated cell death by sensitizing T cells to other death pathways and thereby prevents the accumulation of abnormal Thy1⁺B220⁺CD4⁻CD8⁻ T cells.

Collectively, this study provides new insights into the biology of NF- κ B: (i) essential role of distinct NF- κ B complexes in different cell types during NKT cell development, (ii) regulation of RelB complexes by alternate NIK/p100 pathway in a signal- and cell type-dependent manner, and (iii) protection of FasL mutant mice from developing generalized lymphoproliferative disorder by the T cell-specific inhibition of NF- κ B.

Regulation der Entwicklung und Funktion verschiedener T-Zell Subtypen durch die Mitglieder der Rel/NF- κ B Familie

ZUSAMMENFASSUNG

Die Familie der Rel/NF- κ B Transkriptionsfaktoren spielt eine wichtige Rolle in der Entwicklung und Funktion von Lymphozyten, sowie bei der Regulation der angeborenen und erworbenen Immunantwort. Es konnte gezeigt werden, dass NF- κ B für die Entwicklung der meisten T-Zellen nicht essentiell ist, wobei seine Funktion für die Entwicklung verschiedener Subtypen, wie z.B. natürlicher Killer-T-(NKT)-Zellen, bisher noch nicht untersucht worden ist. Ähnlich wie die meisten T-Zellen entwickeln sich NKT-Zellen im Thymus, obwohl auch einige Thymus-unabhängige NKT-Zellen vorkommen können. Thymus-abhängige NKT-Zellen sind entscheidende Regulatoren der angeborenen und erworbenen Immunantwort und sind an verschiedenen Funktionen des Immunsystems, von Autoimmunität bis Tumorabstoßung beteiligt. Sie exprimieren den NK1.1-Marker, einen invarianten $V\alpha 14$ - $J\alpha 18$ T-Zell-Rezeptor (TCR), und werden positiv selektiert durch CD1d, welches dem Klasse-I-Haupthistokompatibilitätskomplex (major histocompatibility complex = MHC) ähnelt. Die molekularen Ereignisse unterhalb von CD1d sind bislang noch kaum verstanden.

Diese Studie belegt die Bedeutung bestimmter Mitglieder der Rel/NF- κ B-Familie sowohl in nicht-hämatopoetischen als auch in hämatopoetischen Zellen für die Entwicklung Thymus-abhängiger NKT-Zellen. Die Aktivierung von NF- κ B über den klassischen $I\kappa B\alpha$ -regulierten Signaltransduktionsweg wird in NKT-Vorläufern benötigt, um ein effizientes Reifen von NK1.1⁻ zu NK1.1⁺ NKT-Zellen zu gewährleisten. Das Rel/NF- κ B-Familien-Mitglied RelB hingegen ermöglicht in stromalen Zellen des Thymus die Entwicklung sehr früher NKT-Vorläufer. NF- κ B-induzierende Kinase (NIK) in Stroma-Zellen des Thymus ist ebenfalls für die NKT-Zell-Entwicklung notwendig. Diese Studie zeigt, dass NIK sowohl die konstitutive als auch die Signal-induzierte DNA-Bindung von RelB, nicht aber von RelA reguliert. Hierbei ist die NIK-induzierte DNA-Bindung von RelB von einer Spaltung des inhibitorischen p100 zu p52 abhängig, einem alternativen Weg der NF- κ B-Induktion. Dies zeigt, dass Rel/NF- κ B Komplexe, die über den klassischen $I\kappa B\alpha$ -Weg in NKT-Vorläufern aktiviert werden und der alternative NIK/p100/RelB Signaltransduktionsweg in Thymus-Stromazellen unterschiedliche Stadien der NKT-Entwicklung regulieren.

Neben der Lymphozyt-Entwicklung reguliert NF- κ B zusätzlich das Überleben und die Proliferation peripherer T- und B-Zellen. Am Ende einer Immunantwort werden aktivierte, periphere T-Zellen durch Aktivierungs-induzierten Zelltod (AICD) eliminiert, überwiegend vermittelt durch den FasL/Fas Signalweg. Mäuse, deren FasL/Fas-Signaltransduktionsweg aufgrund von Mutationen in FasL bzw. Fas defekt ist, haben AICD-Beeinträchtigungen und entwickeln eine generalisierte lymphoproliferative Erkrankung (*gld*) bzw. Lymphoproliferation (*lpr*). *Gld* und *lpr* Mäuse haben einen ähnlichen Phänotyp, der durch progressive Akkumulation abnormer Thy1⁺B220⁺CD8⁺CD4⁻ T-Zellen, Splenomegalie, Lymphadenopathie und erhöhtes Serum Ig gekennzeichnet ist und mit einem Autoimmunsyndrom und frühem Tod einhergeht. Diese Studie zeigt, dass Inhibition von NF- κ B durch transgene Überexpression einer nicht-abbaubaren $I\kappa B\alpha$ -Mutante in T-Zellen von FasL-mutierten *gld* Mäusen die Akkumulation abnormer Thy1⁺B220⁺CD8⁺CD4⁻ T-Zellen verhindert, die Serum-Ig-Spiegel erniedrigt und vor der Entwicklung von Splenomegalie und Lymphadenopathie schützt. Durch die Blockierung von NF- κ B in T-Zellen von *gld* Mäusen wird das Fehlen von FasL/Fas-vermitteltem Zelltod durch eine Sensibilisierung der T-Zellen gegenüber alternativen Zelltod-vermittelnden Signaltransduktionswegen kompensiert und dadurch die Akkumulation von Thy1⁺B220⁺CD8⁺CD4⁻ T-Zellen verhindert.

Zusammengefasst liefert diese Studie neue Einsichten in die Biologie von NF- κ B: (i) Belege für eine essentielle Rolle von NF- κ B-Komplexen in verschiedenen Zelltypen während der NKT-Entwicklung, (ii) Regulation von RelB-Komplexen durch einen alternativen NIK/p100 Weg in Signal- und Zelltyp-abhängiger Weise, (iii) Schutz von FasL-Mutanten Mäusen vor einer generalisierten lymphoproliferativen Erkrankung durch T-Zell-spezifische Inhibition von NF- κ B.

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CURRICULUM VITAE

Abbreviations

ABBREVIATIONS

Ab(s)	Antibody (dies)
Ag	Antigen
AICD	Activation induced cell death
aly	alymphoplasia
BM	Bone marrow
bp	base pair
BrdU	Bromodeoxy uridine
oC	Degrees Celsius
cDNA	Complementary DNA
Ci	Curie
cpm	Counts per minute
DC	Dendritic cell
DCR	Decoy receptor
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DN	double-negative
DP	double-positive
DTT	Dithiothreitol
E	Embryonic day
EAE	Experimental allergic encephalomyelitis
ECL	Enhanced chemilluminescence
EDTA	Ethylenediamine-N,N-tetraacetate
EGTA	Ethylenguanidine- N,N-tetraacetate
ELC	EBV-induced molecule 1 ligand chemokine
ELISA	Enzyme linked immunoabsorbent assay
EMH	Extramedullary hematopoiesis
et al.	Lat. et ali, and others
FasL	Fas ligand
FCS	Fetal calf serum
g	gram

Abbreviations

GalCer	Galactosyl Ceramide
gld	generalized lymphoproliferative disorder
GMCSFR	Granulocyte-macrophage colony-stimulating factor receptor
h	hour (s)
HVEM	Herpes virus entry mediator
HRP	Horseradish peroxidase
IFN	interferon
Ig	Immunoglobulin
IL	Interleukin
IkB	Inhibitor of NF-kB
IKK	IkB kinase
IP	immunoprecipitation
kD	Kilodalton
LIGHT	homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes
LN	Lymph node
lpr	lymphoproliferation
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTbR	LTb receptor
LZ	Leucine zipper
MAPKKK	Mitogen activated protein kinase kinase kinase
MEF	Mouse embryonic fibroblast
M	Molar
m	milli-(10 ⁻³)
mAb	monoclonal Ab
mg	milligram
MHC	Major histocompatibility complex
min	minute (s)
ml	milliliter

Abbreviations

mM	millimolar
mRNA	Messenger RNA
m	micro-(10 ⁻⁶)
mg	microgram
ml	microliter
mM	micromolar
n	nano-(10 ⁻⁹)
N	Number
NEMO	NF- κ B essential modulator (IKK γ)
NF- κ B	Nuclear factor kappa B
ng	nanogram
NIK	NF- κ B inducing kinase
NK	Natural killer
NKT	Natural killer T
NLS	Nuclear localization signal
OD	Optical density
O/N	Overnight
PAGE	Polyacrylmide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PP	Peyer's Patch
PVDF	Polyvinylidenedifluoride
RHD	Rel homology domain
RNA	Ribonucleic acid
RT	Room temperature
rpm	rotation per minute
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SDS	Sodium-lauryl-sulphate
Ser	Serine
SP	spleen

Abbreviations

TBE	Tris-boric acid-EDTA
TCR	T cell receptor
TEMED	N,N,N',N' tetramethylene-diamine
Th	Thymus
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRAIL	TNF related apoptosis inducing ligand
U	Unit (s)
UV	Ultra-violet light
V	Volt
v/v	volume per volume
WCE	Whole cell extract
w/v	weight per volume

1 INTRODUCTION

1.1 The Rel/NF- κ B family of transcription factors.

Nuclear factor kappa B (NF- κ B) was first discovered in 1986 in B cells as a factor that binds to an enhancer element of the immunoglobulin (Ig) κ light chain gene and has been shown to be necessary for the transcriptional activation of this gene (1-3). Shortly after its initial discovery it became evident that NF- κ B is expressed ubiquitously and that it consists a complex of two subunits with molecular weights 50 kD (p50) and 65 kD (p65). Under resting conditions, NF- κ B is present in an inactive cytoplasmic form bound to inhibitory proteins termed I κ B (4-7). Release of NF- κ B from I κ B inhibition and its nuclear translocation upon treatment with inflammatory cytokines tumor necrosis factor (TNF) and interleukin 1 (IL-1), revealed the importance of NF- κ B in the immune system (7, 8). Soon after, a wide range of inducers including bacteria or bacterial products, viruses, stress conditions, mitogens and growth factors, hormones, environmental hazards and many chemical agents were shown to activate NF- κ B. Almost all activating signals converge on the release of NF- κ B from its inhibitor I κ B, resulting in its nuclear translocation and activation of transcription of its target genes (4). Over 150 target genes of NF- κ B were identified, most of which participate in host immune and inflammatory responses and they include genes encoding chemokines and cytokines, stress and acute phase response proteins, factors involved in antigen presentation such as major histocompatibility complex (MHC), cell adhesion molecules, cell surface receptors, regulators of apoptosis, growth factors, transcription factors and many more (8). In addition to this inducible activity, NF- κ B has been found in a constitutively active form in the nucleus of mature B cells, neuronal cells and macrophages suggesting a role for NF- κ B in maintaining the differentiated state of these cell types (4). The diversity in the genes regulated by NF- κ B activity in various cell types suggests a central role for NF- κ B in the mammalian immune system.

Identification and cloning of the genes encoding the p50 subunit (also called NF- κ B1) p65 (also called RelA), p52 (also called NF- κ B2), RelB, v-rel and its cellular counter part c-rel, revealed a high degree of homology in their DNA binding domains and established the presence of a multigene NF- κ B family (6, 7, 9). *Drosophila melanogaster* a fruit fly,

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was found to express three NF- κ B molecules, Dorsal, Dif and Relish, with a high degree of homology to their mammalian counterparts illustrating the evolutionarily conservedness of the NF- κ B signaling system (**Fig. 1.1**) (4, 5). Each family member contains an approximately 300 amino acid conserved N-terminal DNA-binding and dimerization domain, termed Rel homology domain (RHD), which also contains the nuclear localization signal (NLS) and mediates the interaction with I κ B proteins (**Fig 1.1**) (4, 5). Almost all members of this family can be found as homo or heterodimers *in vivo* with the exception of RelB, which does not form homodimers. NF- κ B dimers bind to the consensus sequence 5'GGGGYNNCCY3', where N can be any nucleotide and Y is a pyrimidine (4). The genes for NF- κ B1 and NF- κ B2 encode the precursor proteins p105 and p100, which are proteolytically processed to give rise to p50 and p52, respectively (4). Both p50 and p52 lack transactivation domains, while RelA, RelB and c-rel contain a non-homologous transactivation domain in their C-termini and are not proteolytically processed to generate their active forms (6, 10).

NF- κ B activity is under the tight regulation by the inhibitory proteins called I κ B. Isolation of the gene encoding one of the I κ B proteins, I κ B α , led to the discovery of another multigene family consisting of I κ B α , I κ B β , I κ B ϵ , I κ B γ , and Bcl3 in mammals and Cactus in *Drosophila* (4-6, 11). All of these I κ B proteins contain multiple copies of an ankyrin-repeat motif, which binds to the RHD of NF- κ B members and masks the NLS causing their cytoplasmic retention. I κ B α , I κ B β , and I κ B ϵ contain N-terminal regulatory elements required for stimulus dependent degradation, a key step in NF- κ B activation. Interestingly, the C-termini of p100 and p105 also contain ankyrin repeats and can function as I κ B proteins retaining NF- κ B in the cytoplasm (**Fig. 1.1**) (4, 5).

Although there is a possible functional redundancy among the I κ B proteins, their differential affinity to each of the NF- κ B dimer, the number and sequence differences in the ankyrin repeats and the expression pattern would determine the specificity of regulation of NF- κ B activity by individual I κ B proteins (4, 5, 12). For instance I κ B α , I κ B β and I κ B ϵ were shown to preferentially bind to RelA or c-Rel. In contrast Bcl-3 binds p50 or p52 homo-dimers (4).

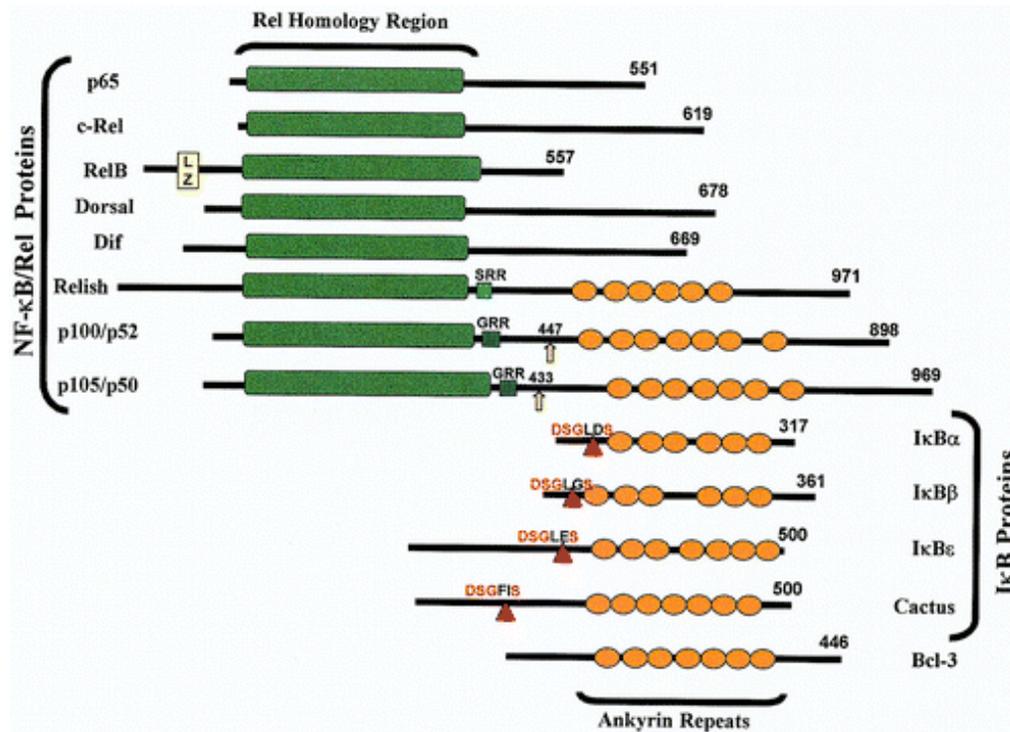


Fig. 1.1: The Rel/NF-κB and IκB proteins.

Schematic representation of mammalian and *Drosophila* members of the Rel/NF-κB and IκB families of proteins. The numbers of amino acids in each protein are listed on the right. The arrows point to the C-terminal residues of p50 and p52 (following processing of p105 and p100, respectively). The N-terminal motif required for inducible degradation of IκBα, IκBβ, IκBε, and Cactus is indicated. Conserved residues are colored in red and the ankyrin repeats are indicated by orange circles; LZ, leucine zipper; GRR, glycine-rich region; SRR, serine-rich region. {adapted from Karin et al., 2000 (5)}

1.1.1 Activation of NF-κB: the consensus pathway.

In most resting cells, NF-κB is kept in an inactive form in the cytoplasm via the interaction with IκB proteins. Once a cell receives any of the multitude of extra-cellular signals, such as TNF or IL-1, IκB proteins are rapidly degraded, resulting in the nuclear translocation of NF-κB, which is then active and can mediate transactivation of its target genes. Almost all signals that activate NF-κB converge on a serine specific IκB kinase (IKK) complex. Activation of IKK leads to the phosphorylation of two conserved serines residues in the N-terminus of IκB proteins (Ser 32, 36 for IκBα, Ser19, Ser23, for IκBβ, and Ser157, Ser161 for IκBε). The events governing the degradation of IκBα are well characterized. Following the phosphorylation of two serine residues, IκBα is recognized by the F-box protein β-TrCP, resulting in the polyubiquitination of IκBα at lysines 21 and 22. IκBα is then targeted to degradation by the 26S proteasome, resulting

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in the release of NF- κ B. The NLS of NF- κ B is exposed and is translocated to the nucleus (**Fig. 1.2**) (5, 9). This mechanism defines the classical pathway of NF- κ B activation which predominantly activates p50/RelA complexes (5, 11). Other mechanisms of NF- κ B activation involving the degradation of I κ B α without the requirement of phosphorylation at Ser32 and Ser36 have also been described (5). In addition, the NF- κ B1 and NF- κ B2 precursors p105 and p100 respectively, were shown to inhibit the NF- κ B via their C-terminal ankyrin repeats and were shown to be proteolytically processed by the ubiquitin-proteasome system (4, 5). Moreover, NF- κ B function has also been shown to be regulated by I κ B α independent mechanism, which is mediated by modulatory phosphorylation of individual NF- κ B subunits (5, 13).

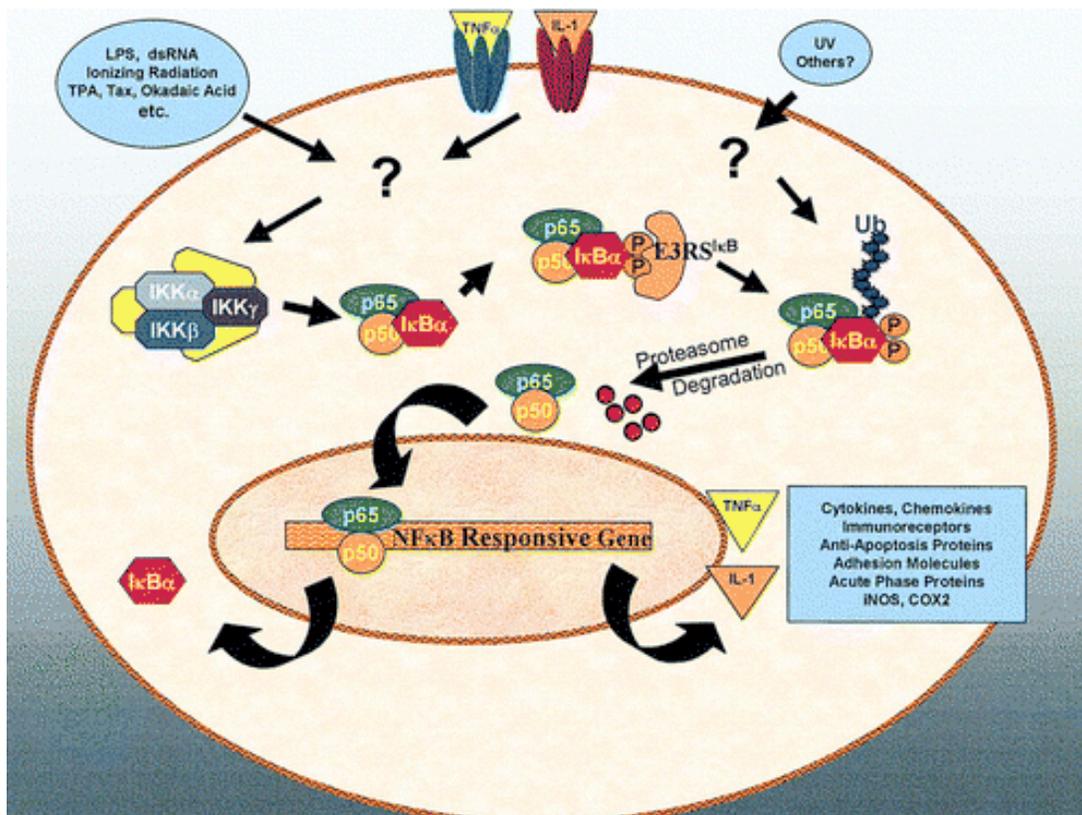


Figure 1.2: The consensus pathway of NF- κ B activation.

Schematic model of NF- κ B activation. Various stimuli, including the proinflammatory cytokines TNF and IL-1, activate IKK, which then phosphorylates I κ B α , leading to its recognition by E3RS κ B, a receptor component of a SCF type E3. This leads to the polyubiquitination of I κ B α targeting I κ B α for rapid degradation by the 26S proteasome. As a result NF- κ B is released and translocates to the nucleus. In the nucleus NF- κ B regulates transcription of target genes, including I κ B α , which functions to terminate NF- κ B activity {adopted from Karin et al., 2000 (5)}.

Introduction

In addition to cytoplasmic retention of NF- κ B, I κ B α has been shown to terminate NF- κ B activation by entering into the nucleus and causing the dissociation of NF- κ B from DNA and its export to the cytoplasm (5, 9, 14).

Once in the nucleus, distinct NF- κ B dimers exhibit differential affinity to different promoters and different DNA-binding specificities, resulting in fine tuning of NF- κ B activity (15). After binding to the DNA, NF- κ B members recruit various other factors, including proteins of the basal transcriptional machinery, such as TFIIB and TFIID. The best characterized NF- κ B subunit in this context is RelA, which in addition to TFIIB, TFIID, can also interact with p300, CREB-binding protein (CBP) of the transcriptional co-activator class of proteins. Moreover, RelA also interacts with proteins such as c-Jun suggesting the cross-talk of NF- κ B pathway with other signaling pathways (6).

1.1.2 The I κ B kinase (IKK) complex

After the establishment of NF- κ B activation via I κ B α phosphorylation, the IKK complex has been identified as a high molecular weight complex of 700-900 kD, suggesting that it is a multi-component protein complex. However a smaller molecular weight complex of 300 kD was also shown to have IKK activity (5). Later it was demonstrated that the IKK complex consists of three components, IKK α or IKK1 (85 kD), IKK β or IKK2 (87 kD) and IKK γ 48 kD) (5, 11). IKK α and IKK β are highly homologous proteins with 50% sequence identity and contain N-terminal kinase domains as well as leucine zipper (LZ) and helix-loop-helix (HLH) motifs (**Fig. 1.3**). IKK α and IKK β are the catalytical subunits of the IKK complex. They can form homo or heterodimers by virtue of their LZ motifs and their dimerization is essential for the kinase activity. The third component IKK γ , is the regulatory subunit of IKK. It lacks a kinase domain and exists as dimer or a trimer (5). It is also called NF- κ B essential modulator (NEMO, IKK associated protein 1 (IKKAP1) or 14.7 interacting protein (FIP-3) (5, 16).

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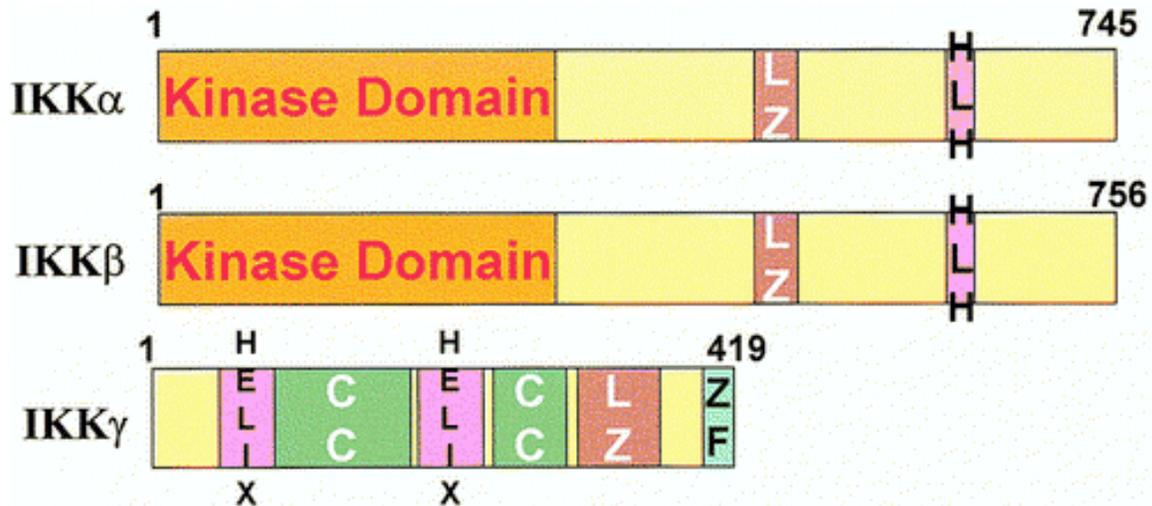


Figure 1.3: The subunits of the IKK complex

Schematic diagram showing the known subunits of IKK and their putative functional and structural motifs. CC, coiled coil; Helix, helix; HLH, helix-loop-helix; LZ, leucine zipper; ZF, zinc finger. adapted from Karin *et al.*, 2000 (5).

The kinase domains of IKK α/β are similar to other known serine/threonine kinases. The conserved lysine in this region (Lys-44) is important for the kinase function of IKK α/β . Moreover phosphorylation of Ser 176, 180 in the case of IKK α and Ser 171, 181 in the case of IKK β are required for IKK activation. A large number of protein kinases including different protein kinase C (PKC) isozymes and the mitogen-activated protein kinase kinase kinase (MAPKKK) family members such as NIK, AKT/PKB, MEKK1, MEKK2, MEKK3, were found to be inducible activators of the IKK complex (5). Among these, NIK has been shown to directly phosphorylate IKK α . It is likely that different IKK kinases dock on to IKK γ and activate the IKK complex by phosphorylating IKK α and or IKK β . It has been suggested that different NF- κ B inducers may activate distinct IKK kinases (5, 17).

The high degree of sequence similarity between IKK α and IKK β and the fact that both kinases can effectively phosphorylate I κ B in vitro, suggested that these two kinases have functional redundancy in NF- κ B activation. Indeed, activation of the IKK complex and phosphorylation and degradation of I κ B α downstream of proinflammatory stimuli, such as TNF and IL-1, does not require IKK α (5). On the other hand, IKK γ is indispensable for IKK activation downstream of numerous stimuli that activate NF- κ B (5). Gene inactivation experiments however, have revealed, distinct non-redundant roles for IKK α and IKK β . IKK $\beta^{-/-}$ embryos die at embryonic day (E) 12.5-14.5 as a result of massive

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liver apoptosis, which is similar to the phenotype observed in RelA-deficient mice. IKK β ^{-/-} cells do not activate NF- κ B in response to TNF or IL-1, suggesting that IKK α can not compensate for IKK β . In contrast, IKK α ^{-/-} mice die within 4 hours after birth and they lack limbs, tails and ears and have defective keratinocyte differentiation. Similar to IKK β ^{-/-} mice IKK γ ^{-/-} mice die at E12.5-13 due to massive liver apoptosis (5).

1.1.3 Individual Rel/NF- κ B family members have distinct and redundant functions

Despite the significant homology in their sequence, the Rel/NF- κ B family members have distinct as well as redundant functions as revealed by the phenotypes observed in mice deficient for individual Rel/NF- κ B proteins. The pathological changes and cell lineages affected in different Rel/NF- κ B deficient mice as shown in **table I and II** illustrates the distinct requirement of individual Rel/NF- κ B proteins for certain functions and a range of redundancy among these proteins for certain other functions (18-23).

Table I. Pathological changes in mice deficient for individual Rel-NF- κ B proteins.

NF- κ B-deficient mice	Phenotype observed
<i>nfkbl</i> ^{-/-}	Non-remarkable
<i>nfkbl2</i> ^{-/-}	Disorganized B and T-cell areas in spleen and lymph nodes. Absence of marginal zone in spleen. Absence of peyer's patches.
<i>nfkbl</i> ^{-/-} <i>nfkbl2</i> ^{-/-}	Increased mortality (50% survival after 3 weeks). Osteopetrosis, thymic and lymph node atrophy, disorganized splenic structure, myeloid hyperplasia and *EMH.
** <i>relA</i> ^{-/-}	Embryonic lethality at day 15-16 of gestation due to massive apoptosis in liver.
*** <i>relB</i> ^{-/-}	Increased mortality (50% survival after 6-7 weeks). Multiorgan inflammation, atrophy of thymic medulla, splenomegaly due to EMH, disorganized B and T cell areas in spleen, myeloid hyperplasia in spleen and bone marrow, lack of lymph nodes, peyer's patches.
<i>c-rel</i> ^{-/-}	Non-remarkable.

*EMH, extramedullary hematopoiesis. ***infr*^{-/-}*relA*^{-/-} mice were generated, which can survive embryonic lethality. They lack lymph nodes and reveal defective spleen architecture. ****relB*^{-/-}*nfkbl*^{-/-} mice reveal increased severity that is observed in *relB*^{-/-} mice. {Attar MR et al., 1997, Weih F and Caamano J., 2003 (18, 19)}.

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Table II. Cell lineages affected in Rel/NF- κ B-deficient mice.

NF- κ B deficient mice	B Cells	T cells	Others
<i>nfkb1</i> ^{-/-}	Defective response to mitogenic activation. Impaired antibody production.	Impaired proliferative response upon activation of TCR.	
<i>nfkb2</i> ^{-/-}	Reduced number in spleen and lymph nodes.	Increased IL-2 and GM-CSF production upon activation.	Reduction of marginal metallophilic macrophages in spleen.
<i>nfkb1</i> ^{-/-} <i>nfkb2</i> ^{-/-}	Progressive reduction in the population.	Absence of CD4 ⁺ and CD8 ⁺ T-cells in the periphery.	Decrease in osteoclasts and myeloid hyperplasia.
<i>relA</i> ^{-/-}	Reconstituted from fetal liver produce lower IgG1 and IgA. Impaired proliferative responses.	Reconstituted from fetal liver. Defective proliferative response upon TCR activation.	Increased apoptosis of hepatocytes due to TNF.
<i>relB</i> ^{-/-}	Normal maturation. Impaired proliferative responses. Present in inflammatory infiltrates.	Normal development. Auto-reactive T cells due to defective negative selection.	Reduced thymic medullary epithelial and dendritic cells. Impaired antigen presenting function of splenocytes. Myeloid hyperplasia.
<i>c-rel</i> ^{-/-}	Impaired proliferative responses.	Impaired proliferative responses. Decreased production of IL-2, IL-3 and GM-CSF upon activation.	

Mice doubly deficient for *nfkb1*^{-/-}*c-rel*^{-/-} reveal normal development of T and B cells. T cells from these mice have defective proliferative response and survival. Combined lack of c-Rel and relA results in defective maturation and increased apoptosis of B cells, highly reduced peripheral T cell numbers. *relb*^{-/-}*nfkb1*^{-/-} mice reveal impaired B cell development. {Attar MR et al., 1997, Grossmann M et al., 2000, Zheng Ye et al., 2003 (18, 21, 22)}.

1.2 T lymphocyte development.

Development of T cells occurs in the thymus. Committed lymphoid progenitors arise in the bone marrow and early progenitors that are destined to become T cells enter the thymus via the blood (24-26). In the thymus lymphoid progenitors develop into early T cell precursors that are characterized by the lack of expression of the T cell receptor (TCR), CD4, and CD8 and are termed double-negative (DN; CD4⁻ CD8⁻). These DN precursors are further sub-divided into four stages of differentiation designated DN1-4 and they are identified by their surface expression of CD44 and CD25 (see **Fig. 1.4** for details) (26, 27). DN3 cells first express the pre-TCR α -chain, which pairs with a rearranged TCR β -chain to form the pre-TCR (26, 28, 29). The pre-TCR $\alpha\beta$ on the cell surface of DN3 cells in association with several other proteins (CD3/ ζ complex), allows

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further maturation of T cells (26, 30). This process is called the TCR β selection because, cells that do not express functional TCR β , can not provide the signaling requirements for maturation from DN3 stage to further stages of T cell development. Moreover, several key molecules and kinases including zap-70, fyn and lck and adapter proteins such as slp-76 etc., are involved in the pre-TCR $\alpha\beta$ signaling since mutations in these molecules would result in the arrest at the DN3 stage of the T cell development (26). Engagement of the pre-TCR was shown to be required to rescue the early DN progenitors from cell death and to stimulate their proliferation (31). The transcription factor NF- κ B has been shown to be involved in these early stages of T cell development, downstream of pre-TCR signaling (25, 32). However complete lack of NF- κ B activation does not significantly effect thymic development of T cells (25, 33-35).

The thymocytes that pass the TCR β chain selection (late DN3 and DN4) undergo 6-8 cell divisions, after which recombination at the TCR α locus results in the production of functional TCR α chain. The expression of pre-TCR α is lost during this stage, which results in the cell surface expression of mature TCR $\alpha\beta$ assembled with CD3/ ζ proteins. Simultaneously, thymocytes start to express co-receptor proteins, first CD8 followed by CD4 (26, 36). Eventually, a large number of immature CD4⁺CD8⁺ double-positive (DP) thymocytes are formed that constitute 85-90% of the lymphoid compartment of the thymus (**Fig. 1.4**).

1.2.1 TCR-mediated positive and negative selection of DP thymocytes.

From the large number of DP thymocytes only few cells will be selected to develop into mature T cells. The interaction of the thymocytes by virtue of their TCR with the self-peptide-MHC ligands would govern the four distinguishable processes, which include death by neglect, negative selection, positive selection and lineage-specific development (26, 36, 37). Most (about 90%) of thymocytes express TCRs that interact poorly with the self-peptide-MHC ligands, so that the intra-cellular signals required for the survival are poorly generated and are subjected to death by neglect. From the remaining 10% thymocytes very few of them express TCRs which reveal very strong high affinity interaction with the self-peptide-MHC ligands. Such thymocytes are subjected to cell death by a process called negative selection. Thymocytes expressing TCRs that recognize self-ligands and generate signals of strength that is intermediate to those resulting in negative selection or death by neglect, would initiate a multi-step process called positive

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selection which ultimately results in the maturation of DP thymocytes into either CD4⁺ or CD8⁺ lineage (Fig. 1.4 and for reviews see 26, 36). The lineage decision between becoming a CD4⁺ T cell or a CD8⁺ T cell from the DP precursors, is still a matter of debate. The commitment to CD4 or CD8 lineage may either depend, on a random choice or instructions given by the type of signal received by the developing thymocyte (26).

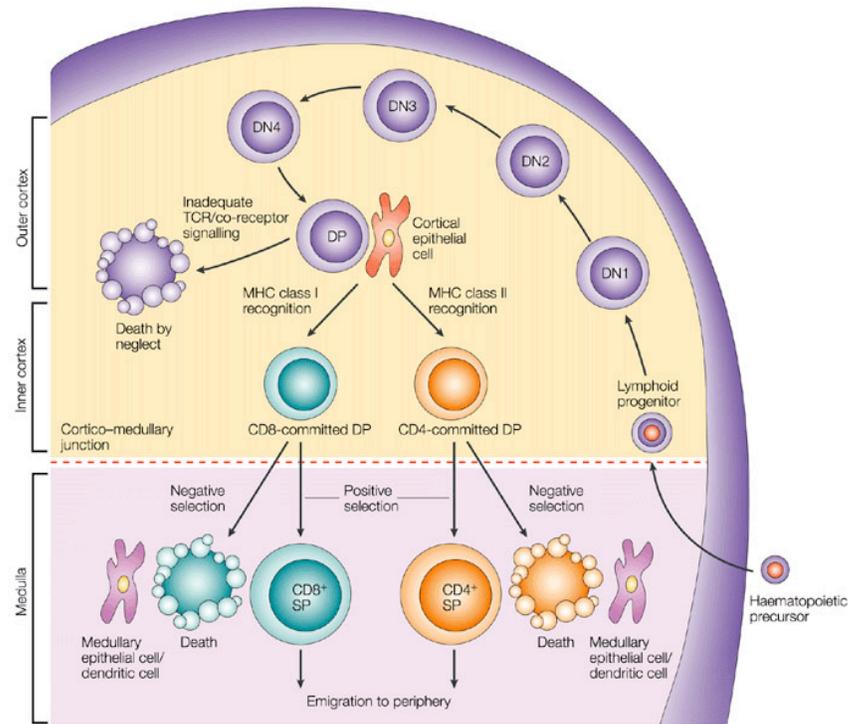


Fig. 1.4: Overall Scheme of T-cell development in the thymus

Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. Early committed T cells lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN; no CD4 or CD8) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation (DN1, CD44⁺CD25⁻; DN2, CD44⁺CD25⁺; DN3, CD44⁻CD25⁺; and DN4, CD44⁻CD25⁻). As cells progress through the DN2 to DN4 stages, they express the pre-TCR, which is composed of the non-rearranging pre-TCR α chain and a rearranged TCR β chain. Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP) transition and replacement of the pre-TCR α chain with a newly rearranged TCR α chain, which yields a complete $\alpha\beta$ -TCR. The $\alpha\beta$ -TCR⁺CD4⁺CD8⁺ (DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC class-I and class-II molecules associated with self-peptides. The fate of the DP thymocytes depends on signaling that is mediated by interaction of the TCR with these self-peptide-MHC ligands. Too little signaling results in delayed apoptosis (death by neglect). Too much signaling can promote acute apoptosis (negative selection); this is most common in the medulla on encounter with strongly activating self-ligands, particularly presented by medullary dendritic cells. The appropriate, intermediate level of TCR signaling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self-peptide-MHC-class-I complexes become CD8⁺ T cells, whereas those that express TCRs that bind self-peptide-MHC-class-II ligands become CD4⁺ T cells; these cells are then ready for export from the medulla to peripheral lymphoid sites. SP, single positive.

{Adapted from Germain R., Nat. Rev. Immunology 2002 (26)}

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In addition to the complex network of signaling events during the entire stages of T cell development, several transcription factors including TCF1/LEF1, Sox4, Ets1, LKLF, CREB, NFAT, GATA etc are involved in the different stages of T cell development (25). Although a requirement of NF- κ B has been shown during the pre-TCR signaling, mice lacking NF- κ B have no defect in the development of T cells (25).

1.3 Natural killer T cells.

Natural killer T (NKT) cells are a minor subset of mature T lymphocytes, which are characterized by the co-expression of Natural Killer (NK) cell surface markers such as NK1.1 and the TCR, as well as by the secretion of high amounts of cytokines, in particular interleukin-4 (IL-4) and interferon-gamma (IFN- γ). NKT cells are most frequent in thymus, liver, spleen and bone marrow (BM), and represent a smaller proportion in lymph nodes, blood and lungs. NKT cells develop in the thymus and they are either CD4⁺CD8⁻ (60%) or CD4⁻CD8⁺ (40%). Collectively NKT cells represent up to 03-1% of total thymocytes and up to 20% of the mature heat stable antigen low or negative (HSA)^{low-neg} thymocyte fraction (for reviews see 38-40). Although majority of the NKT cells develop in the thymus, there is evidence for the extra-thymic development (41, 42), suggesting that both thymus-dependent and thymus-independent NKT cells exist. However, in athymic *nu/nu* mice (nude), the numbers of NK1.1⁺ T cells are extremely low, suggesting that the majority of the NKT cells develop in thymus (41).

Several features of thymus-dependent NKT cells distinguish them from the CD4⁺ or CD8⁺ mainstream conventional T cells. Whereas the conventional T cells are positively selected by the classical MHC class I or class II molecules, NKT cells are positively selected by CD1d, a non-classical MHC class I-like, β 2-microglobulin-associated molecule, which predominantly presents lipid antigens (38, 43, 44). Moreover, thymus-dependent NKT cells express an invariant V α 14-J α 18 TCR, referred to as V α 14*i* TCR, which is predominantly associated with the V β 8.2 chain (38, 41). The thymus-dependent NKT cells, by virtue of their V α 14*i* TCR, recognize the marine sponge-derived glycolipid, α -galactosyl ceramide (α -GalCer) and readily respond to this lipid antigen (45, 46). On the other hand, thymus-independent NKT cells, which are defined by NK1.1 and TCR expression, do not express the V α 14*i* TCR, do not react with CD1d and thus are unresponsive to α -GalCer (47, 48). Similar to mouse V α 14*i* NKT cells, humans have

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V α 24i NKT cells, characterized by invariant TCR comprised of V α 24-J α 15. However, unlike, mouse V α 14i NKT cells, human V α 24i NKT cells are less frequent and can be CD8⁺ (41). One striking feature of both mouse V α 14i and human V α 24i NKT cells is their surface phenotype (CD44^{hi}CD62L⁺CD69⁺), which is characteristic of recently activated or memory T cells (41). Interestingly, even mice raised under germ-free conditions have V α 14i NKT cells with an activated phenotype, indicating that these lymphocytes are *in vivo* autoreactive (41).

1.3.1 Use of α -GalCer-loaded CD1d tetramers in identifying V α 14i NKT cells.

By virtue of their invariant V α 14i TCR, thymus-dependent NKT cells react strongly with the glycolipid antigen alpha-galactosyl-ceramide (α -GalCer). Similar to conventional peptide-loaded-MHC tetramers, α -GalCer-loaded CD1d-tetramers have been generated (for more details see materials and methods section). Until recently NKT cells are identified by the co-expression of NK1.1 and TCR, which may not be a direct measure for thymus-dependent NKT cell numbers. Moreover, NKT cells can develop in the absence of NK1.1 expression (49). Therefore, as an alternative, one can use α -GalCer-loaded CD1d-tetramers and TCR β to exclusively identify NKT cells that express V α 14i TCR (50, 51, 52). (These so-called V α 14i NKT cells would hereafter be referred to simply as NKT cells unless mentioned specifically)

1.3.2 Development of NKT cells.

In the mouse unlike conventional T cells, NKT cells appear late in ontogeny and can be detected only one week after birth, using α -GalCer-loaded CD1d-tetramers (41). Later, the absolute number and proportion of CD1d-tetramer positive cells increases progressively and reaches a plateau by 6 weeks of age (41). This increase in the proportion of NKT cells is mainly due to proliferative expansion as indicated by the incorporation of BrdU by tetramer-positive cells (41).

Several independent observations indicate that the developmental requirements for NKT cells and conventional TCR $\alpha\beta$ T cells are fundamentally different. First, whereas thymic epithelial cells are required for the positive selection of conventional T cells, BM-derived immature CD4⁺CD8⁺ DP thymocytes that express CD1d are required for the positive selection of NKT cells (36, 53). Second, several mutations/gene deletions that impair

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development of conventional T cells have no effect on the development of NKT cells. In the other case, NKT cell development is more impaired by genetic alterations than development of conventional T cells (41). For example, NKT cells develop normally in mice that express dominant-negative mutants of Ras and/or MEK, which inhibits positive selection of conventional T cells (41). On the other hand, NKT cell development is very sensitive to mutations such as *fyn* and *Ets1* (54-56), whereas conventional T cell development is not impaired by the lack of these genes. Several other gene products have a particular influence on the generation of normal numbers of NKT cells, including IL-15 and its receptor (41) as well as membrane lymphotoxin (LT) $LT\alpha 1\beta 2$, which signals through LT β receptor ($LT\beta R$) (57-59) (for a list of genes that are required for NKT cell development see **Table III**).

Genetically modified mice	Change in cell number		
	T cells	NKT cells	NK cells
Common γ -chain ^{-/-}	--	-	--
<i>Il2rβ</i> ^{-/-}	+++	--	--
<i>Il15</i> ^{-/-}	+++	--	--
<i>Il15rα</i> ^{-/-}	+++	--	--
GMCSFR- β ^{-/-}	+++	--	+++
<i>Ets1</i> ^{-/-}	+++	--	--
<i>Irf1</i> ^{-/-}	+++	--	--
<i>Fyn</i> ^{-/-}	+++	--	+++
<i>Lt$\alpha\beta$</i> ^{-/-}	+++	--	--
aly/aly	+++	--	--
<i>Lfa1</i> ^{-/-}	+++	-- (liver only)	+++
<i>Jα18</i> ^{-/-}	+++	--	+++
<i>Cd1d</i> ^{-/-}	+++	--	+++
dnRas/dnErk Tg	--	+++	+++
C β FG loop ^{-/-} Tg	+++	--	+++
Ly49a Tg	+++	-- (D ⁺)	+++

aly, alymphoplasia, mutation of the NF- κ B-inducing kinase; C β FG loop^{-/-} Tg, transgenic mice having a deletion of the FG loop of the T-cell receptor β -chain; dn, dominant negative; *Ets1*, a member of the Ets family of winged helix-turn-helix transcription factors; GMCSFR- β , granulocyte-macrophage colony-stimulating factor receptor β -chain; *Il*, interleukin; *Irf1*, interferon regulatory factor 1; *Lfa1*, lymphocyte function-associated antigen 1, an integrin composed of CD11a and CD181; *Lt $\alpha\beta$* , lymphotoxin $\alpha\beta$; NK, natural killer.

Table III. Genes that are involved in the development of V α 14i NKT cells.
 {Adapted from Kronenberg M. and Gapin L., 2002 (41)}.

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These observations suggest that mouse NKT cells belong to a special lineage of T-lymphocytes, raising the question whether NKT cells develop similar to mainstream T cells from DP thymocyte precursors or from a separate pre-committed precursor. So far there is no evidence that such a pre-committed precursor could give rise to the mature CD1d-tetramer positive NKT cells (41). On the other hand, it has been suggested that NKT cell development is similar to conventional T cell development until the DP stage, at which the CD1d/V α 14-J α 18 specific signal would instruct the DP precursors to develop into NKT lineage (38). Recent findings suggest that NKT cells are derived from DP thymocytes (60) supporting this model (**Fig. 1.5**). However, the molecular events activated downstream of CD1d/V α 14-J α 18 TCR are poorly understood. In particular the questions such as how do these CD1d-reacting DP precursors acquire the NK1.1 marker, what factors are involved in the proliferative expansion and establishment of a pool of thymic and peripheral NKT cells, and what are the molecular events required for the completion of positive selection signal, remains to be studied.

Although positive selection of NKT cells has been shown to be mediated by CD1d expressed on DP thymocytes, the autoreactive phenotype of NKT cells raised a question as to whether NKT cells undergo negative selection. Recent findings that presentation of α -GalCer by dendritic cells (DC) results in negative selection, (61), indicate that NKT cells do undergo negative selection similar to conventional T cells.

1.3.3 Developmental intermediates of NKT cells

Recent reports indicate that the positive selection of DP precursors by CD1d, results in the generation of intermediate NK1.1⁻ NKT precursors (62, 63). These NK1.1⁻ NKT precursors were shown to progress through additional intermediate stages of NKT cell development characterized by the expression of CD44, such as CD44^{lo}NK1.1⁻ and CD44^{hi}NK1.1⁻ (**62, 63**). Both CD44^{lo}NK1.1⁻ and CD44^{hi}NK1.1⁻ cells undergo massive proliferation before they become CD44^{hi}NK1.1⁺ mature NKT cells. Mature CD44^{hi}NK1.1⁺ NKT cells are a non-dividing sessile population (63). Moreover, it has been shown that the early CD44^{hi}NK1.1⁻ precursors leave the thymus and enter other peripheral organs such as spleen and liver to establish the pool of mature NK1.1⁺ NKT cells (**see Fig. 1.5**). The proliferative expansion appears to be a requisite for the establishment of mature NKT cell pools both in the thymus and in the periphery. It is

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important to study, whether the signal for the proliferation of NK1.1⁻ NKT cells is given by the CD1d/V α 14-J α 18 TCR or by other molecules. Also, it is not known which are the factors that regulate the transition from DP cells to the early NK1.1⁻ NKT precursors and from NK1.1⁻ to the mature NK1.1⁺ NKT cells.

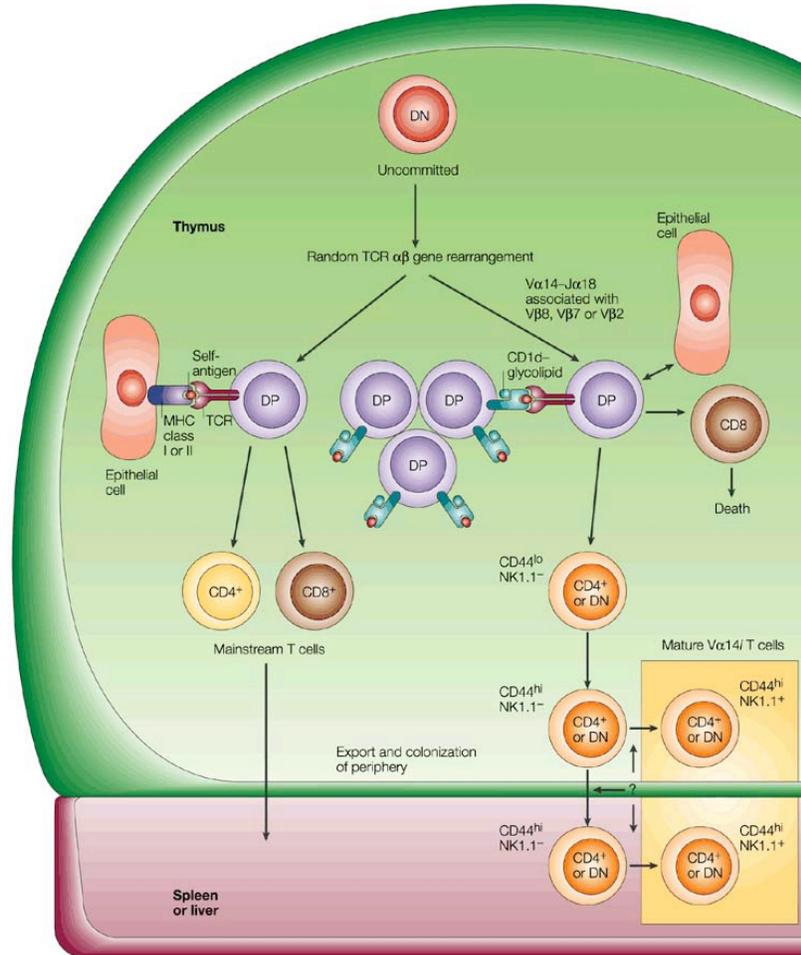


Fig. 1.5: Schematic view of the development of V α 14i T cells in the thymus.

Development of both mainstream-T and NKT cells from the DP thymocytes. An uncommitted precursor thymocyte randomly rearrange and express a CD1d-specific V α 14i TCR, at the double-positive (DP) stage, and will be positively selected by CD1d-expressing DP thymocytes. The diagram indicates the additional requirement of a radiation-resistant cell, which could be an epithelial cell. The positively selected V α 14i T cells will mature into double-negative (DN) or CD4⁺ NKT cells, whereas the expression of CD8 might induce negative selection by increasing the affinity of the TCR for CD1d. After selection, NK1.1⁻ V α 14i T cells go through CD44^{lo} and CD44^{hi} stages, during which these cells proliferate and expand and are exported to the periphery. A hypothetical second signal is required for the acquisition of the mature NK1.1⁺ phenotype. J, joining region; V, variable region.

{Adapted from Kronenberg M. and Gapin L., 2002 (41)}.

1.3.4 Immune responses of NKT cells.

Both mouse and human NKT cells have cytolytic activity (41) and they rapidly produce large amounts of various cytokines including IL-4 and IFN- γ , upon TCR activation (38,

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39). NKT cell-derived cytokines in turn, activate several other cell types, including NK cells (41), conventional T cells, macrophages, B cells and can recruit myeloid dendritic cells (DCs) (41). The invariant nature of V α 14-J α 18 TCR of NKT cells also suggest that they recognize one particular type of antigens, similar to the receptors involved in innate immune responses such as Toll-like receptors (TLRs). Once activated by virtue of their invariant TCR, NKT cells produce a range of cytokines, which activate other cell types involved in innate immune responses such as DC, NK and B cells. In many cases innate immune responses are in the first instance triggered by DC, B cells macrophages etc., by producing cytokines such as IL-12, which activates NKT cells via IL-12 receptor. Activated NKT cells, in turn, produce IFN- γ , which activates DC and NK cells, resulting in a positive feed back loop leading to a strong innate immune response (64, 65). Indeed, it has been shown that during certain viral infections, an initial release of IL-12 by other innate immune cells activates NKT cells, which releases high amounts of IFN- γ resulting in efficient viral clearance (66). An interesting question is whether the invariant TCR expressed by NKT cells also activates NF- κ B similar to other innate receptors such as TLRs (67,68).

The capability to produce both Th1 and Th2 cytokines enables NKT cells to push the adaptive immune responses into either a Th2 direction, in the case of IL4 and/or IL-13 production or into a Th1 direction in the case of IFN- γ production. In addition to the regulation of adaptive responses, NKT cells have been shown to protect against infections such as malaria, Hepatitis B virus and *Leishmania major* and to suppress autoimmune disorders such as diabetes and experimental allergic encephalomyelitis (EAE). Moreover, NKT cells have been shown to be key regulators of tumor rejection (41). In particular treatment with α -GalCer has been shown to protect against tumor metastasis and several autoimmune disorders (69). Activation of NKT cells with α -GalCer has also been shown to enhance CD8 T cell mediated immunity against malaria infection (70).

1.3.5 NF- κ B and lymphotoxin Signaling in development of NKT cells.

Analysis of alymphoplasia (*aly/aly*) mice, which have a point mutation in the C-terminal end of the NF- κ B inducing kinase (NIK) (71), revealed an essential role for signaling through NIK in thymic stromal cells for NKT cell development (72). The membrane-

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bound form of LT (LT α 1 β 2) signals exclusively through LT β R and has also been shown to be involved in NKT cell development (57, 58). Signaling through LT β R and TNF receptor (TNFR) results in activation of NF- κ B (73, 7, 8). Moreover, signaling through LT β R is defective in the absence of NIK (74-76). Lack of functional NIK in *aly/aly* mice results in impaired development of thymic medulla with reduced numbers of medullary epithelial cells and disturbed thymic architecture (77). It has been suggested that thymic medullary epithelial cells are essential for NKT cell development (72). Mice deficient for RelB also have defective development of thymic medulla and reduced numbers of medullary epithelial cells (78, 79). Interestingly, a recent report indicates that signaling through LT β R is essential for the proper development of thymic medullary epithelial cells and maintenance of thymic architecture (80). In addition to its role in NKT development, LT signaling has been shown to play important role in development and maintenance of secondary lymphoid organs, such as spleen, lymph nodes (LN) and Peyer's patches (PP) (19, 81, 82). LT belongs to the TNF superfamily of cytokine genes. Four members of this family LT α , LT β , TNF and LIGHT together with their receptors, LT β R, TNF receptor-I (TNFR-I) and TNFR-II and herpes virus entry mediator (HVEM) form the TNF-LT system, which has been shown to be the major regulator of development and maintenance of secondary lymphoid organs (83). TNF is a type II transmembrane protein and becomes soluble after cleavage and release by TNF-converting enzyme (TACE). LT α 3 homotrimer is a soluble cytokine. TNF and LT α 3 can signal through either TNFR-I or II (**Fig. 1.6** and 83). LT β is also a type II membrane protein and together with LT α it forms membrane-bound LT α 1 β 2 heterotrimer, which signals exclusively via LT β R (83). LIGHT binds to both LT β R and HVEM (**Fig. 1.6**). While the ligands of the TNF-LT system are predominantly expressed by hematopoietic cells such as thymocytes, their receptors are expressed by both hematopoietic and non-hematopoietic cells, with the exception of LT β R which is predominantly found on non-hematopoietic stromal cells (83). Gene knock-out studies revealed that mice deficient for LT, LT β R and *aly/aly* mice have defective lymphoid organ development (19, 81, 82). Mice lacking distinct Rel/NF- κ B proteins also reveal defective lymphoid organ development (19). This suggests that signaling through the TNF-LT system via distinct receptors and NIK may regulate different components of the NF- κ B members during the

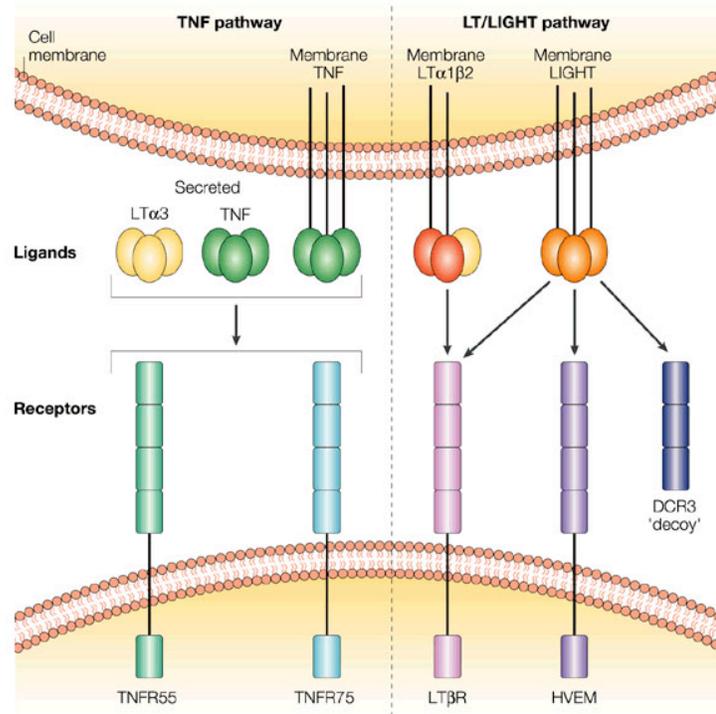


Fig. 1.6: Ligands and receptors of the tumour-necrosis factor/lymphotoxin system.

Two fundamental pathways can be defined. The TNF pathway is activated by tumour-necrosis factor (TNF) or lymphotoxin-3 (LT3)-induced signaling through the two TNF receptors (TNFRs). The LT/LIGHT system is composed of a typical TNF family receptor, LT receptor (LTβR), that binds to two ligands, the LT hetero-trimer and homo-trimeric LIGHT. LIGHT also binds to two additional receptors in the TNF family, decoy receptor 3 (DCR3) and herpes-virus entry mediator (HVEM). HVEM was originally discovered as the receptor for herpes-virus entry and probably has a role in T-cell function. DCR3 (decoy receptor 3) is a soluble receptor that binds LIGHT and FasL, which is involved in down-regulating these pathways.

{Adapted from Gommerman JL et al., Nat. Rev. Immunol., 2003 (83)}.

development of NKT cells, thymic medullary epithelial cells, as well as secondary lymphoid organs.

1.4 Role of NF-κB in peripheral T cells.

Although T cell development in the thymus does not require NF-κB, complete inhibition of NF-κB activation either by deletion of IKKβ or by transgenic over-expression of mutant non-degradable form of IκBα (*mIκBα^{ts}*) in T cells, results in highly reduced numbers of peripheral T cells (33-35, 84). This reduction is more severe for CD8⁺ compared to CD4⁺ T cells (33, 35). Moreover, peripheral T cells from *IKKβ^{-/-}* and transgenic mice overexpressing *mIκBα* in T cells, reveal impaired proliferative responses

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and defective survival (33, 35, 84). Mice lacking individual Rel/NF- κ B proteins have normal survival and numbers of peripheral T cells. On the other hand, peripheral T cells from *relA*^{-/-}, *c-rel*^{-/-} and *nfkbl*^{-/-} mice reveal impaired proliferative responses to upon TCR activation (18, 20). Moreover, *c-rel*^{-/-} T cells have impaired expression of IL-2 (18, 20). This indicates that distinct Rel/NF- κ B proteins have non-overlapping functions in the regulation of T cell proliferation as well as cytokine production, and redundancy in their function in determining the normal numbers and survival of peripheral T cells.

TCR and CD28 co-receptor engagement, strongly induces NF- κ B, which mainly consists of p50-RelA and p50-cRel heterodimers (85), suggesting that, the major NF- κ B function in T cells is comprised of heterodimers containing RelA or c-Rel. Although RelB is expressed in T cells, it is not clear whether RelB has any distinct role in T cells. Moreover, it is not clear whether RelB heterodimers have overlapping functions with heterodimers of RelA or c-Rel, in determining the normal numbers, proliferation and survival of peripheral T cells. It is therefore necessary to have a mouse model where one can study the function of RelB in T cells.

1.5 Generalized lymphoproliferative disorder (gld) and lymphoproliferative disorder (lpr).

A key feature of lymphocyte development and homeostasis in mammals is the process of programmed cell death or apoptosis of activated lymphocytes. Usually, the acquired immune response to foreign antigen in animals involves the generation of a pool of activated effector cells through a process of activation, proliferation and differentiation followed by lymphocyte death, which is mainly mediated by signaling through the death receptors including Fas (CD95) and TNFR (Fig. 1.7B). In particular, activation of the Fas (CD95) death receptor by Fas ligand (FasL/CD95L) has been shown to play an important role in the apoptosis of activated T and B cells (86-88).

FasL and Fas are members of the TNF and TNFR family, respectively. While FasL is a type II membrane protein that is expressed predominantly on activated lymphocytes (89) Fas is a type I membrane protein that is expressed on various cell types (89, 90). Binding of FasL homo-trimers to the Fas receptor activates an apoptotic signal via the conserved intracellular death domain, resulting in the rapid killing of the Fas-bearing cell (88).

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The importance of lymphocyte apoptosis induced by the FasL/Fas pathway has been illustrated by spontaneous mutations in the genes encoding FasL leading to generalized lymphoproliferative disorder (*gld*) and Fas leading to lymphoproliferation (*lpr*) in mice (91-93). Mice that are homozygous for the *gld* (*gld/gld*) or *lpr* (*lpr/lpr*) mutations develop similar disorders including splenomegaly and lymphadenopathy. In addition, they have increased serum Ig levels and develop an autoimmune syndrome, including the production of autoantibodies, glomerulonephritis, and arthritis, leading to premature death between 5 and 9 months of age (91). It is important to note that humans with mutations in FasL or Fas also develop lymphadenopathy, splenomegaly, and autoimmune symptoms similar to those observed in *gld* or *lpr* mice (94, 95).

While splenomegaly and lymphadenopathy are caused by progressive accumulation of thymus-derived, Thy-1⁺CD4⁻CD8⁻CD3⁺B220⁺ abnormal T cells in peripheral lymphoid organs including spleen and lymph nodes, autoimmune syndrome is caused by B cells (91). Since thymic selection of T cells was shown to be normal in *gld/gld* or *lpr/lpr* mice (96) it is likely that CD4⁺ cells, CD8⁺ cells, or both could be the potential origin for the abnormal T cell population. Although evidence has been there for the requirement of CD4⁺ T cells and CD8⁺ T cells in the accumulation of Thy-1⁺CD4⁻CD8⁻CD3⁺B220⁺ abnormal T cells, it is still not clear, as to what is the exact source for these so called abnormal T cells in *gld/gld* or *lpr/lpr* mice (97-98). In the context of an immune response, TCR triggering initially results in the proliferation and Ag-specific clonal expansion of T cells. TCR triggering also delivers signals required to kill activated T cells at the end of the immune response, a process called activation-induced cell death (AICD), which mainly depends on a functional FasL/Fas signal transduction pathway (99, 100).

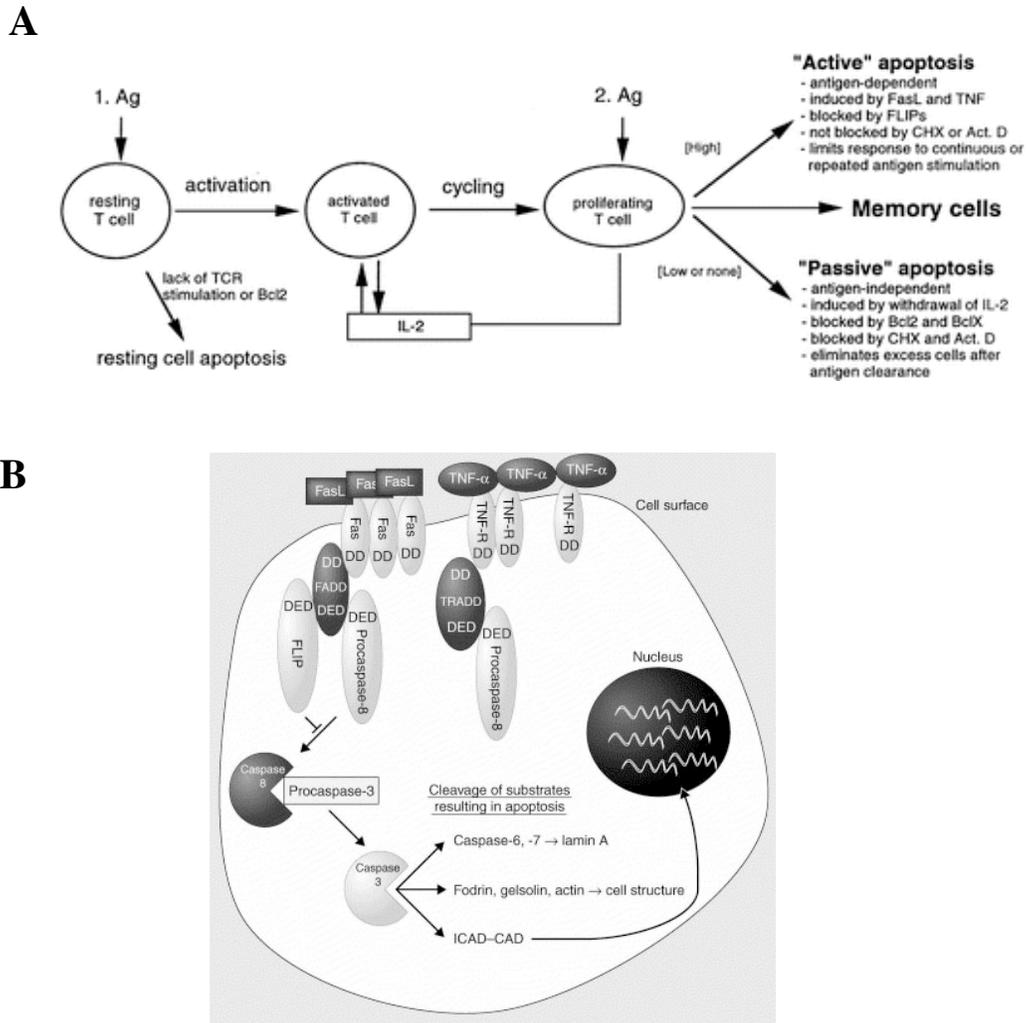


Fig. 1.7: Schematic representation of the events of Activation induced cell death of T cells.

(A) T cell response to antigen leads to activation, production of IL-2 and proliferation, which is due to cell cycle progression caused by IL2. Cycling T cells become highly susceptible to apoptosis. Active apoptosis occurs if strong secondary TCR engagement is encountered. Passive apoptosis occurs after cessation of antigen and IL-2 stimulation. A small number of cells escape the death pathways and these cells become memory population. (B) Illustration of death receptor driven apoptosis in activated T cells. Oligomerization of death receptors (such as Fas and TNF-R) by their ligands induces recruitment of adaptor proteins such as FADD and TRADD. These adaptor proteins bind to the cytoplasmic tail of receptors through homologous DD interactions. Procaspase-8 (non-activated caspase-8) is recruited into the complex via homologous death effector domain (DED) interactions. High levels of FLIP out-compete procaspase-8 for binding to FADD and thereby prevent initial caspase activation. Once activated, caspase-8 cleaves and activates caspase-3, which in turn cleaves other caspases (e.g. caspase-6 and -7), the inhibitor of caspase-activated DNase (ICAD) and molecules important in cell integrity. These in turn act on molecules such as lamin A and affect cell structure; once released from ICAD, CAD affects the cell nucleus.

{A. Adapted from Hildeman DA et al., 2002 (99); B. Adapted from Lenardo M et al., 1999 (100)}.

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These kinetics of T cell activation, proliferative expansion and apoptotic death at the end of the immune response are illustrated in **Fig. 1.7A and B**. Due to the mutation in the FasL and Fas gene, elimination of activated T cells is impaired and appears to be the primary defect in *gld* and *lpr* mice respectively (101, 102).

TCR triggering, also leads to the induction of members of the Rel/NF- κ B family of transcription factors, which play an important role in proliferation and survival of lymphocytes (103, 104). In addition to TCR stimulation, treatment of T cells with TNF also results in the activation of NF- κ B, which protects T cells from TNF mediated death. Since, Fas pathway is a poor activator of NF- κ B activation of Fas usually results in cell death rather than survival (7, 8, 105). The predominant NF- κ B inhibitory molecule in T cells is I κ B α (106). Non-phosphorylatable mutants of I κ B α as described before have been generated and shown to inhibit signal-dependent NF- κ B activation in many systems, including in T cells of transgenic mice. Inhibition of NF- κ B in T cells results in impaired proliferation and increased apoptosis upon activation. In particular, NF- κ B is required to protect T cells from apoptosis induced by death pathways other than Fas (33, 35, 84). Therefore, it would be interesting to study the consequences of T cell-specific inhibition of NF- κ B on the development of *gld* phenotype.

1.6 Aims of the project.

Deletion of individual members of the Rel/NF- κ B family or complete inhibition of NF- κ B in T cells by overexpression of mutant I κ B α does not effect the thymic development of mainstream T cells. However, role of NF- κ B in the development of other subtypes of T cells such as NKT cells has not been studied. LT signaling and NIK are involved in NF- κ B activation and have been shown to be required for NKT cell development. Therefore, the major aim of this project is to understand (i) role of individual Rel/NF- κ B family members in the development of NKT cells. Towards this goal *nfkb1^{-/-}*, *nfkb2^{-/-}*, *relB^{-/-}* mice and *mI κ B α ^{tg}* mice in which NF- κ B is completely inhibited in T cells by the overexpression of mutant non-degradable form of I κ B α , will be analyzed by flowcytometry; and (ii) to understand the mechanism by which NIK regulates activation of NF- κ B. Towards this goal, embryonic fibroblasts from wild-type and *aly/aly* mice will be used as a model system to study the requirement of NIK for NF- κ B activation downstream of LT and TNF signaling.

The function of RelB in T cells is less understood. Previous reports indicate that RelB is less susceptible for inhibition by I κ B α , which is the predominant inhibitor of NF- κ B in T cells. Therefore, consequences of RelB overexpression in *mI κ B α ^{tg}* mice will be studied with the aim to understand RelB function in T cells.

Mice with mutations in the genes for FasL and Fas develop generalized lymphoproliferative disorder (*gld*) and lymphoproliferation (*lpr*), respectively. These disorders are predominantly due to defective activation induced cell death of peripheral T cells. NF- κ B regulates the proliferation of T cells and protects peripheral T cells from apoptosis. Therefore, *mI κ B α ^{tg}* mice will be crossed to *gld/gld* mice to generate *mI κ B α ^{tg}gld/gld* mice with the aim of studying the consequences of inhibition of NF- κ B within T cells, on the development of generalized lymphoproliferative disorder.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Mice

Generation of *nfkb1^{-/-}*, *nfkb2^{-/-}*, *relB^{-/-}*, *light^{-/-}*, *lta^{-/-}*, *ltb^{-/-}*, *tnf^{-/-}*, *lta^{-/-}tnf^{fl}*, *ltbr^{-/-}*, *tnfr^{-/-}*, and *relb^{tg}* mice has been previously reported (107-115). *hvem^{-/-}* mice were kindly provided by Dr. Klaus Pfeffer, Institute of Medical Microbiology, University of Dusseldorf, Dusseldorf, Germany. *ltbr^{-/-}* and *hvem^{-/-}* mice were crossed to generate *ltbr^{-/-}hvem^{-/-}* double-deficient mice. Alymphophasia (*aly/aly*) were kindly provided by Dr. Thomas Böhm, Max-Planck-Institute of Immunobiology, Freiburg, Germany. Mice carrying the autosomal recessive mutation *gld* (B6Smn.C3H-*Fas^{gld}*) were obtained from The Jackson Laboratory, Bar Harbor, ME. C57BL/6-Ly-5.1 mice were obtained from Charles River Laboratories. Transgenic mice expressing the non-degradable mutant IκBα in T cells (*mIkBα^{tg}*) was generated and kindly provided by Dr. Rolf-Peter Ryseck, Department of Oncology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, USA. Briefly, the non-degradable mIkBα carrying alanines instead of serines 32 and 36 was generated by the method of Kunkel. The mutated cDNA was then cloned into plasmid pTLC by inserting into the *Bam*HI site under the control of 3.2 kb of the mouse proximal *lck* promoter. The initiation codon of the cDNA is preceded by the β-globin initiation signal, to maximize translation efficiency, and a T7 tag. A 2.1 kb fragment of the human growth hormone gene downstream of the cDNA provides introns and the polyadenylation signal sequence. A 2.1 kb fragment of the locus control region of the human CD2 gene at the 3' end confers copy number-dependent and insertion site-independent levels of expression (115). Generation of transgenic mice was essentially as described before (115). Transgenic *gld/gld* mice that overexpress the non-degradable molecule in T cells (*mIkBα^{tg}gld/gld*) were generated by crossing the *mIkBα^{tg}* mice with B6Smn.C3H-*Fas^{gld/gld}* mice. Generation of *relB^{tg}* mice was described before (115) and is essentially similar to *mIkBα^{tg}* mice. Double-transgenic mice overexpressing, both RelB and mIkBα in T cells were generated by crossing *mIkBα^{tg}* mice with *relB^{tg}* mice. All animals were housed and bred under the standard conditions with water and food ad libidum in the pathogen free mouse facility of the Forschungszentrum Karlsruhe, Institute of

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Toxicology and Genetics. For timed pregnancies, mice were mated and the noon of the day vaginal plugs were observed was accepted as 0.5 days post coitus (d.p.c).

2.1.2 Chemicals and reagents

All the general lab chemicals used in this study were purchased from the following list of companies.

Carl Roth GmbH + Co

E. Merck

Life Technologies

Serva Fein Biochemica GmbH & Co

Sigma Chemical Co

α -GalCer was a kind gift from Chi-Huey Wong

2.1.2.1 α -GalCer-loaded CD1d-tetramers.

α -GalCer-loaded CD1d-tetramers were kindly gifted by Dr. Mitchell Kronenberg. They are similar to conventional MHC-tetramers, which are used to detect antigen specific T cell populations, from millions of T cells with different antigenic specificity. While conventional T cells recognize peptides that are bound to MHC molecules on the antigen-presenting cells (APCs), V α 14i NKT cells recognize lipid antigen (α -GalCer) bound to CD1d. This recognition is specific for both the MHC allele and the peptide/lipid antigen. Therefore it is possible to identify antigen-specific cells by staining with soluble peptide-MHC or lipid-CD1d complexes that are attached to fluorochromes. Since monomeric peptide-MHC or lipid-CD1d complexes bind poorly to the TCR, multimers (tetramers) are necessary for efficient detection of antigen-specific T cells (51, 52 and for a review see 116). For a brief method of MHC-tetramer preparation see below (**Fig. 2.2**).

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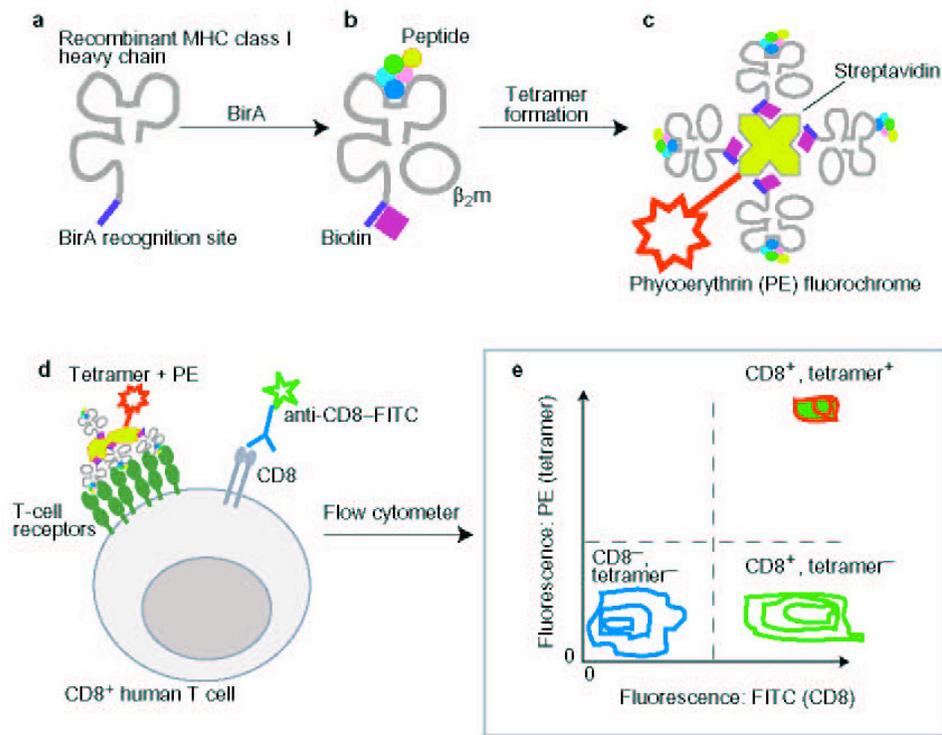


Fig. 2.2: Schematic representation of preparation of MHC tetramers and tetramer analysis to detect antigen-specific T cells that have specific T cell receptors on their cell surface .

(a) Soluble versions of the heavy chain of major histocompatibility complex (MHC) class I molecules are synthesized in E.coli bacteria. (b) The molecules adopt an appropriate conformation following the addition of $\beta 2$ microglobulin ($\beta 2m$) and a synthetic peptide that represents the epitope that is recognized by the TCR of interest. This peptide is able to bind to the MHC molecule. In addition, the enzyme BirA is used to attach a biotin molecule to the specific BirA-recognition sequence, which has been incorporated into the carboxyl terminus of the MHC molecule. (c) Four MHC-biotin complexes are linked to a single streptavidin molecule, using the specific biotin-avidin interaction, to form a tetramer. The streptavidin molecule is tagged with a fluorochrome (e.g. phycoerythrin; PE). (d) Tetramers are mixed with the cell population that is to be analyzed (eg. $CD8^+$ T cells or total T cells). Only T cells with TCRs that are specific for the MHC-peptide combination that is present in the tetramer are able to bind the tetramer and such cells will be labeled with the PE fluorochrome (shown in red on the picture in panel 'e'). A different fluorochrome (eg. Fluorescein isothiocyanate; FITC, shown in green on the picture) tagged mAb. that is specific to a different T cell marker can be used to specifically detect particular class of T cells. After staining with both the tetramer and the T cell-specific mAb, cells are analyzed by flow cytometry to detect the cells that are reactive to a particular antigen on the tetramer. Empty tetramers (without the peptide) are used generally as a control in these experiments.

NOTE: In the case of α -GalCer-CD1d-tetramers, in steps a and b, CD1d molecules and α -GalCer are used instead of classical MHC and peptide. The rest of the protocol is similar.

(Adapted from Expert Reviews in Molecular Medicine ISSN 1462-3994)

2.1.3 Oligonucleotides

Unless otherwise stated, all oligonucleotides were from MWG Biotech GmbH and Genset Oligos. All oligonucleotides were synthesized under standard conditions as 0.01 μ M.

Below are the sequences of the oligonucleotides used in this study for RT-PCR analysis:

LT α -Forward	5'-ATGACACTGCTCGGCCGTCT-3'
LT α -Reverse	5'-CTACAGTGCAAAGGCTCCAAA-3'
LT β -Forward	5'-TTGTTGGCAGTGCCTATCACTGTCC-3'
LT β -Reverse	5'-CTCGTGTACCATAACGACCCGTAC-3'
LT β R-Forward	5'-TTATCGCATAGAAAACCAGACTTGC-3'
LT β R-Reverse	5'-TCAAAGCCCAGCACAATGTC-3'
IL-15-Forward	5'-TCA GCA GAT AAC CAG CCT AC-3'
IL-15-Reverse	5'-TTT CTC CTC CAG CTC CTC AC-3'
IL-15R α -Forward	5'-GAA GAG AAG ATA CAA GGG CAG-3'
IL-15R α -Reverse	5'-ACA CAC ACA CAC ACA CAT AC-3'
IRF-1-Forward	5'-AAG CCA CCA TGC CAA TCA C-3'
IRF-1-Reverse	5'-CTA TCT TCC CTT CCT CAT CCT C-3'
GMCSF-R β -Forward	5'-ATG CTC TCC GGT GGT GAA-3'
GMCSF-R β -Reverse	5'-TGG CGC AGT ATG AGG TGT CT-3'
Vav-1-Forward	5'-GGG AGC GAG ACA ACA AGA AG-3'
Vav-1-Reverse	5'-TTA CAG GGA AAC CAG CCG AC-3'
fyn-Forward	5'-CCA TCC CGA ACT ACA ACA AC-3'
fyn-Reverse	5'-CCC TTT CAT ATC ATC CCA ATC AC-3'
Ets-1-Forward	5'-ACT ACC CTT CTG TCA TTC TCC-3'
Ets-1-Reverse	5'-TTT CCT CTT TCC CCA TCT CC-3'
Cathepsin-S-Forward	5'-TGG TGT GCT CTG TGG CAA TGG A-3'
Cathepsin-S-Reverse	5'-TGA AAG CCC AAC AGG CAC CAC-3'
V α 14	5'-GTT GTC CGT CAG GGA GAG AA-3'
J α 18	5'-CAA TCA GCT GAG TCC CAG CT-3'

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C β -Forward	5'-CAC TGA TGT TCT GTG CA-3'
C β -Reverse	5'-GAG GAT CTG AGA AAT GTG ACT CCA-3'
Actin-Forward	5'-AGAGGTATCCTGACCCTGAAGTACC-3'
Actin-Reverse	5'-CCACCAGACAACACTGTGTTGGCAT-3'

2.1.4 Cells and Cell Culture Media.

Unless otherwise stated, all tissue culture media and PBS used to wash cells were purchased from Invitrogen GmbH together with the supplements such as Penicillin/Streptomycin and Glutamax. Trypsin was purchased from Difco Laboratories and was diluted to 0.25% in 15 mM sodium citrate and 134 mM potassium chloride, which were kept as stocks at -20°C.

Wild-type and *aly/aly* primary mouse embryonic fibroblasts (MEFs) were prepared in house.

2.2 METHODS

2.2.1 Preparation of Mouse Embryonic Fibroblasts.

All the primary MEFs were prepared at E15.5 or E16.5, which was determined according to the plug date of the female mouse. Head and internal organs of the embryos were removed on a petri plate. Equipment used was cleaned with PBS and 70% ethanol between processing of each embryo. The trunk of the embryo was kept in a multi well plate on ice in 1 ml PBS. Embryos (one per plate) were dissected in 10 ml 0.05% trypsin (diluted from 0.25% stock with plain DMEM medium) in 10-cm dish with fine scissors. After incubation at 37°C for 10 min, the solution was pipetted up and down 5 times with 20 ml glass pipette. This was repeated with 10 ml and 5 ml glass pipettes with 10 min incubations at 37°C in between. Once complete, trypsinization was terminated by adding 2 ml FCS. Cell suspension and clumps were transferred into a 15-ml Falcon tube and incubated on ice to sediment the clumps for 5-10 min. Approximately 10 ml of the supernatant was transferred into a 50-ml Falcon through 100 μ m cell strainer (Falcon) leaving clumps behind. After centrifugation at 2000 rpm for 5 min at RT, the pellet was washed once with 10 ml complete medium, the suspension was transferred to a new falcon tube through the cell strainer and cells were counted using Trypan blue (Sigma) (10 μ l cell suspension + 10 μ l Trypan blue solution, cell number per ml was determined

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after counting at least 2 big squares on the hemocytometer using the following formulae: number of cells counted / number of squares x 2 (dilution factor) x 10^4). Typical yield per an embryo is 8-10 (10^6) cells. Finally, the pellet was resuspended according to the count and plated up to 10 (10^6) cells per 15 cm plate. The next day, fibroblasts adhere and non attached cells were removed by removing the medium and adding fresh medium. In 2 or 3 days, the desired confluency was reached for splitting. Primary cells prepared as described above were expanded and frozen down in aliquots of 2×10^6 /ml in freezing solution (90% FCS and 10% DMSO) (see long term storage of cells below).

2.2.2 Preparation of single cell suspensions from lymphoid organs.

Different lymphoid organs were first collected and the tissues were pushed through a metal sieve into a 6 cm petri dish with 5 ml of FACS buffer (I) using a plunger from a 5 ml syringe. The metal sieve was rinsed with 5 ml of FACS buffer and the cell aggregates that were collected into the petri dish were passed through 10 ml syringe with a 21 Gauge needle for 3-5 times. After transferring into a 15 ml or 50 ml Falcon tubes, cells were centrifuged. Cells were then resuspended in ACK lysis buffer (II) and left at room temperature (RT) for 5 minutes (min). 5 ml of cold FACS buffer was added and centrifuged. Supernant was removed and the cells were washed with 10 ml of FACS buffer and centrifuged. The cells were finally resuspended in 10 ml of FACS buffer and passed through cell strainer with 70 μ m pore size. An aliquot of 10 μ l cells was taken into trypan blue solution for counting trypan blue negative cells using hemocytometer. All the centrifugation steps were performed at 1500 revolutions per minute (rpm) for 5 min at 4°C.

FACS buffer (I) : PBS with 2% fetal calf serum (FCS)

ACK lysis buffer (II) : 0.15 mM NH_4Cl , 1 mM KHCO_3 , 0.1 mM Na_2EDTA pH 7.2-7.4 adjusted with 1 N HCl, filter-sterilized and stored at RT.

2.2.3 Preparation of mononuclear cells from Liver.

Freshly collected liver was passed through a metal sieve into a 6 cm petri plate containing 5 ml FACS buffer. The metal sieve was rinsed with 5 ml of FACS buffer and the cell aggregates were passed through 10 ml syringe with 21 Gauge needle as described above. Cells were passed through a cell strainer of 100 μ m pore size and collected into 15 ml Falcon tubes and centrifuged at 1500 rpm for 10 minutes at 4°C. During the centrifugation, percoll solution (amersham) was diluted in PBS to prepare 40% and 60%

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solutions. Cells were resuspended in 6 ml of 40% percoll solution and layered carefully onto 60% percoll solution in 15 ml falcon tubes and were centrifuged at 900 g for 20 min at 4°C. The mononuclear cells and red blood cells from the junction between the 60% and 40% percoll solutions were collected carefully using a pastuer pipette into another 15 ml falcon tube. About 10 times excess of FACS buffer was added to wash the cells and centrifuged at 1800 rpm for 5 min. The pellets were resuspended in 5 ml ACK lysis buffer and incubated for 5 min at RT. 5 ml of cold FACS buffer was added and the cells were centrifuged at 1500 rpm for 5 min at 4°C. Pellets were resuspended in 2 ml FACS buffer and passed through cell strainer of 70 µm pore size. An aliquot of cells was used for counting.

2.2.4 Purification of lymphocyte sub-types using magnetically activated cell sorting (MACS) columns.

Purification of different lymphocytes was performed essentially according to the manufacturers specifications (Miltenyi Biotec), with minor modifications.

2.2.4.1 Purification of B Cells.

Approximately 100×10^6 splenocytes were stained with 20 µl of purified anti-B220 mAb (Pharmlingen) in 1 ml of FACS buffer on ice for 25 min and then washed with 10 times excess of MACS buffer (I). After centrifuging at 1300 rpm for 5 min at 4°C, cells were resuspended in 600 µl of MACS buffer and mixed with 100 µl of anti-rat-IgG MicroBeads (Miltenyi Biotec) and incubated for 15 min at 6-10°C. After the incubation magnetic beads were washed away by adding 10-12 ml of cold FACS buffer and centrifuged at 1300 rpm for 5 min. Pellet was resuspended in 1 ml of cold MACS buffer and passed through cell strainer (70 µm pore size) on to the MACS positive selection column (LS; Miltenyi Biotec) and purified the B cells as per the manufacturer's specifications. An aliquot of cells from each step of purification procedure was used for flow cytometric analysis to check the purity of B cells from the eluate.

MACS Buffer (I): 0.5% Bovine Serum Albumin (BSA) in PBS without Ca^{2+} and Mg^{2+} and 2 mM EDTA.

2.2.4.2 Purification of CD4⁺ and untouched T cells.

For purifying CD4⁺ T cells, approximately 100 x 10⁶ splenocytes in 1 ml of MACS buffer were stained with 100 µl of anti-CD4 MicroBeads for 15 min at 6-10°C. The rest of the protocol is essentially same as described for B cell purification. For purifying untouched T cells, lymph node cell suspensions were incubated with anti-MHC class-II MicroBeads and T cells were purified by passing cells through MACS negative selection columns (LD; Miltenyi Biotec) and following the manufacturer's specifications.

2.2.5 Analysis of cells by flow cytometry.

Flow cytometry was performed using a Becton Dickinson LSR flow cytometer. All the monoclonal antibodies (mAbs) used for flow cytometric analysis were purchased from BD biosciences. PE-conjugated mAbs were diluted 1:200 and all other mAbs were diluted 1:100 before use. Single cell suspensions were washed twice with staining buffer (PBS, 2%FCS) and incubated with Fc BlockTM (clone 2.4G2). The following fluorescently conjugated mAbs were used: anti-NK1.1-PE (clone PK136), anti-TCRβ-FITC (clone H57-597), anti-CD24/HAS-biotin (clone M1/69), anti-CD4-FITC/Biotin (clone RM4-5), anti-CD8α-PE/Biotin (clone 53-6.7), anti-CD1d-FITC (clone 1B1), anti-CD44-Biotin (clone IM7), anti-Ly5.2-Biotin (clone 104), anti-ly5.1-Biotin, anti-B220-PE (clone RA3-6B2), anti-Thy1.2-FITC (clone 53-2.1), anti-CD3ε-PE (clone 145-2c11). Biotinylated mAbs were detected with Streptavidin-PerCP (diluted 1:200). α-GalCer loaded CD1d-tetramers were kindly provided by Dr. Mitchell Kronenberg.

Staining with all the mAbs was done as follows. Cells were incubated in 96 well plates (CellStar, Griener) on ice with different combinations of mAbs in PBS containing 2% FCS (FACS buffer) for 30 minutes (min). After the incubation, cells were centrifuged for 5 min at 1500 revolutions per minute (rpm) and were washed two times with FACS buffer. Cells were then fixed with 4% formaldehyde in PBS for 10 min at room temperature (RT). After washing for two times cells were resuspended in FACS buffer for analysis or for storage at 4°C.

For staining with α-Galcer-loaded CD1d-tetramer the tetramer stock solution was diluted 1:200 in FACS buffer and cells were resuspended in 27 µl of diluted solution and were

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incubated for 30 min on ice. Cells were washed two times and analyzed directly by flow cytometry.

Apoptotic cells were detected by Annexin-V-FITC (Roche Diagnostics Mannheim, Germany) according to the manufacturer's specifications. Briefly, 24 well petri plates (CellStar, Greiner) were coated with anti-CD3 and anti-CD28 mAbs (BD Bioscience), after diluting to 10 mg/ml each in 50 mM Tris-Cl pH 9.0 (Ab., coating buffer) for 12 hours at 4°C or 2 hours (h) at 37°C. Plates were rinsed once with PBS and then used. Upto 1×10^6 cells/ml in lymphocyte medium were added to the wells and stimulated for 36 h at 37°C in the incubator. Cells were initially harvested into eppendorfs, centrifuged for 5 min at 1400 rpm and resuspended in 200 µl of incubation buffer (I) and were transferred to 96 well plates. The plates were centrifuged to remove the supernatant and then cells were stained with annexin-V staining solution (II). After incubating for 15 min cells were washed twice with 200 µl of incubation buffer and finally resuspended in 100 ml of incubation buffer for flow cytometric analysis.

Incubation buffer (I): 10mM HEPES/NaOH, pH 7.4, 120 mM NaCl, 5 mM CaCl₂

Annexin-V staining solution (II): 10 ml of annexin-V-FITC stock (Roche) and 20 µl of propidium iodide stock (50 µg/ml) were diluted in 1500 µl incubation buffer which is sufficient for 15 samples.

Data were analyzed using CELLQuest Pro™ software (BD Bioscience).

2.2.6 CELL CULTURE METHODS

2.2.6.1 Media and Maintenance

All cells were maintained at 37°C in an incubator C200 (Labotect GmbH) in 5% CO₂ and 95% humidity. Cells were grown on appropriate sized petri plates (Greiner Labortechnik). Below are the complete media used for different cell types:

Primary or established fibroblasts: DMEM (Invitrogen), 10% fetal calf serum (Bio Whittaker), Penicillin (100 U/ml) / Streptomycin (100 µg/ml), Glutamax I (1x, 2 mM) (L-Alanyl-L-Glutamine)

Starvation Medium for fibroblasts: DMEM, **0.5%** fetal calf serum (Bio Whittaker) or **0.5% CS** for NIH 3T3 cells, Penicillin (100 U/ml) / Streptomycin (100 µg/ml), Glutamax I (1x, 2 mM)

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Lymphocyte culture medium: RPMI, 10% FCS, Penicillin (100 U/ml) / Streptomycin (100 µg/ml), Glutamax I (1x, 2 mM), 2 mM β-mercaptoethanol.

Heat inactivated (at 56°C for 30 min) serum was aliquoted to 50 ml, Pen /Strep and Glutamax I (100x, 200 mM) stocks were aliquoted to 5 ml respectively and stored at -20°C. For routine complete medium preparation, one aliquot of each were used per 500 ml of plain DMEM or RPMI 1640 medium. All the media and trypsin were stored at 4°C and prewarmed to 37°C in a water bath prior to use.

For routine culture of fibroblasts, 15 cm petri plates were preferred. Fibroblasts were allowed to grow to 80-90% confluency for 3 days and then were splitted by trypsinization. For this procedure, 0.25% trypsin/0.5 mM EDTA solution was used. After the removal of medium, cells were washed once with PBS (-Ca/Mg, Invitrogen) and trypsin was added just enough volume to cover the cells. After incubation at 37°C for 5 min, trypsin was diluted by the addition of complete medium and cells were transferred to appropriate falcon tubes to be pelleted by centrifugation at 1500 rpm for 5 min at RT. The pellet was then reconstituted with fresh complete medium and re-plated at desired density.

2.2.6.2 Long Term Storage.

Growing cells were trypsinized, pelleted and washed with PBS thouroughly to remove the traces of trypsin and medium. Cells were then resuspended in freezing buffer (90% FCS, 10% DMSO), at a density of 2-3 million cells/ml., and aliquated into cryovials. DMSO was purchased from Fluka Chemie. Vials were placed in a cell freezing box (Nalgene™) and left at -80°C O/N for gradual freezing. Frozen cell vials then were transferred to liquid nitrogen for long term storage. Repropagation was performed by placing the vial with frozen cells briefly at 37°C and gradually thawing the cells by the addition of complete medium. The cell suspension was transferred to a Falcon tube, pelleted and washed with complete medium for the removal of DMSO and was resuspended in 10 ml complete medium to be plated in 10-cm tissue culture plates.

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2.2.6.3 Stimulation and Harvesting of cells.

Prior to stimulation, fibroblasts in this study were starved in 0.5% FCS containing DMEM medium for 24 h after reaching 80% confluency. Following stimulations were then performed:

Recombinant Murine TNF: 20 ng/ml (Promokine)

Recombinant Human TNF: 20 ng/ml (Promokine)

Agonistic anti-LT β R antibody (AC.H6, pure Armenian hamster monoclonal antibody): 1 μ g/ml (Biogen)

Ha 4/8 (anti-KLH Armenian hamster Ig): 1 μ g/ml (Biogen); isotype matched control for AC.H6

These reagents were added into the starvation medium for appropriate time points. Cells were harvested by routine trypsinization (see media and maintenance section) using starvation medium during the procedure. Pellets were processed either for whole cell extract (WCE) preparation and nuclear/cytoplasmic extract preparation.

For the culture and stimulation of thymocytes, peripheral T cells or B cells, 24 well plates were used and cultured in lymphocyte medium described before. The following stimulations were used.

Thymocytes and peripheral T cells:

- i) **anti-CD3 and anti-CD28:** 24 well Plates were pre-coated with anti-CD3 and anti-CD28 mAbs (10 μ g/ml each) in Tris-Hcl pH 9.0 for 2 hours at 37°C or over night (ON) at 4°C. Prior to use, pre-coated plates were flipped over to remove the coating solution and rinsed once with sterile PBS. Up to 10 x 10⁶ cells were then in 1 ml lymphocyte medium were then added to the wells and incubated at 37°C for the desired time points. After the stimulation cells were harvested into eppendorf tubes, pelleted by centrifuging at 1500 rpm for 5 minutes at 4°C and were washed two times with PBS. These cells were then used either for nuclear/cytoplasmic extract preparation or for RNA preparation.
- ii) **PMA + PHA stimulation:** PMA stocks were prepared at in DMSO. In 24 well plates, cells were stimulated in 1 ml lymphocyte medium containing 2 ng/ml PMA and 5 μ g/ml PHA for the desired time points and were harvested as described above to prepare nuclear/cytoplasmic extracts and RNA. For the un-stimulated cells, 2 μ l of DMSO was added.

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- iii) **α -GalCer stimulation:** 1mg/ml stocks were prepared in DMSO and up to 10×10^6 thymocytes in 1 ml lymphocyte medium were stimulated with 200 ng/ml of α -GalCer in lymphocyte medium for 16 hours at 37°C and were harvested to prepare RNA. For the un-stimulated cells 2 μ l of DMSO was added per one ml of the culture medium.

B cells:

Lipopolysaccharide (LPS) stimulation: LPS stocks were made at 1mg/ml in PBS. Up to 10×10^6 B cells in 1 ml lymphocyte medium were stimulated with different concentration of LPS and were harvested for preparing nuclear/cytoplasmic extracts.

2.2.7 Extraction and analysis of RNA.

RNA Isolation From Frozen Tissue Samples.

Tissues were snap frozen in liquid nitrogen as soon as the animals were sacrificed. At this step, samples can be stored in Eppendorf tubes at -80°C for further use. Tissues were either homogenized in 2 ml eppendorfs in 750 μ l Peq GOLD RNA Pure™ reagent (Peq Lab) or in 15 ml corex tubes in 3 ml RNA Pure reagent. The tip of the homogenizer (IKA Labortechnik T25 basic) was cleaned with water and 70% ethanol between samples. As soon as tissues were placed into tubes containing the reagent, homogenization was performed at highest speed.

Following homogenization, samples were left at RT for 5 min to ensure the complete dissociation of nucleoprotein complexes. Chloroform was added (0.2 ml / ml RNA Pure reagent), samples were vortexed vigorously for 10-20 sec and were centrifuged at 12000 g for 15 min at 4°C. 3/4th of the colorless upper aqueous phase was transferred to a new tube and isopropanol was added (0.5 ml/ml RNA Pure reagent) and mixed thoroughly and rapidly. Samples were left at RT for 5-10 min and then were centrifuged at 13000 g for 15 min at 4°C. RNA forms a pellet on the side and the bottom of the tube. Supernatant was removed and pellets were washed twice with 1 ml 75% ethanol. Samples were centrifuged at 13000 g for 5 min at 4°C, supernatants were discarded and pellets were air dried for 5-10 min. Finally the pellets were resuspended in appropriate volume of ddH₂O and were placed on a thermoshaker (Eppendorf Thermomixer 5436) for 10 min at 55°C

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for dissolution. Samples were further used for concentration estimation and gel electrophoresis.

Extraction of Total RNA From Cells.

Cells were pelleted in eppendorf tubes by centrifuging at 1500 rpm for 5 minutes at 4°C. After washing with PBS, cells were pelleted again and resuspended in 1 ml RNA Pure reagent by vortexing till the pellet was dissolved. At this step samples can be stored at -20°C for up to one week. The extraction of RNA was performed as with the frozen tissue samples (see above section) from this step onwards. When the starting material was less than 5×10^5 cells, after the transfer of the aqueous phase to a new tube, 2 µl glycogen (20 µg) (Peq Lab) was added.

Determination of RNA Concentration and Agarose Gel Electrophoresis of RNA Samples.

RNA concentration was determined spectrophotometrically (Eppendorf spectrophotometer) by taking readings at 260 nm against a blank, ddH₂O. A solution of 50 µg/ml DNA or 40 µg/ml RNA in a 1 cm quartz cuvette will give an absorbance of 1 at 260 nm. The concentrations were calculated according to absorbance values and total volume of RNA solution. Protein or phenol contamination during the extraction procedure was assessed by A_{260}/A_{280} ratio with a value of 1.7 or greater as an indication of a good purity.

The quality of RNA samples and genomic DNA contamination were assessed by agarose gel electrophoresis. Aliquots of RNA samples (minimum 0.5 µg) were mixed with RNasin (4 U/sample) and 10x gel loading buffer and were loaded on 0.8% (w/v) agarose gels prepared in 1x TAE buffer and containing ethidium bromide to a final concentration of 0.3 µg/ml for visualization of nucleic acids. Gels were observed by illumination with UV light and photographs were taken using an Eagle Eye photocamera system (Stratagene).

10x gel loading buffer: 0.25% bromophenolblue, 0.25% xylene cyanol FF, 15% Ficoll in water; kept at RT, final 1x concentration.

1x TAE buffer: 0.04 M Tris-acetate, 0.01 M EDTA

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First Strand cDNA Synthesis of Total RNA Samples.

First strand complementary DNA (cDNA) was synthesized from total RNA by using Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen) as described by the manufacturer. One ng-5 µg total RNA was added in an Eppendorf tube together with 1 µl oligo (dT)₁₂₋₁₈ (500 µg/ml) (New England Biolabs) and 1 µl 10 mM dNTP mix (Peq Lab) (10 mM each dATP, dGTP, dCTP, dTTP at neutral pH). The volume was completed to 12 µl with sterile ddH₂O. The reaction mixture was heated to 65°C for 5 min and quickly chilled on ice. Following brief centrifugation, 4 µl 5x first-strand buffer, 2 µl 0.1 M DTT (dithiothreitol) (Invitrogen) and 1 µl RNaseOUT recombinant ribonuclease inhibitor (Promega) (40 U/µl) were added and the samples were incubated at 42°C for 2 min. Then, 1 µl (200 U) of Superscript II was added, mixed by pipetting up and down and samples were incubated at 42°C for 50 min. Following heat inactivation at 70°C for 15 min, cDNA samples were ready for amplification by PCR. The samples were diluted 5 fold (final volume 100 µl) with sterile ddH₂O before they were used as template for amplification.

Semiquantitative Reverse-Transcriptase Polymerase Chain Reaction

The reaction mix was prepared by mixing the following per reaction:

5.7 µl ddH₂O
3 µl glycerol (Calbiochem, molecular biology grade)
2 µl 10x PCR buffer (-Mg) (Invitrogen)
0.6 µl MgCl₂ (50 mM, Invitrogen)
1.5 µl dNTPs (2.5 mM each) (Peq Lab)
0.1 µl [α -³²P] dCTP (3000 Ci/mmol; 10 µCi/µl, Amersham)
1 µl sense primer (250 ng/µl for 25mer)
1 µl antisense primer (250 ng/µl for 25mer)
0.1 µl Taq polymerase (0.5 U, Invitrogen)

The above mix was distributed as 15 µl/reaction into PCR tubes and 5 µl cDNA (from 100 µl final diluted volume) was added and mixed. PCR was performed with MJ Research PTC 225 thermal cycler using the following programme:

94°C 2 min
94°C 45 sec
Determined Ta 45 sec
72°C 45 sec for a total of optimised cycle number
8°C forever

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For determination of the appropriate cycle number, cDNA samples (for most of the primer pairs as a positive sample, cDNA synthesized from total mouse thymus RNA was used) were either left undiluted or diluted 2-fold, respectively and reactions were amplified for 18, 20, 22, 25 and 30 cycle numbers. The radiolabeled PCR products were separated by 6% polyacrylamide gels. Following electrophoresis (200-250 Volts 2-3, h), gels were dried at 80°C for 30 minutes using gel drier (Bio-Rad) and exposed using phosphorimager imaging plate (Fuji film FLA-3000). Quantifications were done using Aida software and the linear range was determined. For subsequent analyses with a particular primer pair, the determined cycle number was used and quantifications were always done as described above.

6% polyacrylamide gels: 35 ml ddH₂O, 10 ml acrylamide: bisacrylamide (30 %) (Rotiphorese), 5 ml 5x TBE, 300 µl 10% (w/v) APS, 40 µl TEMED for a final volume of 50 µl.

5x TBE: 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0)

2.2.8 Extraction and analysis of Proteins.

2.2.8.1 Whole Cell Extract (WCE) Preparation.

Cells were trypsinized and pelleted by centrifugation (2000 rpm 5 min 4°C) and the pellet was washed once with phosphate buffered saline (PBS). The volume of the pellet was estimated, supernatant was quickly removed and the pellet was resuspended on ice with 5-fold volume of ice cold suspension buffer. Then an equal volume (5-fold of pellet volume) of 2x SDS gel loading buffer was added as soon as possible without pipetting up and down. The concentration of loading buffer becomes 1x in the final volume. The sample was incubated at 95°C for 10 min and then kept on ice. The chromosomal DNA was either sheared by sonication (full power between 30 sec and 2 min, Branson sonifier) or by repeated passages through 23 Gauge needle and 1 ml syringe. Following centrifugation at 10000 rpm for 10 min at RT, the supernatant was transferred to a new tube and kept at -80°C for further use. An equal volume of samples (20-40 µl) was then loaded for SDS-PAGE analysis.

Proteinase inhibitors: One tablet in 2 ml ddH₂O gives 25x stock (Roche).

Suspension buffer: 0.01 M Tris.Cl pH 7.6, 0.1 M NaCl, 0.001 M EDTA pH 8.0, stored at RT without proteinase inhibitors and just before use inhibitor solution was added to a final concentration of 1x.

2x SDS gel loading buffer: 100 mM Tris-Cl pH 6.8, 200 mM DTT (5x dilution from 1M stock), 4% SDS (sodium dodecyl sulphate, electrophoresis grade), 0.2% bromophenol blue and 20% glycerol; this buffer is stored at RT without DTT.

2.2.8.2 Preparation of Nuclear / Cytoplasmic Fractions.

Cells were trypsinized and pelleted in an Eppendorf tube. Supernatant was removed and cells were gently resuspended in 400 µl buffer A. The sample was incubated on ice for 15 min and then 25 µl 10% NP-40 was added, vortexed vigorously for 10 seconds and centrifuged at 13000 rpm for 1 min at 4°C. At this step, the supernatant contains cytoplasmic RNA and proteins and pellet contains nuclei and cell debris. An aliquot of supernatant, 200-300 µl, was transferred to a new tube as **cytoplasmic** fraction. The nuclear pellet was washed once with 200 µl buffer A gently, buffer was removed and 60 µl of buffer C was added without resuspending. The sample was then placed on an Eppendorf shaker for 15 min at 4°C to ensure vigorous mixing such that the pellet remains intact and floats around. Following centrifugation at 13000 rpm for 5 min at 4°C, 50 µl of **nuclear** fraction was recovered and protein concentration was determined. Cytoplasmic and nuclear fractions were stored at -80°C. In order to use these fractions for SDS-PAGE, appropriate amounts of protein extracts (minimum 10 µg) were taken and the volume of the samples to be analysed was brought to the same final volume by adding complete buffer C. The same final volume of 2x SDS loading buffer was then added (final 1x), samples were incubated at 95°C for 5 min and left to cool to RT before loading.

Buffer A: 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1x proteinase inhibitors and 2.5 mM DTT

Buffer C: 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 25% glycerol, 1x proteinase inhibitors and 2.5 mM DTT

Both buffers were stored at 4°C without proteinase inhibitors and DTT.

2.2.8.3 Whole Tissue Extract (WTE) preparation.

Freshly isolated spleen and thymus were snap frozen immediately as the animal was sacrificed in liquid nitrogen and were either processed immediately or stored at -80°C. Mortar and pestil were cooled on dry ice and few ml of liquid nitrogen was added to the mortar. Tissues were dropped into the liquid nitrogen in the mortar and were homogenized using the pestil that has been kept on dry ice. The tissue powder was collected into eppendorf tubes and an approximate volume of the powder was estimated and was resuspended in 3 x volume of Buffer-C (*) and vortexed vigorously for 30

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seconds. The samples were kept on ice until further use. In an appropriate container, methanol was added to dry ice and the water bath was kept ready at 37°C. The samples were subjected to 3-4 freeze-thaw cycles using dry ice + methanol and the water bath at 37°C with vigorous vortexing between each freeze-thaw cycle. Samples were then kept on a shaker at 4°C for 30 min and then were centrifuged at 13,000 g for 5 min at 4°C. The supernatant was collected into fresh eppendorfs and stored at -80°C until further use.

*Buffer-C: Same as the one used for making nuclear extracts with 0.25% NP40. Proteinase inhibitor and DTT were added freshly before use.

2.2.8.4 Protein Concentration Determination.

Protein concentration was determined by BioRad microassay procedure following the manufacturer's instructions. Bovine IgG was used as a protein standard. Several dilutions of standard were prepared (1-25 µg/ml). In dry cuvettes, 800 µl diluted standards or appropriate amount of samples (2-3 µl cytoplasmic or nuclear extract) diluted in 800 µl ddH₂O were placed. 200 µl Dye Reagent Concentrate (5x stock, BioRad) was added and gently mixed. For blanks, 800 µl ddH₂O was used. After 5 min RT incubation, OD₅₉₅ measurement was performed and a standard curve (concentration in µg/ml versus OD₅₉₅) was plotted to determine the concentrations of extracted samples.

2.2.8.5 SDS-PAGE.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Sambrook & Russel, 2001. Resolving (8%) and stacking (5%) gels were prepared as below:

<i>gel</i>	<i>8% resolving gel</i>		<i>5% stacking</i>
	9.7 ml	ddH₂O	6.8 ml
	8 ml	acryl.: bisacryl. (30%)	1.7 ml
(pH 8.8)	11.7 ml	1 M Tris HCl (pH 6.8)	1.3 ml
	300 µl	10% SDS	100 µl
	300 µl	10% APS	100 µl
	24 µl	TEMED	10 µl
	30 ml	Final volume	10 ml

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Resolving gel solution was prepared and poured between 2 glass plates using the Joey casting system (Peq Lab). The glass plates were marked to provide approximately 9 cm resolving and 2 cm stacking gel. A straight boundary between resolving and stacking gels was prepared by pouring 70% ethanol. After polymerization was complete, ethanol was poured off, the surface was washed with ddH₂O and dried with Whatman paper. Then 5% stacking gel solution was poured on top and an appropriate comb was inserted. Following polymerization, comb was removed and the system was casted to P9DS apparatus (Peq Lab). Tris-glycine buffer (1x) was used as the running buffer and the gel was run until the bromophenol blue in the SDS gel loading buffer migrated out.

1x Tris-Glycine buffer: 25 mM Tris, 250 mM glycine, 0.1% SDS

2.2.8.6 Western Blotting.

Transfer of Proteins from Gel to Membrane

The transfer was performed by Trans/Blot Cell transfer system (Bio-Rad). For each gel, 4 sheets of Whatman papers and one membrane were pre-cut as the dimensions of the gel. PVDF membrane (ImmobilonTM-P, Millipore) was activated for 15 seconds in methanol, washed in ddH₂O for 2 min and then placed in transfer buffer for 5 min. Meanwhile the gel was removed and different bands of the loaded prestained Benchtop protein marker (Invitrogen) was recorded by placing a transparent file on the gel and marking the bands. This record was later to be used to mark the corresponding molecular weights on the autoradiogram. The gel was placed on 2 sheets of prewet Whatman papers. The membrane was placed on top of the gel, the start was cut on the edge for orientation and air bubbles were prevented by rolling over a glass pipette gently. The sandwich was completed by placing 2 extra sheets of Whatman papers and was assembled into the transfer chamber filled with transfer buffer, which was freshly prepared for each transfer. All transfers were carried out at 20 V at RT for 7 h on a magnetic stirrer to ensure continuous mixing of the buffer.

Transfer buffer: 39 mM Glycine, 48 mM Tris base, 0.037% SDS (electrophoresis grade), 20% (v:v) methanol; pH 8.3

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Immunoblotting

Following transfer, membrane was placed as protein side up into a container filled with blocking solution and was blocked either for at least 1 h at RT or O/N at 4°C. Once complete, the membrane was sealed in a nylon bag and primary antibody diluted in blocking solution was added. Following 1 h RT incubation, the membrane was rinsed rapidly twice and then washed 3 times, 10 min each, with TBS-T. The membrane then was incubated similarly with appropriate horseradish peroxidase (HRP) conjugated secondary antibody for 1 h at RT. Following washes as described above, chemilluminescence detection was performed by ECL Western blotting detection system (Amersham) according to the manufacturer's instructions. The exposures were performed with Hyperfilm ECL (Amersham).

10x TBS: 250 mM Tris, 1.5 M NaCl, pH adjusted to 8.0 with HCl.

TBS-T: 1x TBS in ddH₂O, 0.1% (v:v) Tween-20.

Blocking solution: 5% (w:v) nonfat dried milk (Saliter) in TBS-T.

The following antibodies were used for the western blotting analysis of proteins.

Ab.	Catalogue No	Dilution
anti-RelB	Santa Cruz	1:500/1:200
anti-p52	Santa Cruz	1:300
anti-LDH	Santa Cruz	1:300
anti- β actin	Sigma	1:50000
anti-I κ B α	In house	1:1000

Secondary antibodies

<i>Antibody</i>	<i>Company</i>	<i>Dilution</i>
anti-mouse Ig-HRP	DAKO Diagnostika	1:2000
anti-rabbit Ig-HRP	DAKO Diagnostika	1:5000
anti-goat Ig-HRP	DAKO Diagnostika	1:2000

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2.2.9 Electromobility Shift Assay (EMSA).

2.2.9.1 Preparation of Probes.

Following single stranded oligonucleotides were used:

Oct-1 up: 5'-GAT CCT GTC GAA TGC AAA TCA CTA GAA A-3'

Oct-1 lo: 5'-GAT CTT TCT AGT GAT TTG CAT TCG ACT C-3'

Igk up: 5'-GAT CCA GAG GGG ACT TTC CCA CAG GA-3'

Igk lo: 5'-GAT CTC CTC TGG GAA AGT CCC CTC TG-3'

2.2.9.2 Annealing of Oligonucleotides.

Following reaction mix was prepared:

2 μ l of each single stranded oligo (100 pmol/ μ l)

200 μ l TE (pH 7.5)

2 μ l NaCl (5 M)

(1 pmol/ μ l oligo concentration in the final volume of 206 μ l)

The reaction was incubated at 80°C for 15 min and left at RT O/N.

2.2.9.3 Radioactive Labeling of the Annealed Oligonucleotides.

Following reaction mix was prepared:

10 μ l ddH₂O

2 μ l of annealed oligo (2 pmol total)

2 μ l 10x REACT 2 buffer (Invitrogen)

2 μ l dNTPs (0.5 mM each without dCTP)

3 μ l α -³²P-dCTP (3000 Ci/ mmol)

1 μ l Klenow fragment (6.5 U, Invitrogen) as a total volume of 20 μ l and was incubated at RT for 20 min.

2.2.9.4 Purification of the Probe.

10 μ l of blue dextran solution (0.7% (w/v) in water) was added to the labeling mix. Sephadex G-50 (Sigma) in TE buffer was used to prepare 1 ml column in 1 ml syringe the bottom of which was plugged with glass wool. Labeling mix with blue dextran was added on the column and unincorporated radioactivity was left in the column by eluting 200-400 μ l blue solution (DNA complexed with blue dextran) in TE. One μ l of this eluate was measured using scintillation counter 1211 Minibeta (Wallac). A total of 20000-50000 cpm per reaction was used for bandshift assay.

2.2.9.5 Bandshift Assay.

Following solutions were used:

5x Binding Buffer (BB): 50 mM Tris-HCl, 250 mM NaCl, 5mM EDTA, 25% glycerol; kept at RT and before use DTT is added to a final concentration of 25 mM.

Buffer C with 1x proteinase inhibitors and 2.5 mM DTT

Unspecific competitors: Sonicated calf thymus DNA (1 $\mu\text{g}/\mu\text{l}$) for Igk bandshift or dI:dC homopolymer (1 $\mu\text{g}/\mu\text{l}$; Sigma) for Oct-1 bandshift assays.

Rabbit Anti-Rel/NF- κ B Polyclonal Antisera for EMSA

KD 57 or rabbit serum (Sigma): preimmune serum (PI)

KD 57-8: anti-p50

KD 38-2: anti-p52

KD 13-3: anti-p65 (RelA)

KD 6-8 or RR 2-8: anti-RelB

All working solutions and binding reactions should be prepared on ice, 5x BB and buffer C were prepared on ice before setting up the binding reactions. Below are the components of a binding reaction:

5 μl **nuclear extract solution** (2-4 μg extract, volume completed to 5 μl with buffer C)

4 μl **5x BB** (final 1x)

Competitor (0.4 μg (0.4 $\mu\text{l}/\text{reaction}$) calf thymus (1/10) or 2 μg (2 $\mu\text{l}/\text{reaction}$) dI:dC (1/2) for 4 μg nuclear extract)

1 μl **antiserum** (for antibody challenge reactions only)

Oligo (2-3 μl depending on the count, 20000-50000 cpm total)

ddH₂O to complete the volume to **20 μl**

Preferentially, premixes were prepared for each oligo shift by adding 5x BB, competitor and ddH₂O the total amounts of which were determined according to the total number of reactions. Then, the mix was distributed to precooled tubes, followed by addition of appropriate volumes of buffer C and protein extract for each sample. For antibody challenge reactions, 1 μl of appropriate antiserum (see above for antisera used) was added and the reactions were incubated on ice for 10 min. The radioactively labeled probe was then added and the reactions were kept at RT for 20 min. Once complete, 2 μl 10x gel loading buffer per reaction was added and the reaction mix was loaded onto 5% gel. While the binding reactions were incubated, the wells of the gel were washed and prerun was performed at 150 V for 10 min using 0.25x TBE as running buffer. A typical run is at 150 V for 2-3 h at RT. Then the gel was dried at 80°C for 30 min and exposure was performed using Hyperfilm MP (Amersham).

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5% polyacrylamide gels: 8.3 ml acrylamide:bisacrylamide (30%), 2.5 ml 5x TBE (final 0.25x), 39.2 ml ddH₂O, total volume 50 ml.

2.2.10 Enzyme Linked Immunoabsorbent Assay (ELISA) for determination of Serum Ig and anti-DNA Ab levels.

1.2.10.1 Serum Ig levels.

Ninety-six-well flat bottom plate (Immulon 2, Dynatech Lab.) was coated with capture antibody (anti-mouse Ig pure, Southern Biotechnology Associates, Birmingham, AL; SBA) by adding 100 µl per well (diluted to 2.5 µg/ml in PBS). The plate was covered with plastic wrap and incubated in a humidified atmosphere O/N at 4°C. Wells were emptied and washed with 200 µl/well PBS-T (I) 4 times at RT. Blocking was performed by filling the wells with 200 µl blocking buffer (II) and incubated for at least 1 h at RT followed by 4 washes with PBST. 2-fold serial dilutions of Ig isotype standard samples (SBA) were prepared by diluting in dilution buffer (III) by keeping lowest concentration 0.781 ng/ml and highest concentration 400 ng/ml. Two wells of the plate were filled with 100 µl dilution buffer as background and 100 µl of standard samples were added to the rest of 10 wells of the row in increasing concentration. Serum samples were also diluted in dilution buffer to prepare two-fold serial dilutions of sera starting with 1:10,000 dilution. Hundred µl of serial dilutions of samples were added to the wells, the plate was covered and incubated for 2 h at RT. Following four standard washes with PBS-T, 100 µl of detection antibody diluted in PBS (1:200) (anti-mouse Ig isotype-specific Ab conjugated to horseradish peroxidase; SBA) was added and an additional 1 h incubation at RT was performed. Wells were emptied and washed 5 times with PBS-T. Meanwhile, ABTS substrate (Boehringer Mannheim GmbH) was prepared by dissolving 1 tablet (5 mg) per 5 ml of 1x ABTS buffer (diluted with ddH₂O from commercially available 10x stock). For a complete 96 well plate, 2 tablets in 10 ml of buffer would be required. Color reaction was performed by adding 100 µl per well ABTS solution and incubation at RT for 5-20 min. Color reaction was stopped by addition of 50 µl quench solution (IV) and readings were taken within 30 min at 405 nm using BioRad Microplate Reader 3550. Data analysis was performed by MPM III Microplate manager Software.

PBS-T (I)	: PBS, 0.05% Tween-20
Blocking buffer (II)	: PBS, 10% FCS
Dilution buffer (III)	: PBS, 1% BSA
Quench solution (IV)	: 1% SDS

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2.2.10.2 Anti-DNA Ab levels.

Linearized plasmid DNA (10 µg/ml) was immobilized on CovaLink plates (Nunc, Copenhagen, Denmark) by treatment at 50°C for 5 h with 150 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 10 mM 1-methylimidazole pH 7.0. Plates were extensively washed with 5x SSC, 0.25% SDS at 55°C and blocked for 1 h at RT with blocking solution (10 mM Tris-Hcl pH 7.4, 140 mM NaCl, 1% BSA, 3 mM EDTA). Sera were diluted 1,000-fold in blocking solution and 100 µl diluted sera were added to the wells and incubated for 1 h at RT. After 3-4 washes with washing solution (10 mM Tris-Hcl pH 7.4, 140 mM NaCl, 1% BSA, 3 mM EDTA, 0.1% NP40 and 1% gelatin), plates were incubated for 1 h with peroxidase conjugated anti-mouse Ig (Dako, Hamburg, Germany) after diluting to 1,3000. Plates were washed for 4 times and developed with a peroxidase detection kit (BioSource International, Nivelles, Belgium) and readings were taken within 30 min at 405 nm using BioRad Microplate Reader 3550.

2.2.11 Proliferation assay of T and B cells.

For T cell proliferation assay, 96 well flat bottomed plates (CellStar Griener) were pre-coated with 50 µl of coating buffer (Tris-Cl pH 9.0) containing 10 µg/ml anti-CD28 mAb and increasing concentrations of anti-CD3 mAb (0.3, 1.0, 3.0, and 10 µg/ml) for 12 h at 4°C or 2 h at 37°C. Plates were emptied and rinsed once with sterile PBS. Up to 1×10^5 T cells in lymphocyte culture medium were added per well and incubated in the incubator for 48 h. Then 1 µCi (^3H)thymidine (Amersham Pharmacia Biotech) was added to each well and cells were incubated for additional 16-18 h. Cells were harvested using a Tomtec harvester and the incorporated radioactivity was measured using a MicroBeta scintillation counter from Wallac ADL.

For B cell proliferation assay, up to 1×10^5 cells in lymphocyte medium containing different concentrations (1, 3, 5, and 10 µg/ml) of LPS were added to 96 well flat bottomed plates and the rest of the procedure was essentially as described for T cell proliferation assay.

Materials and Methods

2.2.12 Generation of Bone marrow chimeric mice.

Bone marrow cells were isolated from femora of 2 to 3 month old wild-type, *relB*^{-/-}, *nfkb1*^{-/-}, *nfkb2*^{-/-} mice. Bones were first thoroughly cleaned and rinsed with 70% alcohol and washed extensively in sterile PBS. The ends of the bones were cut off and the bone marrow cells were flushed out with PBS into a petri dish containing 1-2 ml sterile PBS using a 2 ml syringe with a 27 Gauge needle. The cells were then passed through the same syringe for additional 2-3 times and an aliquot of cells was used for counting the number of cells. Upto 4-6 x 10⁶ cells were injected intra venously (i.v.) per mouse in different combinations of donor and recipient mice as described in the results section. Before injection, recipient mice were irradiated with 2 x 550 rad with 3 h interval and rested for 4-6 h after the second irradiation. After the injection of bone marrow cells, mice were kept under pathogen free conditions and were analyzed after 12-13 weeks for NKT cell development. For the analysis of chimerism, peripheral blood was collected by cardiac puncture at necropsy and immediately transferred into 15 ml falcon tubes containing 10 ml of PBS, 5mM EDTA and centrifuged at 1500 rpm for 5 min at 4°C. The pellets were resuspended in 2 ml of ACK lysis buffer and kept at RT for 5 min and then centrifuged. ACK lysis was repeated and the pellets were washed 2 x with FACS buffer and the leukocytes were resuspended in 100 µl of FACS buffer. The cell suspension was transferred to eppendorf tubes and centrifuged at 2000 rpm for 5 min at 4°C and the pellets were resuspended in PBND buffer containing proteinase K and kept on an incubator shaker at 55°C for 2-3 hours. The lysates obtained were used for analysis by PCR to check the chimerism.

For the generation of mixed bone marrow chimeras, bone marrow cells from wild-type Ly-5.1 and mIkBα^{tg} Ly-5.2 mice was isolated and 3 x 10⁶ cells from each mouse were mixed and injected i.v. into irradiated Ly-5.2 wild-type recipients. The mice were analyzed after 12-13 weeks for NKT cell development.

For the analysis of chimerism of the mixed bone marrow chimeras, thymocytes were stained with anti-Ly5.1 and/or anti-Ly5.2 mAb and were analyzed by flow cytometry.

PBND buffer: PCR buffer with nonionic detergents; 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% v/v NP-40, 0.45% v/v Tween-20, 0.1 µg/ml Proteinase K (3 µl from 10 mg/ml stock); this buffer was kept at 4°C without Proteinase K which was added just before use.

2.2.13 Immunohistochemical analysis.

(This work was performed by Debra Weih).

For analyzing the Ig depositions in kidney, frozen kidney sections of 10 µm thickness were made and air-dried and were fixed in cold acetone. Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide in methanol. Sections were blocked with 10% goat serum, rinsed in PBS and then incubated with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, diluted 1:100) for 2 h at RT. Immunohistochemical staining was visualized with a standard avidin-biotin-peroxidase complex procedure and AEC using products from Vector Laboratories and counterstained with hematoxylin. For negative control slides, the anti-mouse IgG Ab was omitted.

For analyzing apoptotic cells in lymph nodes, lymph node sections were made as described for kidneys and were detected by TUNEL method using the ApopTag reagents from Oncor Intergen (Heidelberg, Germany). Micrographs were taken with a Zeiss Axioskop and a Jenoptik ProgRes 3012 digital camera system.

3 RESULTS

PART ONE

3.1 Role of NF- κ B family members in NKT cell development.

Rel/NF- κ B proteins play an important role in development and function of lymphocytes and in the regulation of innate and adoptive immune responses (4, 67). NKT cells are key regulators involved in both innate immunity and the regulation of adoptive immune responses (68-70). Previous studies have revealed that Rel/NF- κ B family members are not essential for the development of mainstream T cells, but their role in development of other T cell sub-types, such as NKT cells, has not been addressed. Therefore, the possible role of NF- κ B family members in development of NKT cells was examined.

3.1.1 NF- κ B is essential for NKT cell development

To examine the role of individual Rel/NF- κ B subunits in NKT cell development, mice with targeted disruptions of RelB (*relB*^{-/-}), p50/NF- κ B1 (*nfkb1*^{-/-}), and p52/NF- κ B2 (*nfkb2*^{-/-}) were analyzed. The percentage of NK1.1⁺TCR β ⁺ cells in thymus of *relB*^{-/-} mice was severely reduced (~20-fold), compared to wild-type mice (**Fig. 3.1**). Thymic NK1.1⁺TCR β ⁺ cells were also markedly reduced in *nfkb1*^{-/-} mice (~4-fold), whereas *nfkb2*^{-/-} mice showed a milder phenotype with 2- to 3-fold reduced NK1.1⁺TCR β ⁺ cell numbers (**Fig. 3.1**).

Results

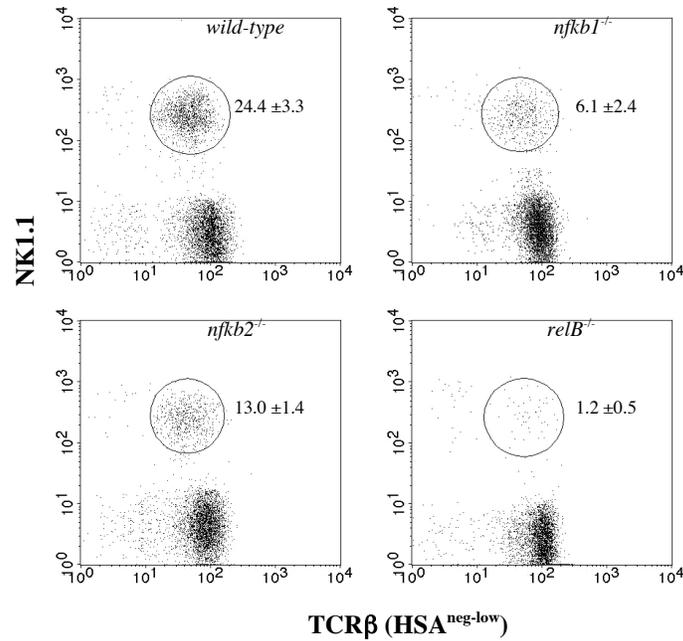


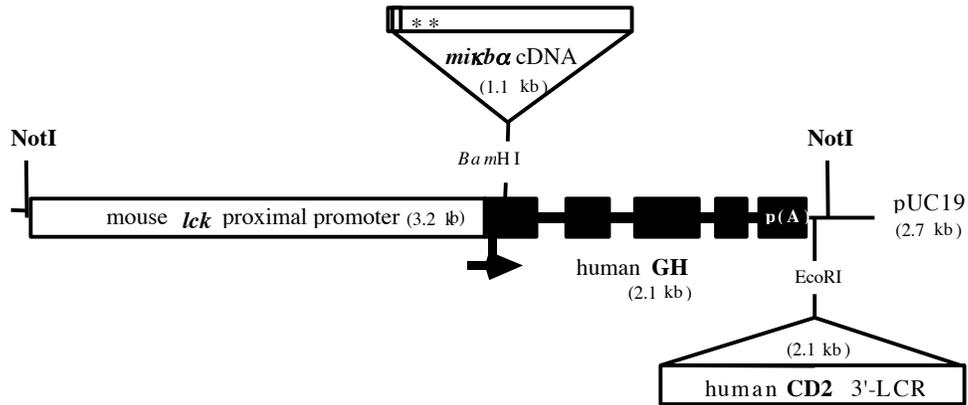
Fig. 3.1: Reduced numbers of thymic NKT cells in NF-κB deficient mice.

Thymocytes from adult wild-type, *nfkb1*^{-/-}, *nfkb2*^{-/-}, and *relB*^{-/-} mice were analyzed for NKT cell percentages after staining with anti-NK1.1 and anti-TCRβ mAbs and analyzed for NK1.1, TCRβ expression by flow-cytometry. Percentages of NKT cells (circles) were increased by gating on HSA^{neg-low} cells. Numbers indicate mean values ±SD from 5-7 mice.

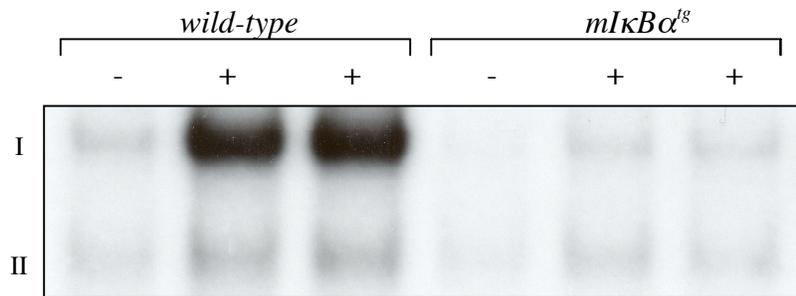
3.1.2 NF-κB is required within thymocytes for NKT cell development.

The defective NKT cell development in *relB*^{-/-}, *nfkb1*^{-/-}, and *nfkb2*^{-/-} mice could be due to the lack of these proteins in all cells of the thymus (including stroma) or due to impaired NF-κB function in hematopoietic thymocytes that develop into mature T cells. To address this question, NKT cell development was analyzed in transgenic mice, in which NF-κB function was inhibited exclusively in thymocytes by the expression of a non-degradable mutant form of the IκBα molecule under the control of the T cell specific *lck* proximal promoter (*mIκBα*^{tg}) (Fig. 3.2A). Thymocytes from *mIκBα*^{tg} mice revealed almost no induction of NF-κB upon PMA + PHA treatment (Fig. 3.2B). Interestingly, although *mIκBα*^{tg} mice revealed normal development of mainstream T cells (33, 35), NKT cell development was impaired in these mice, resulting in a ~4-fold reduced thymic NKT cell population compared to non-transgenic littermates (Fig. 3.2C). Since RelB-deficient mice revealed a more severe defect in NKT cell development than *mIκBα*^{tg} mice, these results collectively suggest that IκBα-regulated NF-κB function, such as NF-κB1-containing complexes, may be required within thymocytes, whereas RelB-containing complexes may be important in cell types other than thymocytes for proper NKT cell development.

A



B



C

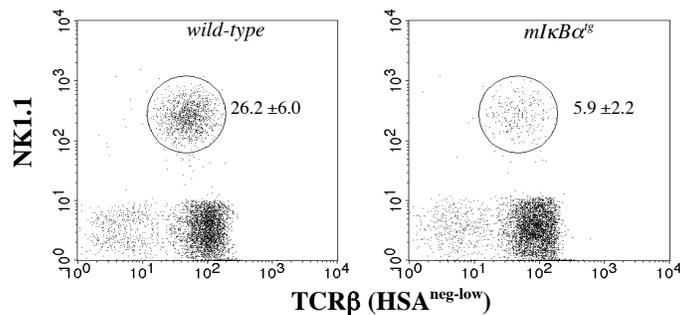


Fig. 3.2: NF- κ B within thymocytes regulates NKT cell development.

(A) Schematic representation of the *mIkB α* transgene. Asterisks indicate serines 32 and 36 mutated to alanines. For further details see Materials and Methods. (B) Expression of *mIkB α* almost completely blocks NF- κ B induction in thymocytes. Thymocytes from wild-type and *mIkB α* ^{tg} mice were either or untreated (-) or treated (+) with PMA (10 ng/ml) + PHA (5 μ g/ml) for 1 hour (h). Nuclear extracts were prepared and analyzed for κ B binding by electrophoretic mobility shift assay (EMSA) using Ig κ probe. (I) p50/RelA heterodimers and (II) p50 homodimers. The integrity of the nuclear extracts was checked by EMSA with an OCT probe (not shown). (C) Defective development of NKT cells in *mIkB α* ^{tg} mice. Thymocytes from adult wild-type and *mIkB α* ^{tg} mice were analyzed for NKT cell percentages as described in Fig. 3.1. Note the reduced numbers of NKT cells in *mIkB α* ^{tg} mice.

Results

3.1.3 Defective NKT cell development in the absence of NF- κ B is not due to impaired expression of NK1.1.

It is known that NKT cell development can occur in the absence of expression of the NK1.1 marker (49). Hence, it is possible that the observed reduction of NK1.1⁺TCR β ⁺ cells in *relB*^{-/-}, *nfkb1*^{-/-}, *nfkb2*^{-/-}, and *mIkB α* ^{tg} mice could be due to the lack of NK1.1 expression. Expression of the V α 14-J α 18 TCR is therefore considered a more stringent measure for the major population of NKT cells in thymus (117). Semi-quantitative RT-PCR analysis revealed reduced V α 14-J α 18 mRNA levels in *relB*^{-/-}, and *mIkB α* ^{tg} thymocytes (Fig. 3.3A). Thymic V α 14i NKT cells can be detected by staining with α -GalCer-loaded CD1d-tetramers and anti-TCR β mAb, even in the absence of NK1.1 expression. Flowcytometric analysis of thymocytes revealed significantly reduced, tetramer⁺ TCR β ⁺ V α 14i NKT cells in *mIkB α* ^{tg} and *relB*^{-/-} mice compared to wild-type controls (Fig. 3.3B). These results reflect a true reduction in the numbers of NKT cells in NF- κ B-deficient mice.

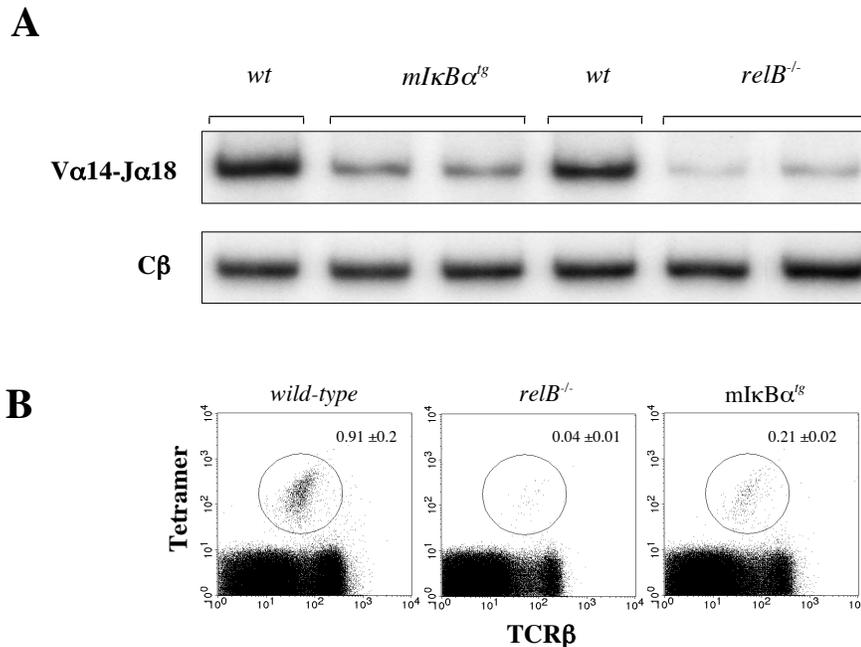


Fig. 3.3: Reduced expression of V α 14-J α 18 TCR in *relB*^{-/-} and *mIkB α* ^{tg} mice.

(A) Total thymic RNA from wild-type, *mIkB α* ^{tg}, and *relB*^{-/-} mice was reverse transcribed and analyzed by RT-PCR for the expression of V α 14-J α 18 TCR. Expression of the C β chain is shown as an amplification control. (B) Thymocytes from wild-type, *mIkB α* ^{tg}, and *relB*^{-/-} mice were stained with α -GalCer loaded CD1d-tetramer and anti-TCR β mAb. Tetramer⁺TCR β ⁺ V α 14i NKT cells were analyzed by flow-cytometry. Circles and numbers indicates the mean value of percentage of V α 14i NKT cells \pm SD from 3 mice.

3.1.4 Selective loss of CD4⁺ NKT cells in the spleen of *relB*^{-/-} and *nfkb1*^{-/-} mice.

Spleen and bone marrow (BM) are other major lymphoid organs where NKT cells are found. Despite the significant reduction in thymic NKT cells, only a moderate reduction in total NKT cell numbers was observed in spleen and BM of *relB*^{-/-}, *nfkb1*^{-/-}, and *mIκBα*^{tg} mice (data not shown). *nfkb2*^{-/-} mice were not included in this analysis because of the moderate reduction of NKT cell percentages in the thymus, which is the major site for NKT cell development. When different peripheral NKT cell subpopulations were analyzed, thymus-dependent CD4⁺ NKT cells were clearly reduced in spleens from *relB*^{-/-} (4.5-fold) and *nfkb1*^{-/-} mice (3-fold). Interestingly, *nfkb1*^{-/-} spleens showed a selective 4-fold increase in the percentage of CD8⁺ NK1.1⁺ T cells, whereas this subpopulation was only minimally increased in *relB*^{-/-} mice (**Fig. 3.4**). In the case of *mIκBα*^{tg} mice both CD4⁺ and CD8⁺ NKT cells were equally reduced in the spleen and BM, presumably due to the generally impaired survival of T cells in the periphery of these mice (33, 35). Collectively, these results indicate a specific requirement of NF-κB for the development of thymus dependent Vα14i NKT cells rather than for total NK1.1⁺ NKT cells. This result also suggests that the reduction in the numbers of thymic NKT cells in NF-κB-deficient mice is not due to increased emigration to periphery.

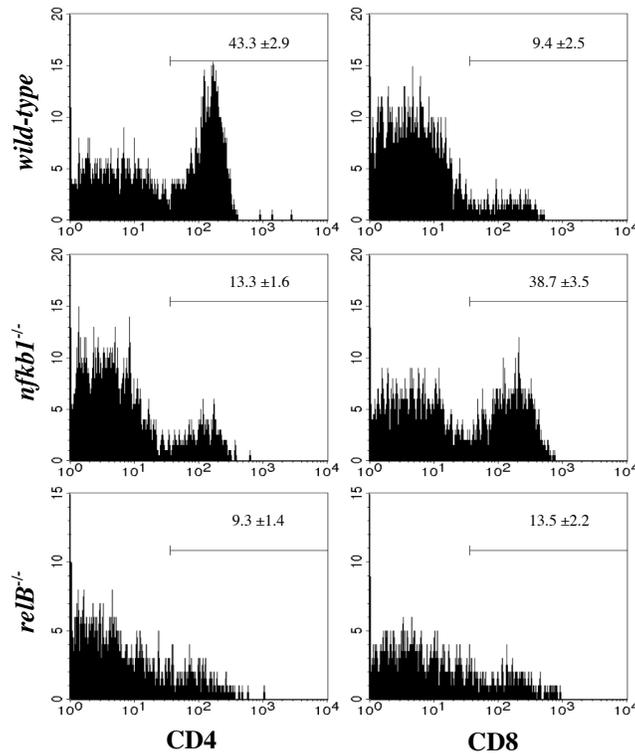


Fig. 3.4: Selective loss of peripheral CD4⁺ NKT cells in *relB*^{-/-} and *nfkb1*^{-/-} mice.

Splenocytes from adult wild-type, *nfkb1*^{-/-}, and *relB*^{-/-} mice were stained with anti-NK1.1, anti-TCR β and either anti-CD4 or anti-CD8 mAbs. NK1.1⁺TCR β ⁺ cells were gated and analyzed for CD4 or CD8 expression by flow cytometry. Histograms show CD4 (left) and CD8 (right) expression levels on NK1.1⁺TCR β ⁺ cells. Note the reduction of CD4⁺ NKT cells in *nfkb1*^{-/-} and *relB*^{-/-} mice and the increase of CD8⁺ NK1.1⁺ T cells in *nfkb1*^{-/-} but not in *relB*^{-/-} animals. Numbers indicate mean values \pm SD from 3-7 mice.

3.1.5 CD1d expression on DP thymocytes is not effected by the lack of NF- κ B function.

Thymic NKT cells are positively selected via the interaction of their V α 14-J α 18 TCR with CD1d, expressed on CD4⁺CD8⁺ DP cortical thymocytes (53). Flow cytometric analysis revealed normal CD1d levels on thymocytes from *relB*^{-/-}, *nfkb1*^{-/-}, *nfkb2*^{-/-}, and *mIkB α* ^{tg} mice (**Fig. 3.5**), indicating that NF- κ B is not required for the expression of CD1d and that the defective development of NKT cells in these mice is not due to impaired CD1d expression.

Results

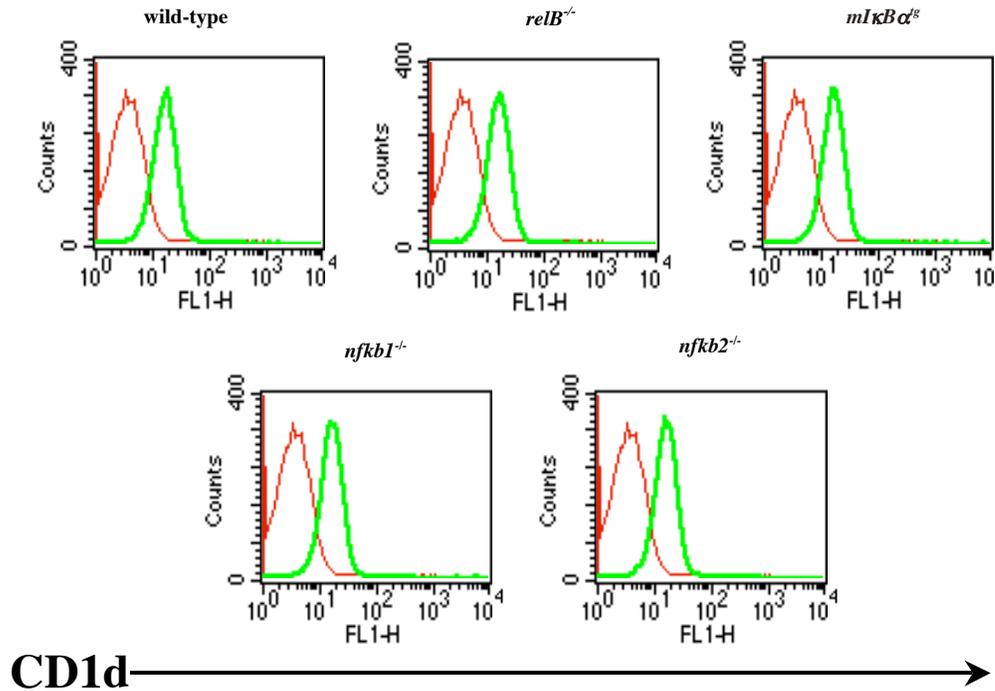


Fig. 3.5: CD1d expression on thymocytes is not regulated by NF-κB.

Thymocytes from wild-type, *relB*^{-/-}, *nfkb1*^{-/-}, *nfkb2*^{-/-}, and *mIκBα*^{tg} mice were stained with anti-CD1d mAb and analyzed by flow cytometry. Green histograms shows CD1d expression, red lines indicate unstained cells.

3.1.6 Subunit-specific requirement of NF-κB family members for the expression of lymphotoxin and IL-15.

Since NF-κB regulates gene expression, both steady state (total thymus) and induced (thymocytes treated with PMA + PHA) mRNA levels of several genes that have been shown to be crucial for NKT cell development (41) were analyzed. Focusing on *relB*^{-/-} and *mIκBα*^{tg} mice, no significant defects were observed in the expression of genes encoding cathepsin S, cathepsin L, Ets-1, Fyn, GM-CSFRβ, IL-15Rα, IRF-1, and Vav-1, compared to wild-type controls (data not shown). In contrast, steady state IL-15 mRNA levels were reduced ~3-fold in *relB*^{-/-} but not in *mIκBα*^{tg} thymus (**Fig. 3.6A**). Since IL-15 expression was not detected in thymocyte single cell suspensions (118 and data not shown) this result indicates that RelB is required for the maintenance of normal IL-15 mRNA levels in thymic stromal cells.

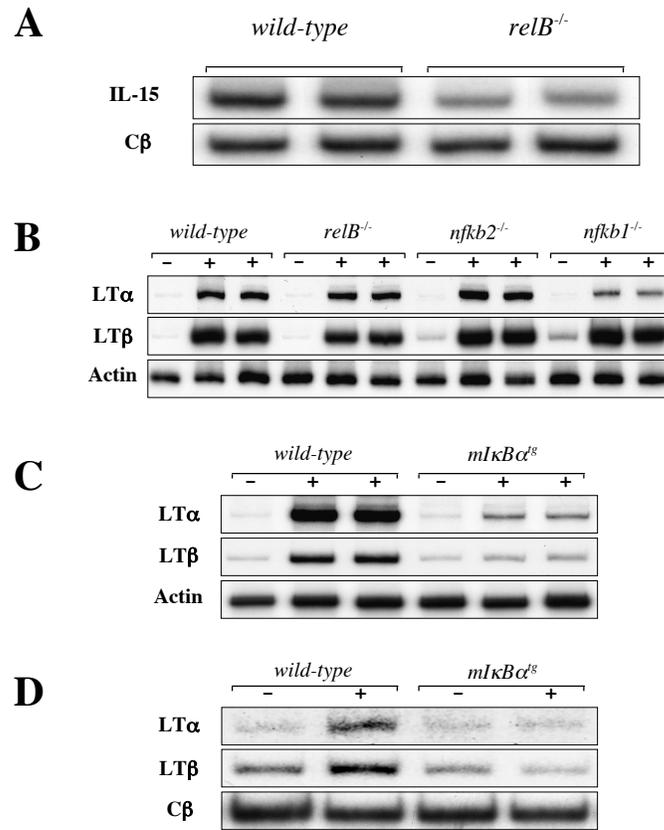


Fig. 3.6: Regulation of IL-15 and LT expression by distinct NF-κB members.

(A) Total thymus RNA from wild-type and *relB*^{-/-} mice was analyzed by RT-PCR for the expression of steady state levels of IL-15. (B) Thymocytes from wild-type, *relB*^{-/-}, *nfkb1*^{-/-} and *nfkb2*^{-/-} mice and (C) from *mIkBα*^{tg} mice were either untreated (-) or treated (+) with PMA + PHA (10 ng and 5 μg/ml, respectively) for 2 h. RNA was extracted and LT expression was analyzed by RT-PCR. (D) NKT cells upregulate LT upon activation via Vα14-Jα18 TCR, in an NF-κB-dependent manner. Thymocytes from wild-type and *mIkBα*^{tg} mice were either untreated (-) or treated (+) with α-Galcer (200 ng/ml) for 16 h. RNA was extracted and LT expression was analyzed by RT-PCR. Expression of either actin or Cβ chain, is shown as an amplification control.

The LT signaling pathway has been shown to be crucial for NKT cell development and both LTα and LTβ are potential NF-κB target genes (8, 57, 58). Therefore thymic LTα and LTβ mRNA expression was examined in *relB*^{-/-}, *nfkb1*^{-/-}, *nfkb2*^{-/-}, and *mIkBα*^{tg} mice. No differences in steady state or inducible LT mRNA levels were detected in thymocytes from *relB*^{-/-} and *nfkb2*^{-/-} mice, indicating that RelB and p52 are not involved in the regulation of LT expression (**Fig. 3.6B**). The p50 subunit of NF-κB, however, was required for full induction of LTα expression, whereas it was dispensable for the induction of LTβ (**Fig. 3.6B**). Blocking of total NF-κB activation in *mIkBα*^{tg} thymocytes almost completely abolished induced expression of both LTα and LTβ without affecting

Results

their steady state mRNA levels (**Fig. 3.6C**). To investigate whether the specific activation of thymic V α 14i NKT cells also induces LT expression, thymocytes were stimulated with α -GalCer. Similar to PMA + PHA treatment, α -GalCer treatment resulted in an NF- κ B-dependent induction of LT α (~4-fold) and LT β (~3-fold) (**Fig. 3.6D**). The lower level of LT mRNA induction upon α -GalCer treatment compared to PMA + PHA treatment was expected since the responding NKT cells represent less than 1% of total thymocytes. Thus, NF- κ B regulates the induced expression of LT in mainstream T cells as well as in response to V α 14-J α 18 TCR signaling in thymic NKT cells.

3.1.7 Distinct NF- κ B complexes are required in hematopoietic and non-hematopoietic cellular compartments for the development of NKT cells.

Development of NKT cells has been shown to involve the role of both thymocytes and thymic stromal cells (53, 72). The requirement of individual Rel/NF- κ B members in hematopoietic and non-hematopoietic cells was therefore examined by radiation chimera analysis. Reciprocal BM transplantations between wild-type and *relB*^{-/-} mice revealed that RelB in radiation resistant thymic stromal cells is required for NKT cell development, since wild-type BM transferred into lethally irradiated *relB*^{-/-} recipients resulted in severely impaired development of NKT cells (**Fig. 3.7**). Interestingly, reciprocal BM transfers between wild-type and *nfkb1*^{-/-} mice revealed that p50/NF- κ B1 is required in hematopoietic cells rather than in the stromal compartment (**Fig. 3.7**). On the other hand, unlike p50/NF- κ B1 but similar to RelB, p52/NF- κ B2 was required in radiation-resistant non-hematopoietic cells for proper NKT cell development (data not shown).

Results

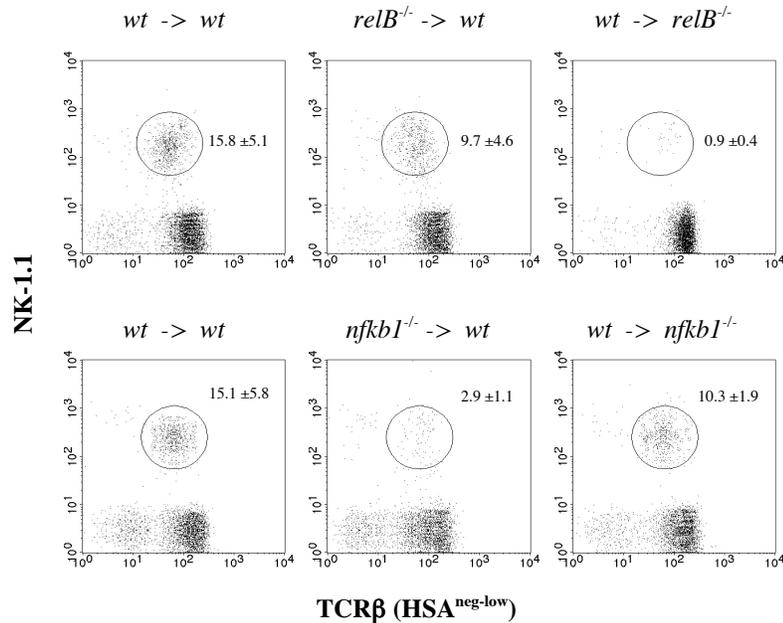


Fig. 3.7: Analysis of NKT cell development in BM chimeras.

Thymocytes were isolated from lethally irradiated mice that were reconstituted with BM as indicated (donor BM -> irradiated recipient). HSA^{neg-low} cells were gated and analyzed for NKT cell percentages as described in Fig. 3.1. Percentages of NKT cells (mean values from 3-7 mice ±SD) are indicated.

3.1.8 Classical NF-κB function within NKT precursors is required for their maturation.

Reciprocal BM transfer experiments between wild-type and *nfkb1*^{-/-} mice clearly demonstrated the requirement of NF-κB in thymocytes for the development of NKT cells. However, it is not clear whether NF-κB is required within NKT precursors or in accessory thymocytes for their maturation. To address this question, *mIkBα*^{tg} mice were used to generate mixed BM chimeric mice. BM cells from Ly-5.1 wild-type and Ly-5.2 *mIkBα*^{tg} mice were mixed at equal proportions and injected into lethally irradiated Ly-5.2 wild-type recipients. Flow-cytometric analysis of thymocytes from mixed BM chimeric mice showed that ~70% of the thymocytes were derived from Ly-5.2 *mIkBα*^{tg} BM, whereas Ly-5.1 wild-type BM contributed the remaining 30% (**Fig. 3.8A**). Interestingly, only Ly-5.1 wild-type BM could generate NKT cells, whereas BM from Ly-5.2 *mIkBα*^{tg} mice was unable to efficiently generate NKT cells despite the co-existence of wild-type thymocytes (**Fig. 3.8B**). Together, these data indicate that thymic NKT cell development is dependent, in a cell-autonomous manner, on classical IκBα-regulated NF-κB activity in NKT precursors and on RelB containing complexes in the thymic stroma.

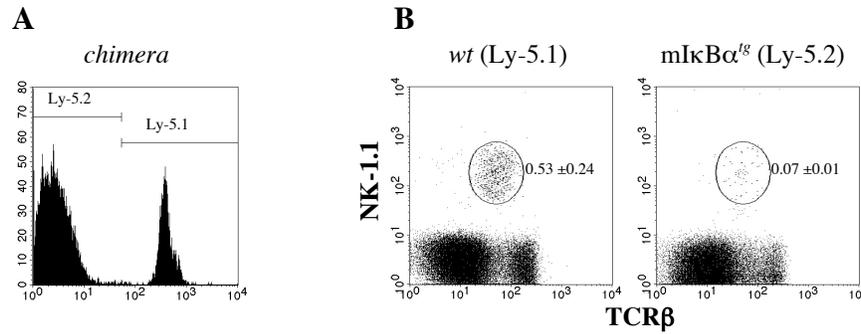


Fig. 3.8: Analysis of NKT cell development in mixed BM chimeric mice.

Competitive BM chimeras were generated by mixing wild-type Ly-5.1 BM with *mIkBα^{lg}* Ly-5.2 BM to reconstitute lethally irradiated wild-type Ly-5.2 mice. Chimerism was checked by flow cytometry (A). NKT cells derived from wild-type (Ly-5.1⁺) and from *mIkBα^{lg}* (Ly-5.2⁺) BM were stained for NK-1.1 and TCRβ expression and analyzed by flow cytometry (B). Percentages of NKT cells among total thymocytes (mean values from 2-3 mice ±SD) are indicated.

3.1.9 Defective development of NKT cells in the absence of NF-κB function is not due to increased apoptosis.

Since NF-κB regulates apoptosis of T cells (104, 130), it is possible that the reduced numbers of NKT cells in NF-κB-deficient mice is due to increased cell death of NKT cells. To address this question, thymocytes from *relB^{-/-}* mice and *mIkBα^{lg}* mice were stained with α-GalCer loaded CD1d-tetramers and with Annexin V, to identify apoptotic cells. The percentage of CD1d-tetramer⁺ and Annexin V⁺ cells among wild-type, *relB^{-/-}* mice and *mIkBα^{lg}* mice was similar (Fig. 3.9), indicating that the defective development of NKT cells in the absence of NF-κB function is not due to increased apoptosis of these cells.

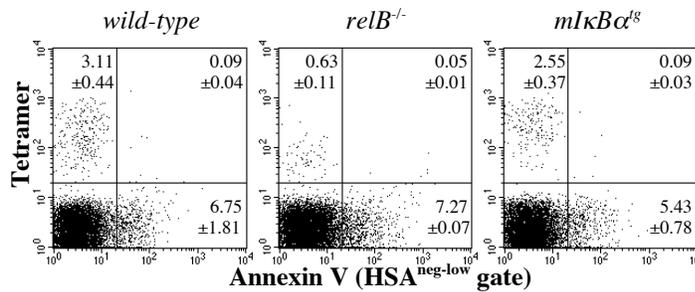


Fig. 3.9: Analysis of apoptotic cells in thymus

Thymocytes from 2-week-old C57BL/6 wild-type, *relB^{-/-}*, and *mIkBα^{lg}* mice were stained with Annexin V and α-GalCer-CD1d-tetramer (tetramer). HSA^{neg-low} cells were gated as in Fig. 3.1 to enrich for NKT cell percentages and analyzed for apoptotic cells among tetramer⁺ NKT as well as mainstream T cells.

3.1.10 Distinct NF- κ B complexes within NKT precursors and thymic stromal cells are required at different stages of NKT cell development.

Similar to mainstream T cells, NKT cells were shown to be derived from CD4⁺CD8⁺ DP thymocytes (60). Recent evidence indicates that positive selection of DP NKT precursors by CD1d results in the generation of NK1.1⁻ NKT precursors, which in turn give rise to mature NK1.1⁺ NKT cells in both thymus and peripheral lymphoid tissues (41, 62, 63; also see Fig. 1.6). These NK1.1⁻ NKT precursors can be detected by staining with α -GalCer-loaded CD1d tetramer and anti-TCR β mAb. Since the frequency of NK1.1⁻ precursors is low in adult compared to young animals, thymocytes from 2-week-old mice were analyzed by flow cytometry. Staining of wild-type thymocytes with α -GalCer-loaded CD1d-tetramer and anti-NK1.1 mAb revealed that CD1d-tetramer⁺ cells at this age consisted predominantly of NK1.1⁻ precursors and a smaller portion of mature NK1.1⁺ NKT cells. In contrast, the NK1.1⁻ precursor population was markedly reduced in *relB*^{-/-} mice whereas mature NKT cells were less severely affected. On the other hand, *mIkB α* ^{tg} mice revealed normal NK1.1⁻ precursor frequency but a 9-fold reduction in the percentage of mature NK1.1⁺ CD1d-tetramer⁺ NKT cells (Fig. 3.10A).

After positive selection, NKT precursors progress through CD44^{lo} and CD44^{hi} stages before they become positive for the NK1.1 marker (see Fig. 1.6 for review see 41). Therefore, CD44/NK1.1 profiles of CD1d-tetramer⁺ thymocytes in 2-week-old wild-type, *relB*^{-/-}, and *mIkB α* ^{tg} mice were compared. While the percentage of mature CD44^{hi}NK1.1⁺ NKT cells was not reduced, the frequency of the intermediate CD44^{hi}NK1.1⁻ NKT population was strongly reduced in *relB*^{-/-} mice. In contrast, intermediate CD44^{hi}NK1.1⁻ NKT precursors were only mildly affected in *mIkB α* ^{tg} mice, while further differentiation into mature CD44^{hi}NK1.1⁺ NKT cells was clearly impaired in these mice (Fig. 3.10B). These differences between wild-type, *relB*^{-/-}, and *mIkB α* ^{tg} mice became even more obvious when numbers of the different subpopulations were calculated (Fig. 3.10C). Fig. 3.10C also shows that RelB is required for the generation of normal numbers of early CD44^{lo}NK1.1⁻ NKT precursors, whereas this population was not reduced in *mIkB α* ^{tg} mice. In conclusion, these data indicate that the early but distinct blocks caused by the lack of RelB in stromal cells and classical NF- κ B in hematopoietic cells result in severely reduced numbers of mature V α 14i NKT cells in adult mice.

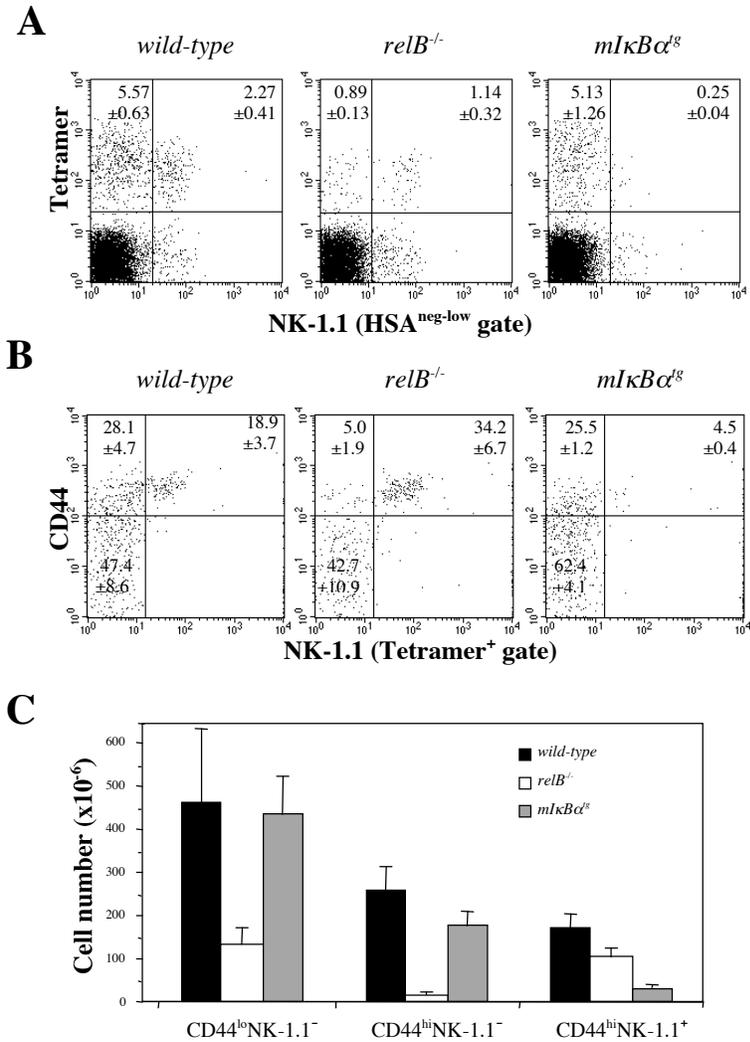


Fig. 3.10: Analysis of developmental intermediates of V α 14i NKT cells in NF- κ B-deficient mice.

(A) Requirement of RelB in stromal cells for the generation of NK1.1⁻ precursors and NF- κ B in NKT precursors for transition of NK1.1⁻ to NK1.1⁺ NKT cells. Thymocytes from 2-week-old C57BL/6 wild-type, *relB*^{-/-} and *mIkBα*^{tg} mice were stained with anti-NK1.1 mAb and α -GalCer-CD1d-tetramer (tetramer). HSA^{neg-low} cells were gated as described in Fig. 3.1 and analyzed for NK1.1⁻tetramer⁺ and NK1.1⁺tetramer⁺ NKT cells by flowcytometry. (B) Thymocytes from 2-week-old wild-type, *relB*^{-/-}, and *mIkBα*^{tg} mice were stained with anti-CD44, anti-NK1.1 mAbs and with α -GalCer-CD1d-tetramer. Tetramer⁺ cells were gated and analyzed for expression of CD44 and NK1.1 on tetramer⁺ thymocytes by flow cytometric analysis. Numbers in quadrants indicate mean values of percentages \pm SD from 3 mice. (C) Numbers of tetramer⁺ cells in 10⁶ thymocytes from 2-week-old wild-type, *relB*^{-/-}, and *mIkBα*^{tg} mice based on their CD44 and NK1.1 expression patterns.

PART TWO

3.2 Differential regulation of RelA and RelB by TNFR-I and LT β R: lymphotoxin signaling in NKT cell development.

RT-PCR results obtained from thymocytes (see Fig. 3.6) indicated a role for classical I κ B α -regulated NF- κ B function, consisting of p50/NF- κ B1 and most likely RelA and c-Rel subunits in the regulation of LT α and LT β , whereas RelB and p52/NF- κ B2 are dispensable for the expression of both LT α and LT β . Membrane bound LT, which signals through LT β R has been shown to be required for proper NKT cell development and signaling through LT β R is defective in the absence of NIK. Since some of the phenotypes observed in *aly/aly*, *ltbr*^{-/-}, *relB*^{-/-} and *nfkb2*^{-/-} mice are similar (see introduction), it is likely that p52/NF- κ B2 and RelB are regulated downstream of LT β R and NIK. Moreover, similar to the stromal requirement of NIK, BM transfer experiments from this study revealed a role for RelB as well as p52/NF- κ B2 in the thymic stroma for NKT cell development (see Fig. 3.7 and data not shown). These observations made it necessary to study if RelB and p52 complexes are regulated by LT signaling downstream of NIK.

3.2.1 Regulation of RelB DNA binding by NIK and LT β R in thymus and spleen

To address whether RelB function is regulated by LT β R and NIK in lymphoid organs, steady state DNA-binding of NF- κ B complexes in whole thymus and spleen extracts from wild-type, *aly/aly*, *ltbr*^{-/-}, and *tnfr1*^{-/-} mice were analyzed by EMSA (**Fig. 3.11**). Thymic extracts from *relB*^{-/-} mice were also included in this analysis as a specificity control (**Fig. 3.11A**). Dissection of different Rel/NF- κ B complexes by supershift assays using antibodies specific for individual Rel/NF- κ B members revealed three qualitatively and quantitatively different complexes. While complex I consisted of RelA heterodimers, complex II consisted of RelB heterodimers. Complex III consisted of p50 and p52 homodimers (**Fig. 3.11** and data not shown). Interestingly, supershift assays revealed severely impaired RelB (complex II), but not RelA DNA-binding in both thymus and spleen extracts from *aly/aly* mice. Thymus and spleen extracts from *ltbr*^{-/-} mice also revealed a less severe reduction in the DNA-binding of RelB but not RelA. Mice lacking

Results

TNFR-I had normal DNA-binding of RelB or RelA in thymus as well as spleen (**Fig. 3.11A and B**).

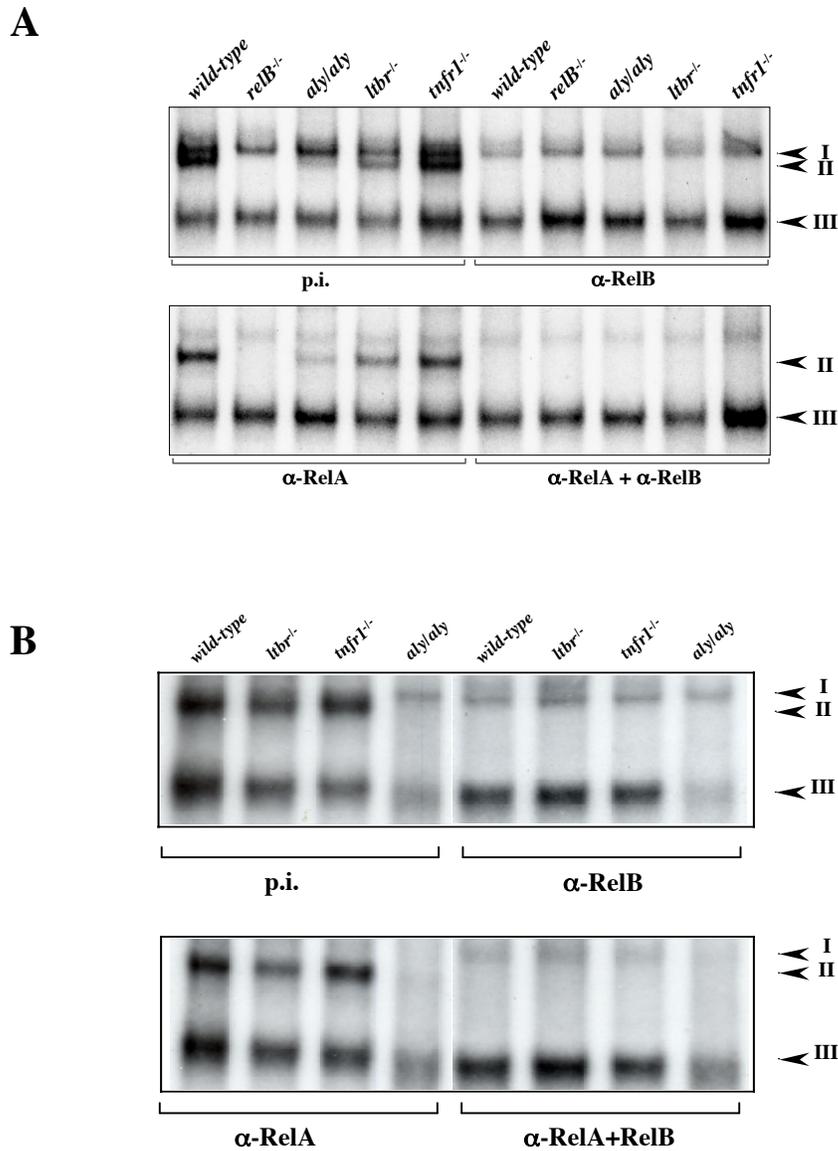


Fig. 3.11: Regulation of RelB DNA binding by NIK and LT β R in thymus and spleen.

(A) Whole thymus extracts from wild-type, *relB*^{-/-}, *aly/aly*, *ltbr*^{-/-}, and *tnfr1*^{-/-} mice were prepared and analyzed for κ B-binding activity in EMSAs. Complexes are indicated by arrowheads and their identity was determined with Abs specific for individual Rel/NF- κ B family members. p.i., pre-immune serum; complex I, RelA heterodimers; complex II, RelB heterodimers. Complex III consists of p50 and p52 homodimers (not shown). (B) Whole spleen extracts from wild-type, *ltbr*^{-/-}, *tnfr1*^{-/-} and *aly/aly* mice were prepared and analyzed for κ B-binding activity as in (A). Note the complete lack and partial reduction of RelB DNA binding in *aly/aly* and *ltbr*^{-/-} extracts, respectively.

These results indicate that within the lymphoid organs, NIK and to a lesser extent LT β R regulate the constitutive DNA binding of RelB but not RelA.

3.2.2 Signaling through LT β R induces DNA binding of RelB in a NIK-dependent manner in primary mouse embryonic fibroblasts.

It has been shown that LT β R activation results in NF- κ B activation and that NIK regulates signaling through LT β R (73-75). The defective RelB DNA-binding observed in whole thymus and spleen extracts from *aly/aly* and *ltbr*^{-/-} mice could be an indirect effect. To check whether activation of LT β R induces RelB DNA-binding in a NIK-dependent manner, primary mouse embryonic fibroblasts (MEFs) from wild-type and *aly/aly* mice were activated by agonistic anti-LT β R mAb AC.H6 (119) and nuclear extracts were prepared. EMSA analysis of these nuclear extracts and dissection of different DNA binding complexes using antibodies specific for individual Rel/NF- κ B members revealed that LT β R triggering results in a rapidly induced (within 1 h) RelA heterodimeric complexes (I) independent of NIK (**Fig. 3.12A and B**). In addition, LT β R triggering of wild-type MEFs also induced faster migrating RelB heterodimeric complexes (II) with slower kinetics. RelB DNA-binding reached maximal levels at 4 h and is maintained up to 8 h and further. Interestingly, induction of RelB complexes (II) upon LT β R triggering was strictly dependent on NIK as revealed by EMSA of nuclear extracts prepared from *aly/aly* MEFs (**Fig. 3.12A and B**). These data demonstrate that signaling through LT β R regulates RelB, but not RelA, DNA-binding activity in a NIK-dependent manner. RelB DNA-binding is more severely impaired in thymus and spleen of *aly/aly* mice compared to *ltbr*^{-/-} mice, indicating that NIK is a key regulator of RelB downstream of LT β R as well as other receptors.

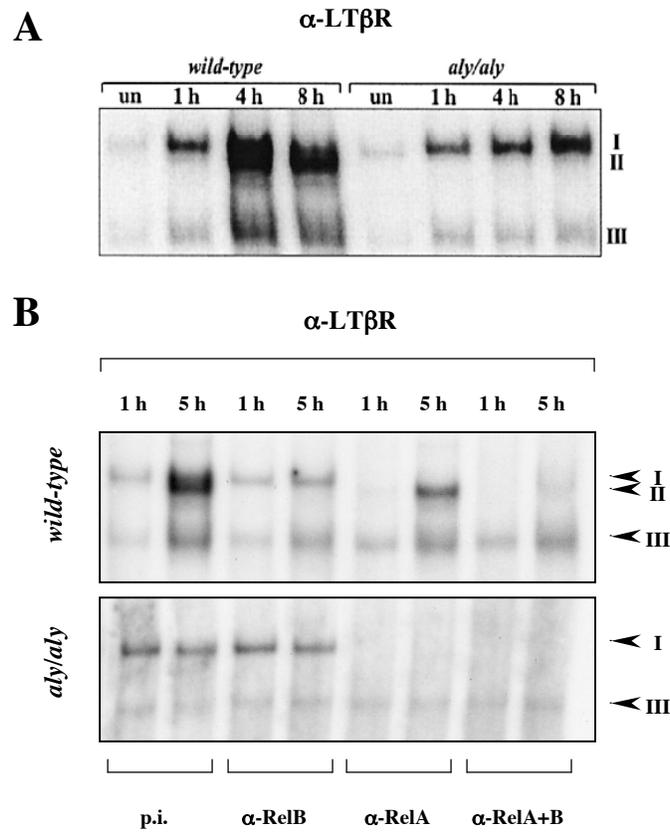


Fig. 3.12: NIK regulates RelB, but not RelA, DNA-binding downstream of LT β R.

Electrophoretic mobility shift assay of NF- κ B complexes induced by LT β R activation in wild-type and *aly/aly* MEFs. (A) Primary MEFs from wild-type and *aly/aly* mice were either un-treated (un) or treated for the indicated time points with agonistic anti-LT β R mAb. Nuclear extracts were prepared and analyzed for NF- κ B induction using Ig κ probe. (B) Dissection of DNA-binding complexes. Nuclear extracts from MEFs stimulated for 1 h and 5 h were analyzed by supershift assay using antibodies specific for individual NF- κ B subunits as indicated. p.i., pre-immune serum. Integrity of nuclear extracts was checked by performing EMSA with an OCT probe (not shown).

3.2.3 TNF induces DNA binding of predominantly classical NF- κ B complexes consisting of p50/RelA independently of NIK.

It has been suggested that NIK is required for TNF-mediated induction of NF- κ B by experiments involving over expression of a dominant-negative form of NIK (120). To analyze the requirement of NIK downstream of TNF primary MEFs from wild-type and *aly/aly* mice were compared. TNF treatment resulted in the rapid induction of NF- κ B which consisted almost exclusively of RelA/p50 complexes (complex I) and no significant difference in the induction was observed between wild-type and *aly/aly* MEFs (Fig. 3.13). This indicates that under physiological conditions, NIK is dispensable for TNFR induced NF- κ B.

Results

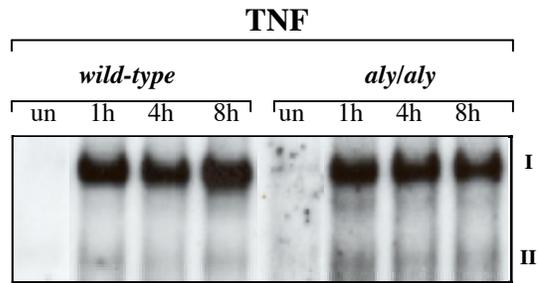


Fig. 3.13: Activation of NF- κ B downstream of TNF is independent of NIK.

Primary MEFs from wild-type and *aly/aly* mice were either un-treated (un) or treated with TNF (20 ng/ml) for the indicated time points. Nuclear extracts were prepared and analyzed for NF- κ B DNA binding by EMSA. The identity of the induced complexes was checked by supershift assays as described in Fig. 3.12. Complex I consisted of RelA heterodimers and complex II consisted of p50 homodimers (not shown). The integrity of extracts was checked by performing EMSA with an OCT probe (not shown).

3.2.4 NIK is required for LT β R-induced processing of p100.

Activation of classical NF- κ B is under the control of I κ B α degradation. In the absence of NIK, I κ B α degradation is normal downstream of both TNFR and LT β R (75). Thus, it is unclear how NIK regulates induction of RelB complexes downstream of LT β R. The p52/NF- κ B2 precursor protein p100 consists of multiple ankyrin repeats at its C-terminus and has been shown to inhibit RelB (121). Moreover, recent evidence indicates that NIK regulates phosphorylation and proteolytic processing of the inhibitory p100 to p52 (122). Thus, LT β R-mediated induction of RelB complexes may be regulated by processing of p100 via NIK. To address this question, primary MEFs from wild-type and *aly/aly* mice were activated with agonistic anti-LT β R mAb as described before. Immunoblotting analysis of whole cell extracts revealed that activation of LT β R results in the proteolytic processing of p100 to p52 in wild-type but not in *aly/aly* MEFs (**Fig. 3.14**). Interestingly, LT β R induced processing of p100 occurred at 4 h after activation and continued up to 8 h and further, correlating with the kinetics of induction of RelB DNA binding (**Fig. 3.14**). In contrast, activation of MEFs with TNF, results in a marked accumulation of p100 and did not trigger its processing to p52 (123). This indicates that signaling through LT β R, but not through TNFR, specifically induces RelB complexes correlating with the differential ability of these receptors to process p100. The levels of RelB protein after LT β R activation were similar between wild-type and *aly/aly* MEFs (**Fig. 3.14**), indicating

Results

that the defective RelB DNA binding downstream of LT β R in the absence of functional NIK is not due to decreased RelB protein levels.

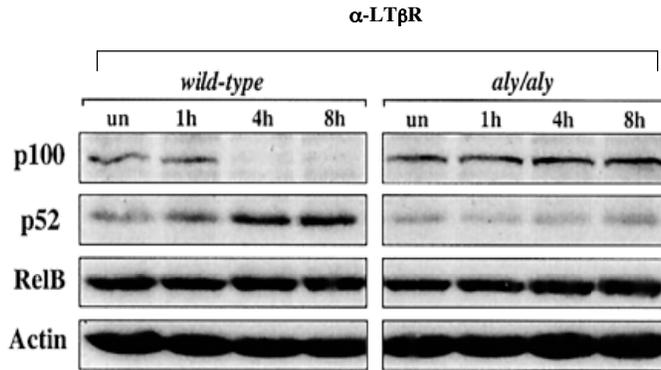


Fig. 3.14: Regulation of p100 processing by NIK downstream of LT β R.

Western blot analysis of p100, p52 and RelB protein levels in whole cell extracts of wild-type and *aly/aly* MEFs after stimulation with agonistic anti-LT β R mAb for the indicated time points. Un: un-treated. β -actin levels are shown as loading control.

3.2.5 NIK is required in a signal-dependent manner for the nuclear translocation of RelB.

Induction of classical NF- κ B is mediated by the signal-dependent degradation of I κ B α followed by the nuclear translocation of p50/RelA. It is therefore possible that similar to classical I κ B α -regulated pathway, LT β R-induced NIK-dependent proteolytic processing of p100 may also regulate nuclear translocation of RelB. Western blotting analysis of nuclear and cytoplasmic extracts revealed increased nuclear translocation of RelB in wild-type but not in *aly/aly* MEFs upon LT β R triggering (**Fig. 3.15A**). Nuclear translocation of RelB occurred after 4 h correlating with p100 processing to p52, indicating that p100 processing regulates the nuclear translocation of RelB downstream of LT β R.

TNF signaling did not induce processing of p100 but rather results in its accumulation (123). Surprisingly, activation of wild-type and *aly/aly* MEFs with TNF resulted in increased nuclear translocation of RelB in a time-dependent manner (**Fig. 3.15B**). However, the majority of the RelB remained cytoplasmic in TNF-treated MEFs (**Fig. 3.15B**). This indicates that RelB nuclear translocation is dependent on NIK and/or processing of p100, in a signal-dependent manner (see also Discussion).

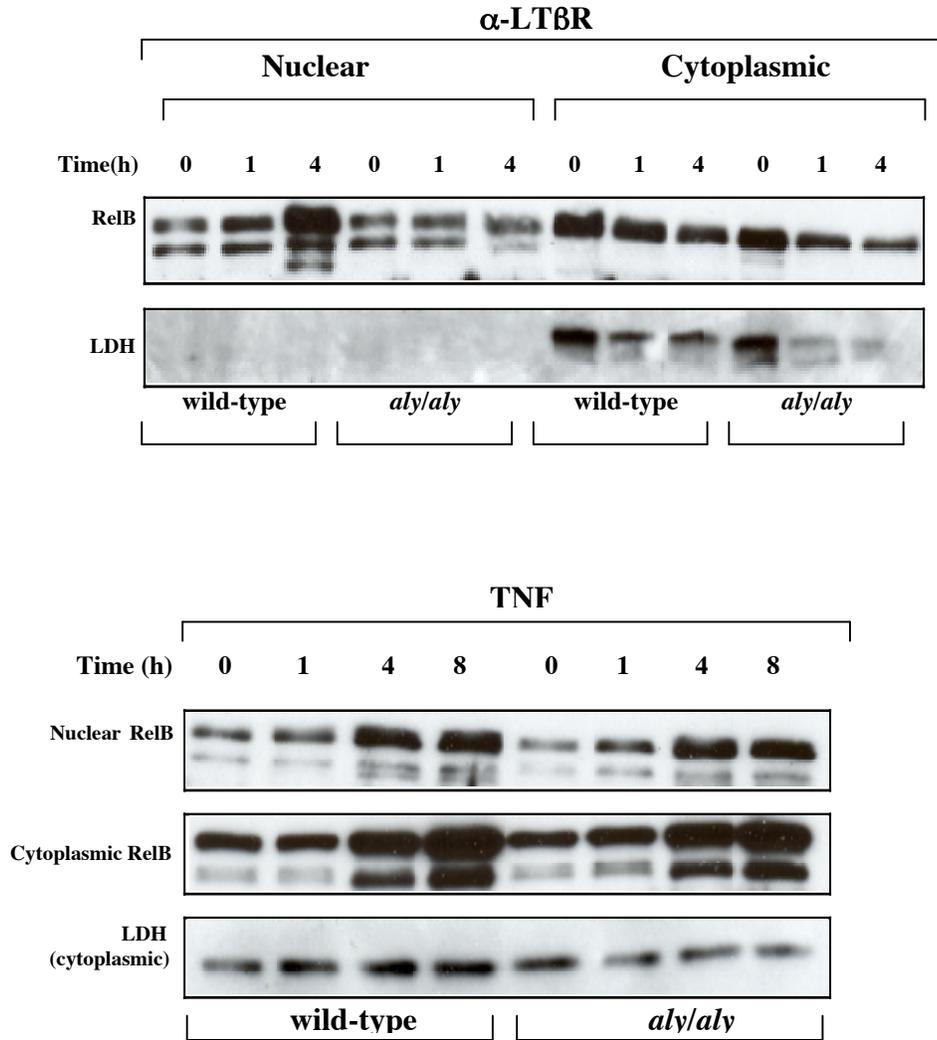


Fig. 3.15: NIK regulates nuclear translocation of RelB downstream of LTβR but not TNFR.

Western blotting analysis of nuclear and cytoplasmic RelB protein levels in primary MEFs from wild-type and *aly/aly* mice upon activation of LTβR or TNFR for the indicated time points. 0 indicates no induction. LDH levels were analyzed to check the purity of nuclear extracts. No LDH was detected in the nuclear extracts prepared from TNF-treated cells (not shown).

Collectively these results demonstrate that in thymocytes IκBα-mediated activation of NF-κB regulates LTα and LTβ expression, while in non-hematopoietic cells, LTβR and NIK-dependent p100 processing regulates DNA-binding of RelB complexes. Thus LT signaling is regulated by distinct NF-κB members both upstream and downstream of LTβR.

Results

3.2.6 Lymphotoxin signaling is not essential for the development of thymic V α 14i NKT cells.

Previous studies have indicated a role for LT signaling and NIK in the development of NKT cells (57, 58, 72). However, no direct evidence exists that clearly demonstrate the requirement of LT β R for the development of NKT cells. Therefore, thymic V α 14i NKT cells were analyzed in wild-type and *ltbr*^{-/-} mice by flow cytometry. α -GalCer loaded CD1d-tetramers were used along with anti-TCR β mAb to stain thymocytes since *ltbr*^{-/-} mice do not express the NK1.1 marker (124). Surprisingly, no defect in NKT cell development was observed in *ltbr*^{-/-} mice (**Table 3.1**), despite the requirement of NIK and previous evidence for an important role of LT α and LT β in NKT cell development. Therefore, *lta*^{-/-}, *ltb*^{-/-} mice and mice deficient for other TNF family members such as *tnf*^{-/-}, *lta*^{-/-}*tnf*^{-/-} double-deficient mice, *light*^{-/-}, *hvem*^{-/-} as well as *hvem*^{-/-}*ltbr*^{-/-} double-deficient mice were analyzed for thymic V α 14i NKT cell numbers. In contrast to previously published data, no dramatic difference in thymic NKT cell percentages was observed between wild-type, *lta*^{-/-}, *ltb*^{-/-}, *tnf*^{-/-} mice, and even *tnf*^{-/-}*lt α* ^{-/-} double-deficient mice (**Table 3.1**). A maximal 3-fold reduced NKT cell percentage was observed in *light*^{-/-} mice, but only one mouse was analyzed and this requires further analysis. Although *ltbr*^{-/-} or *hvem*^{-/-} mice had no significant defect in NKT cell development, *ltbr*^{-/-}*hvem*^{-/-} double-deficient mice had a 3-fold reduction in NKT cell percentages (**Table 3.1**). Collectively, these data indicate that LT signaling is not essential for thymic NKT cell development and that the redundancy among different TNF family ligands and their receptors may compensate for the lack of individual family members during the development of NKT cells (see also Discussion).

Table 3.1. Analysis of thymic NKT cell development in mice deficient for TNF family members and their receptors.

	<i>wt</i>	<i>ltβ</i> ^{-/-}	<i>ltα</i> ^{-/-}	<i>tnf</i> ^{-/-}	<i>tnf</i> ^{-/-} <i>ltα</i> ^{-/-}	<i>light</i> ^{-/-}	<i>ltβr</i> ^{-/-}	<i>hvem</i> ^{-/-}	<i>ltβr</i> ^{-/-} <i>hvem</i> ^{-/-}
Percentage of thymic V α 14i NKT cells	0.50 \pm 0.24	0.34 \pm 0.11	0.25	0.31 \pm 0.10	0.57 \pm 0.03	0.14	0.53 \pm 0.08	0.33 \pm 0.17	0.16 \pm 0.05
N	8	4	1	3	2	1	5	3	3

Thymocytes from mice indicated in the table were isolated and analyzed for V α 14i NKT cell percentages by flow cytometry after staining with α -GalCer loaded CD1d-tetramer and anti-TCR β mAb. Numbers indicate the percentage of thymic NKT cells \pm SD. N, number of mice analyzed.

3.2.7 Signaling through LTβR is required for the migration of thymic NKT cells to the liver.

Migration of thymic NKT cells to liver has been shown to be dependent on LFA-1 expressed on hepatic NK cells (125). Since signaling through LTβR was shown to be required for the development of NK cells, it is possible that *ltbr*^{-/-} mice may have a defective migration of thymic NKT cells to liver. Flowcytometric analysis of Vα14i NKT cells among the mononuclear cells prepared from liver, revealed significantly reduced percentages of these cells in *ltbr*^{-/-} compared to wild-type mice (**Fig. 3.16 A**). This defect in the migration to liver was specific to NKT cells, since the migration of mainstream T cells was not affected (**Fig. 3.16 B**).

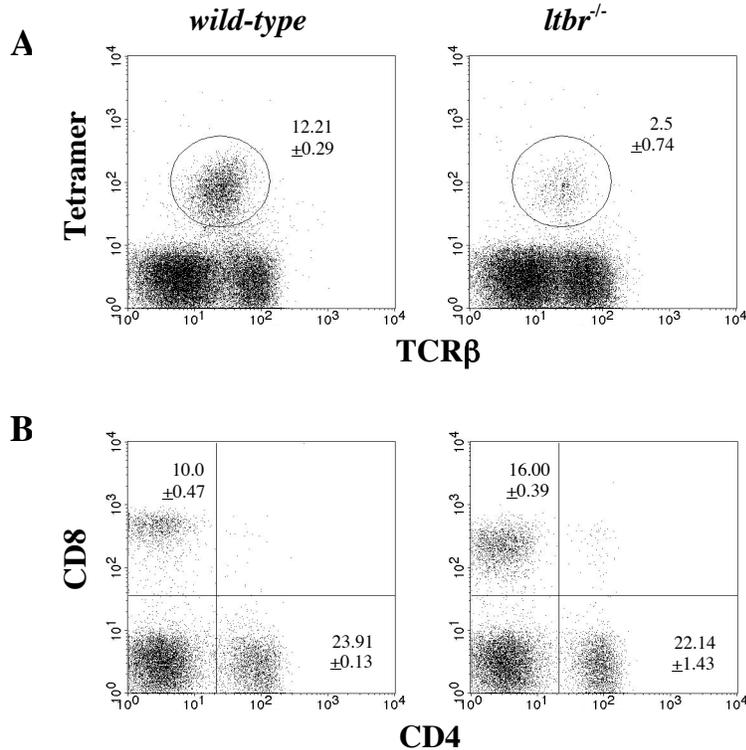


Fig. 3.16: Defective migration of thymic NKT cells, but not mainstream T cells, to the liver in *ltbr*^{-/-} mice.

Hepatic mononuclear cells were isolated by percoll gradient and analyzed by flow cytometry for CD1d-tetramer⁺ NKT cells and mainstream CD8⁺ and CD4⁺ T cells.

PART THREE

3.3 Function of RelB within T cells.

The majority of NF- κ B DNA-binding complexes in T cells consists of RelA/p50 heterodimers and to some extent c-Rel heterodimers (85). Although RelB is expressed in T cells, heterodimers consisting of RelB are very weakly induced upon TCR activation (85 and see **Fig. 3.17A and B**). Since RelB-deficient mainstream T cells develop and function normally, it is not clear whether RelB has any function within T cells (18). To understand the precise function of RelB and its redundancy with other NF- κ B members in T cells, it would be helpful to have T cells, in which only RelB complexes can bind to DNA, while the rest of the NF- κ B activity is inhibited. In the *mIkB α ^{tg}* mice described in the previous sections, NF- κ B DNA-binding activity is almost completely inhibited in T cells (see **Fig. 3.2**). Moreover, it has been shown that I κ B α is a poor inhibitor of RelB (126, 127) and it appears that activation of RelB is predominately regulated by alternate signaling pathways involving NIK-mediated processing of p100 to p52. Therefore, if RelB is overexpressed in T cells of *mIkB α ^{tg}* mice, one possibility is, only RelB DNA binding is induced upon activation due to the capability of mI κ B α to inhibit induction of RelA and c-rel complexes but not RelB complexes. In order to obtain transgenic mice which overexpress both RelB and mI κ B α within T cells, *mIkB α ^{tg}* mice were crossed with another transgenic line overexpressing RelB in T cells under the control of the *lck* proximal promoter (*relB^{tg}*) (Ingmar shall unpublished data and 115). The resulting double-transgenic mice (*relB^{tg}mIkB α ^{tg}*) overexpress both RelB and mI κ B α in T cells.

3.3.1 I κ B α can inhibit DNA binding of RelB.

Since the *mIkB α* transgene blocks classical NF- κ B DNA-binding, *relB^{tg}mIkB α ^{tg}* double-transgenic mice should only have DNA-binding of NF- κ B complexes consisting of RelB heterodimers. To test this, thymocytes from wild-type, *relB^{tg}*, *mIkB α ^{tg}*, and *relB^{tg}mIkB α ^{tg}* double-transgenic mice were treated with PMA + PHA and nuclear extracts were prepared. EMSA and supershift analysis revealed that activation of wild-type thymocytes resulted in strong induction of DNA-binding of RelA/p50 heterodimers (I) and a weak induction of p50 homodimers (III) (**Fig. 3.17A**). Induction of RelB heterodimers in wild-

Results

type thymocytes was almost negligible (**Fig. 3.17B**). As shown in **Fig. 3.2 and 3.17A**, *mIkB α ^{tg}* thymocytes revealed almost no induction of NF- κ B DNA-binding upon treatment with PMA + PHA. Nuclear extracts from *relB^{tg}* thymocytes revealed a very high constitutive (uninduced) faster migrating NF- κ B DNA-binding complex (II) (**Fig. 3.17A**). Supershift assays indicated that complex II consisted exclusively of RelB heterodimers. PMA + PHA activation of *relB^{tg}* thymocytes resulted in massive induction of predominantly RelB complexes. Interestingly, overexpression of RelB resulted in reduced DNA-binding of p50/RelA complexes (**Fig. 3.17B** and Ingmar Sholl unpublished data). Nuclear extracts prepared from *relB^{tg}mIkB α ^{tg}* double-transgenic thymocytes revealed significant reduction in the constitutive DNA-binding of RelB complexes compared to nuclear extracts from *relB^{tg}* thymocytes (**Fig. 3.17A and B**). This indicates that I κ B α can significantly inhibit RelB DNA-binding, which is in contrast to the previously published notion that I κ B α is a poor inhibitor of RelB. Moreover, treatment with PMA + PHA also resulted in significantly reduced induction of RelB-DNA-binding in thymocytes from *relB^{tg}mIkB α ^{tg}* double-transgenic compared to *relB^{tg}* mice. However, the inhibition of RelB DNA-binding by mIkB α was not complete and RelB was still inducible (**Fig. 3.17A and B**). Both basal and inducible NF- κ B DNA-binding activity in thymocytes of *relB^{tg}mIkB α ^{tg}* double-transgenic mice consisted almost exclusively of RelB heterodimers (**Fig. 3.17A and B**). Other NF- κ B DNA-binding activity was very weak and comparable between *mIkB α ^{tg}* and *relB^{tg}mIkB α ^{tg}* double-transgenic mice. (**Fig. 3.17A and B**). Supershift analysis with anti-p50 Ab revealed that p52/RelB heterodimers are also inhibited by mIkB α (**Fig. 3.17C**). These results demonstrate (i) that I κ B α can inhibit RelB DNA-binding and (ii) that thymocytes of *relB^{tg}mIkB α ^{tg}* double-transgenic mice have NF- κ B DNA-binding consisting exclusively of RelB heterodimers.

Results

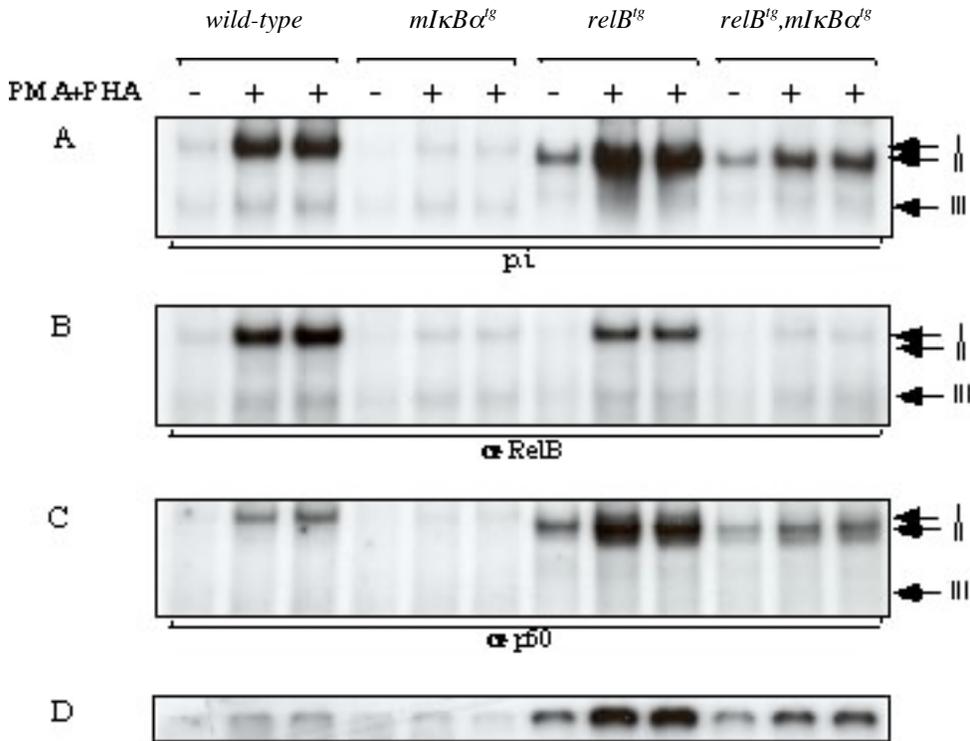


Fig. 3.17: Inhibition of RelB DNA binding by IκBα.

(A) Thymocytes from the indicated mice were either untreated (-) or treated (+) with PMA (10 ng/ml) and PHA (5 μg/ml) for 1 h. Nuclear extracts were prepared and were treated with pre-immune serum (p.i.) and analyzed by EMSA for κB-binding activity. (I) RelA-containing complexes (II) RelB-containing complexes and (III) p50 homodimers. (B) Supershift assay of the above nuclear extracts with anti-RelB Ab and (C) with anti-p50 Ab. (D) Supershifted RelB that remained in the slots of the gel from the EMSA experiment described in panel B is shown. Note the significant inhibition of RelB DNA-binding in extracts from *relB^{tg},mIκBα^{tg}* double-transgenic mice compared to *relB^{tg}* mice.

3.3.2 Gene-specific redundancy in the function of RelB and other NF-κB family members.

Since NF-κB members may have redundant functions in the regulation of gene expression it was examined whether overexpression of RelB can rescue the induction of NF-κB target genes that were down-regulated in *mIκBα^{tg}* thymocytes. For this purpose four genes encoding NF-κB1, NF-κB2, LTα, and LTβ were chosen since their inducible expression was impaired in *mIκBα^{tg}* mice. Interestingly, only the inducible expression of the gene encoding NF-κB2 but not NF-κB1, LTα or LTβ was significantly restored by RelB, indicating that functional redundancy among RelB and other NF-κB members may be is gene specific (**Fig. 3.18** see also Discussion).

Results

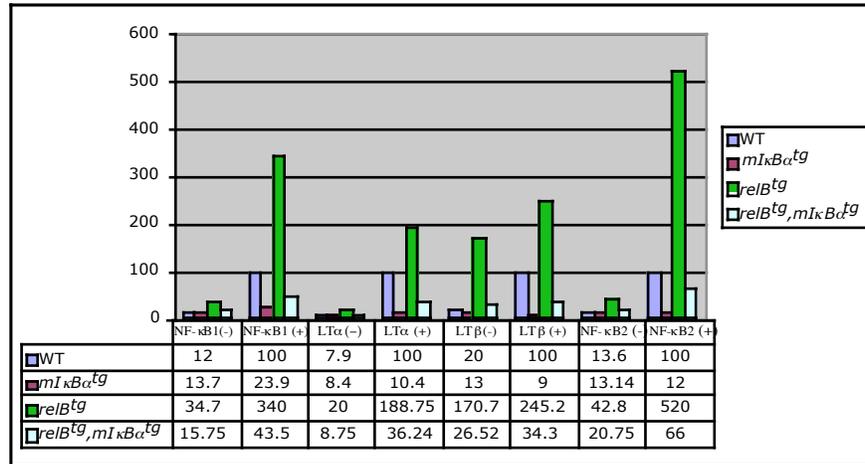


Fig. 3.18: Gene-specific redundancy in the transcription activation function of RelB and other NF-κB members.

Thymocytes from indicated mice were either untreated (-) or treated (+) with PMA (10 ng/ml) and PHA (5 µg/ml) for 2 h. RNA was prepared and analyzed by RT-PCR for the steady state and induced expression of NF-κB1, LTα, LTβ and NF-κB2. Note that RelB heterodimers in *relB^{tg}mIkBα^{tg}* double-transgenic mice, can only restore inducible NF-κB2 gene expression to wild-type levels. Wild-type expression levels were set to 100% and the numbers indicate the levels of expression of the indicated genes after normalizing for actin. Representative of two independent experiments is shown.

3.3.3 Overexpression of RelB in T cells rescues peripheral CD8⁺ T Cell numbers, but not thymic NKT cell numbers in *mIkBα^{tg}* mice.

DNA-binding of only RelB heterodimers in thymocytes of *relB^{tg}mIkBα^{tg}* double-transgenic mice makes these mice a good model system to study functions that RelB heterodimers alone can do in T cells. Since NKT cell development was defective in *mIkBα^{tg}* mice, it was tested whether RelB could rescue NKT cell development in *relB^{tg}mIkBα^{tg}* double-transgenic mice. Flow cytometric analysis of thymocytes from wild-type, *relB^{tg}*, *mIkBα^{tg}* and *relB^{tg}mIkBα^{tg}* double-transgenic mice revealed that *relB^{tg}mIkBα^{tg}* double-transgenic mice had reduced NKT cell percentages similar to *mIkBα^{tg}* mice. This indicates that NKT cell development is strictly dependent on NF-κB function other than RelB heterodimers, and that with respect to NKT cell development RelB has no redundancy with other NF-κB family members (Fig. 3.19A). Interestingly, overexpression of RelB in thymocytes of *relB^{tg}* mice resulted in impaired development of NKT cells with 2-3 fold reduced percentages of thymic NKT cells compared to wild-type mice (Fig. 3.19A).

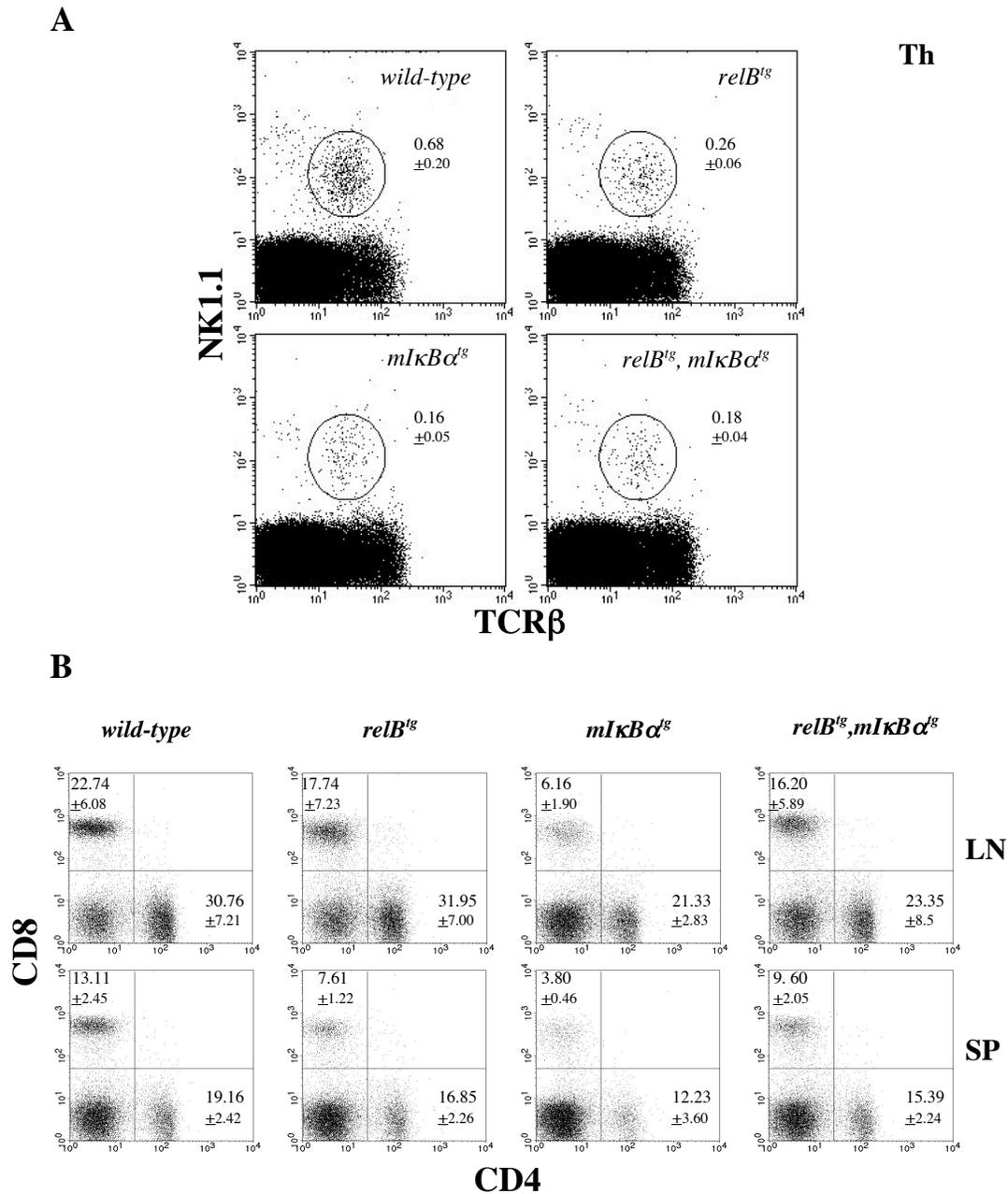


Fig. 3.19: RelB hetero-dimers are sufficient to maintain peripheral mainstream T cell numbers, but do not rescue thymic NKT cell development.

(A) Thymocytes from indicated mice were stained with anti-NK1.1 and anti-TCRβ mAbs and analyzed by flow-cytometry. Circles indicate percentage of NKT cells. (B) Single cell suspensions prepared from LNs and spleen were analyzed for CD4⁺ and CD8⁺ T cell percentages by flow-cytometry. Numbers in quadrants indicate percentages of CD4⁺ and CD8⁺ T cells ±SD. 2-4 mice per each genotype were analyzed and representative dot plots are shown.

Inhibition of NF-κB in T cells results in significantly reduced numbers of peripheral T cells with a more severe reduction in CD8⁺ compared to CD4⁺ T cells as well as severely impaired proliferative responses and increased apoptosis of T cells upon TCR activation

Results

(33, 35 and Fig. 3.19B). Flow cytometric analysis of splenic and LN T cells revealed that over expression of RelB in the thymocytes of *relB^{tg}* mice does not significantly effect the numbers of peripheral CD8⁺ and CD4⁺ T cells (Fig. 3.19B). However, spleen and LN from *relB^{tg}mIkB α ^{tg}* double-transgenic mice had much higher numbers of CD8⁺ T cells compared to *mIkB α ^{tg}* mice (Fig. 3.19B), indicating that RelB heterodimers alone are sufficient to maintain the numbers of peripheral T cells. In contrast, the defective proliferative response of T cells from *mIkB α ^{tg}* mice was not corrected in T cells from *relB^{tg}mIkB α ^{tg}* double transgenic mice (not shown). Collectively, these data indicate that RelB heterodimers have redundant functions with other NF- κ B members in T cells in the maintenance of peripheral T cell numbers.

PART FOUR

3.4 NF- κ B is required within T cells for the development of generalized lymphoproliferative disorder (*gld/gld*).

The primary defect in *gld/gld* mice is the mutation in FasL gene, which should effect Fas mediated apoptosis in all the cells. However, *gld* disorder develops mainly due to T cell abnormalities (97, 98, 128, 129; see also introduction). NF- κ B regulates many functions of T cells including proliferation and protection from apoptosis. To study the consequences of impaired NF- κ B activation in T cells on the development of the *gld* phenotype, *mIkB α ^{tg}* mice were crossed with B6Smn.C3H-FasL^{*gld/gld*} mice to generate *mIkB α ^{tg}gld/gld* mice.

3.4.1 Transgenic *gld/gld* mice with impaired NF- κ B activation in T cells do not develop splenomegaly and lymphadenopathy.

Since splenomegaly and lymphadenopathy primarily characterize the *gld* phenotype, the weights of lymphoid organs from age-and body weight-matched mice of different genotypes were compared as indicated in **Fig. 3.20A**. Spleen and LNs from *mIkB α ^{tg}gld/gld* mice were greatly reduced in size (**Fig. 3.20A**) and cellularity (**Fig. 3.20B**) compared to *gld/gld* mice.

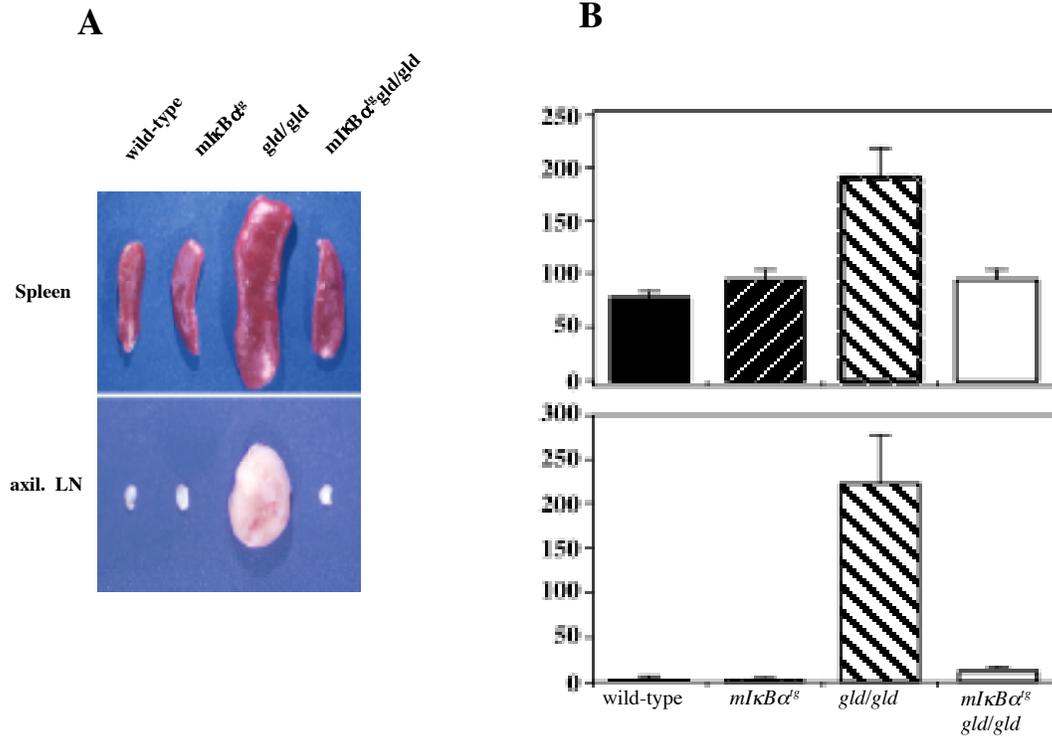


Fig. 3.20: Transgenic gld/gld mice with impaired NF- κ B activation in T cells do not develop splenomegaly and lymphadenopathy.

(A) Gross appearance of spleen and axillary LNs (axil. LN) from 6 to 8 months old wild-type, $mI\kappa B\alpha^{tg}$ gld/gld , and $mI\kappa B\alpha^{tg}gld/gld$ mice. (B) Spleen and mesenteric LN (mes. LN) cellularities from wild-type, $mI\kappa B\alpha^{tg}$, gld/gld , and $mI\kappa B\alpha^{tg}gld/gld$ mice are shown (mean values \pm SEM).

3.4.2 Development/accumulation of abnormal Thy-1.2⁺B220⁺CD4⁻CD8⁻ T cells requires NF- κ B function in thymocytes.

The major cause for the lymphadenopathy and splenomegaly in gld/gld mice is the progressive accumulation of thymus-derived abnormal T cells that express Thy-1.2 and B220, but neither CD4 nor CD8 (91). Flow cytometric analysis revealed that these abnormal T cells constituted up to 55-60% of the lymphocytes in mesenteric LNs from gld/gld mice. In contrast, the percentage of Thy-1.2⁺B220⁺CD4⁻CD8⁻ cells was highly reduced in $mI\kappa B\alpha^{tg}gld/gld$ mice, reaching levels similar to wild-type controls (**Fig. 3.21**).

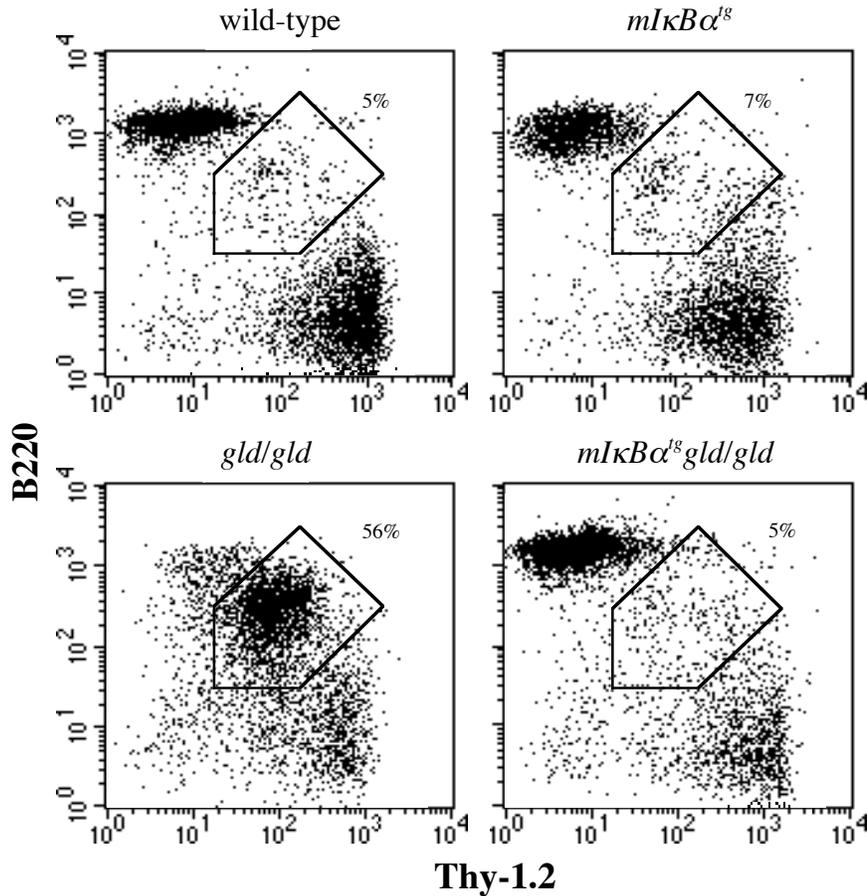


Fig. 3.21: *mIkBα^{tg}gld/gld* mice do not accumulate abnormal Thy-1.2⁺B220⁺ double-positive T cells.

LN cells from 6-month-old wild-type (A), *mIkBα^{tg}* (B), *gld/gld* (C), and *mIkBα^{tg}gld/gld* mice (D) were stained with FITC-conjugated anti-Thy-1.2 and PE-conjugated anti-B220 mAbs. In each case 10⁴ viable cells were analyzed by flow cytometry. Numbers indicate percentages of abnormal Thy-1.2⁺B220⁺ double-positive cells in the marked area. A minimum of four mice was analyzed for each genotype and representative dot plots are shown.

Although the percentages of Thy-1.2⁻B220⁺ B cells were increased (1.5-fold) and Thy-1.2⁺B220⁻ T cells were decreased (1.6-fold) in *mIkBα^{tg}* mice compared to wild-type controls, *mIkBα^{tg}gld/gld* mice revealed a relative increase in the percentage of these normal T and B cells compared to *gld/gld* mice (**Table 3.2**). The lymphocyte subpopulation percentages in mesenteric LNs from the different genotypes are summarized in **Table 3.2**.

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Table 3.2. Percentages of different lymphocyte subpopulations in mesenteric lymph nodes from mice with the indicated genotypes.

Mesenteric lymph nodes (% of total cell number)				
Genotype	Thy-1 ⁺ B220 ⁺	Thy-1 ⁻ B220 ⁺	Thy-1 ⁺ B220 ⁻	CD3 ⁺ CD8 ⁻ CD4 ⁻
wild-type	4.0 ± 0.8	27.2 ± 6.0	63.0 ± 4.5	2.8*
<i>mIkBα^{tg}</i>	6.0 ± 0.9	41.5 ± 3.0	39.5 ± 2.5	3.0*
<i>gld/gld</i>	54.0 ± 4.0	16.5 ± 4.0	26.2 ± 7.5	45.0*
<i>mIkBα^{tg}gld/gld</i>	6.0 ± 0.8	37.2 ± 9.5	36.2 ± 5.5	8.3*

The percentage of each lymphocyte subpopulation in mesenteric lymph nodes was determined from dot plot data of flowcytometric analyses. Values are given as the mean of four mice per genotype ±SEM. *Only one mouse per genotype was analyzed.

The prevention of lymphoproliferation in *mIkBα^{tg}gld/gld* mice was even more pronounced when the absolute number of abnormal T cells was calculated. As shown in **Table 3.3**, the mesenteric LN of *gld/gld* mice had a total of 1.1 x 10⁸ Thy-1.2⁺B220⁺ T cells. This population was decreased 160-fold in *mIkBα^{tg}gld/gld* mice, only 3.5-fold higher than in wild-type controls. Also, the excessive accumulation of normal B and T cells in *gld/gld* mice was almost completely rescued by the *mIkBα* transgene.

Table 3.3. Absolute cell numbers of different lymphocyte subpopulations in mesenteric lymph nodes from mice with the indicated genotypes.

Mesenteric lymph nodes (cell numbers x 10 ⁵)				
Genotype	Thy-1 ⁺ B220 ⁺	Thy-1 ⁻ B220 ⁺	Thy-1 ⁺ B220 ⁻	CD3 ⁺ CD8 ⁻ CD4 ⁻
wild-type	2.0 ± 0.5	15 ± 3.0	38 ± 6.5	1.5*
<i>mIkBα^{tg}</i>	1.0 ± 0	12 ± 4.0	12 ± 3.5	1.5*
<i>gld/gld</i>	1120 ± 285	415 ± 177	533 ± 128	1800*
<i>mIkBα^{tg}gld/gld</i>	7.0 ± 2.5	42 ± 14	41 ± 17	3.0*

Absolute numbers of different lymphocyte populations in mesenteric lymph nodes were determined based on the total cell number shown in **Fig. 3.20B** and the percentage of each subpopulation from dot plot data of flowcytometric analyses. Values are given as the mean of four mice per genotype ±SEM. *Only one mouse per genotype was analyzed.

3.4.3 Lack of NF- κ B induction in peripheral T cells in *mI κ B α^g gld/gld* mice.

The reduction in the percentage of abnormal Thy-1.2⁺B220⁺CD4⁻CD8⁻ T cells in *mI κ B α^g gld/gld* mice compared to *gld/gld* mice could be due to inhibition of NF- κ B in thymocytes and/or peripheral T cells by the *mI κ B α* transgene. Since the *mI κ B α* was expressed under the regulatory elements of *lck* proximal promoter, which predominantly operates in thymocytes, it was not clear whether the transgene can also inhibit NF- κ B in peripheral T cells. To test the functionality of the transgene in peripheral T cells, induction of NF- κ B in splenic CD4⁺ T cells purified from 10-week-old *gld/gld* and *mI κ B α^g gld/gld* mice was compared. At this age, *gld/gld* mice do not accumulate abnormal T cells or develop lymphadenopathy. As shown in **Fig. 3.22**, TCR triggering of CD4⁺ T cells from *gld/gld* mice resulted in increased nuclear NF- κ B activity. In contrast, both basal and induced κ B-binding was reduced in anti-CD3 and anti-CD28 stimulated CD4⁺ T cells from *mI κ B α^g gld/gld* mice. Thus the *mI κ B α* inhibits NF- κ B induction in both thymocytes and peripheral T cells (see also Fig. 3.2A).

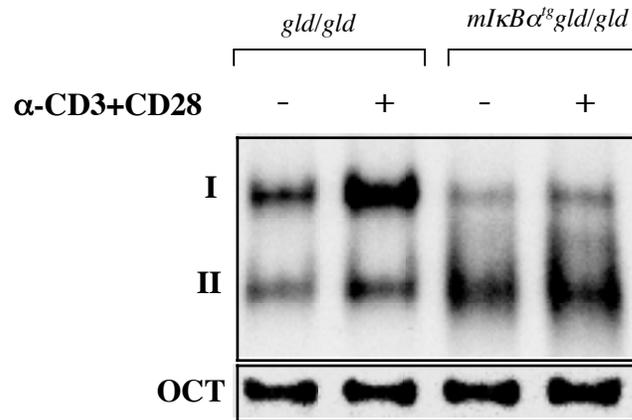


Fig. 3.22: Lack of NF- κ B induction in peripheral T cells from *mI κ B α^g gld/gld* mice. Splenic CD4⁺ T cells from 10-week-old *gld/gld* and *mI κ B α^g gld/gld* mice were either unstimulated (-) or stimulated (+) with plate-bound anti-CD3 and anti-CD28 mAbs (10 μ g/ml each) and nuclear extracts were analyzed for NF- κ B induction by EMSA. Binding to an Oct oligodeoxynucleotide was used to check integrity of the extracts.

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3.4.4 Impaired proliferation of peripheral T cells from $mIkB\alpha^{tg}/gld/gld$ mice upon TCR signaling.

The reduced percentage of abnormal $\text{Thy-1.2}^+\text{B220}^+\text{CD4}^-\text{CD8}^-$ T cells in $mIkB\alpha^{tg}/gld/gld$ mice compared to gld/gld mice could be due to a developmental defect or due to defective progressive and proliferative accumulation as a result of inhibition of NF- κ B in T cells. To address this and to investigate whether the impaired activation of NF- κ B affects the function of T lymphocytes in the periphery, LN T cells from 8-week-old gld/gld and $mIkB\alpha^{tg}/gld/gld$ mice were activated with a constant amount of anti-CD28 and increasing concentrations of anti-CD3 mAb. As shown in **Fig. 3.23A**, T cells from $mIkB\alpha^{tg}/gld/gld$ mice proliferated much less in response to TCR triggering compared to T cells from gld/gld mice.

Inhibition of NF- κ B in T cells results in much stronger reduction of CD8^+ T cells in the periphery compared to CD4^+ cells (see **Fig. 3.19B**). To rule out that the impaired T lymphocyte proliferation in $mIkB\alpha^{tg}/gld/gld$ mice was simply due to reduced numbers of CD8^+ T cells, proliferation of purified splenic CD4^+ single-positive T cells upon TCR stimulation was studied. Similar to LN T cells, splenic CD4^+ T cells from 8-week-old $mIkB\alpha^{tg}/gld/gld$ mice also showed an impaired proliferative response upon anti-CD3 and anti-CD28 stimulation compared to their non-transgenic counterparts (**Fig. 3.23B**).

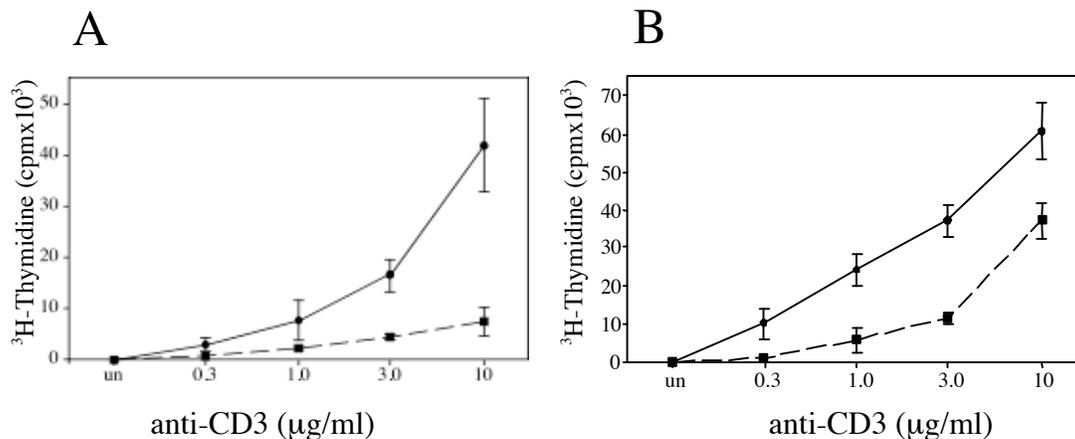


Fig. 3.23: Impaired proliferative response of peripheral T cells from $mIkB\alpha^{tg}/gld/gld$ mice upon TCR signaling.

(A) Proliferation of LN T cells. T cells were purified from mesenteric LNs of 8-week-old gld/gld (circles) and $mIkB\alpha^{tg}/gld/gld$ mice (squares) and stimulated with the indicated concentrations of plate-bound anti-CD3 mAb in the presence of constant amounts of anti-CD28 mAb (10 $\mu\text{g/ml}$). Unstimulated (un) T cells were included as a control. Incorporated ^3H -thymidine was plotted against dilutions of anti-CD3 mAb. Mean values \pm SD of triplicates are shown. Dashed lines indicates $mIkB\alpha^{tg}/gld/gld$ mice whereas continuous lines indicates gld/gld mice (B) Proliferation of purified splenic CD4^+ T cells from 8-week-old gld/gld and

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mIkB α ^{tg}gld/gld mice. Experimental details were essentially as described for panel A.

3.4.5 Peripheral T cells from *mIkB α ^{tg}gld/gld* mice undergo increased apoptosis upon TCR signaling.

The main cause for the accumulation of abnormal Thy-1.2⁺B220⁺CD4⁺CD8⁻ T cells in *gld/gld* mice is the lack of FasL/Fas mediated apoptosis of T cells. It has been shown that NF- κ B protects T cells from apoptosis (104, 130). Therefore, it is possible that the expression of the *mIkB α* transgene may render T cells from *mIkB α ^{tg}gld/gld* more susceptible to programmed cell death. To address this, apoptosis of anti-CD3 and anti-CD28 stimulated CD4⁺ T cells purified from *gld/gld* and *mIkB α ^{tg}gld/gld* spleens was analyzed by Annexin V staining. Interestingly, *mIkB α ^{tg}gld/gld* CD4⁺ T cells showed a \approx 3-fold increase in Annexin V-positive cells compared to non-transgenic *gld/gld* CD4⁺ T cells, despite the defect in the FasL/Fas death pathway (Fig. 3.24A and B). This indicates that peripheral T cells from *mIkB α ^{tg}gld/gld* mice undergo increased apoptosis compared to *gld/gld* mice upon TCR activation.

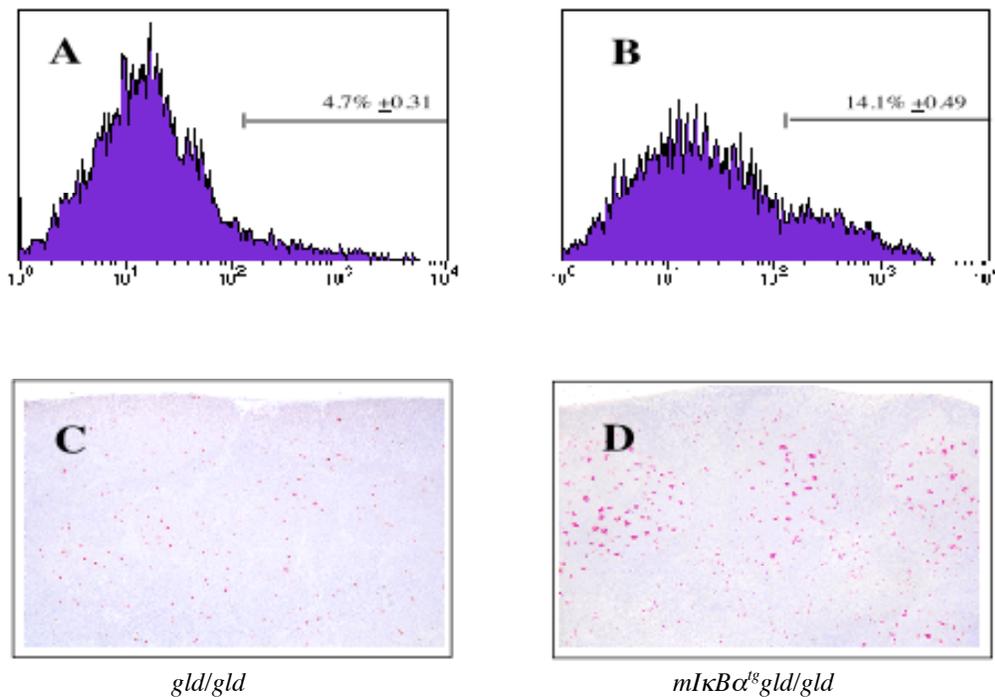


Fig. 3.24: Increased apoptosis of peripheral T cells from *mIkB α ^{tg}gld/gld* mice.

Purified splenic CD4⁺ T cells from *gld/gld* (A) and *mIkB α ^{tg}gld/gld* (B) mice were stimulated with anti-CD3 and anti-CD28 mAbs (10 μ g/ml each). Apoptotic cells were detected after 36 h in culture by flow cytometry after staining with propidium iodide and Annexin V. Percentages indicate propidium iodide-

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negative and Annexin V-positive cells \pm SD. TUNEL staining of mesenteric LN sections revealed increased numbers of apoptotic cells (red) in the paracortex of *mIkB α^g gld/gld* (D) compared to *gld/gld* (C) mice. Original magnification, x10 (TUNEL staining was performed by Debra Weih).

TUNEL staining of LN sections also revealed increased numbers of apoptic cells in *mIkB α^g gld/gld* mice compared to *gld/gld* littermates (**Fig. 3.24C and D**). Taken together, these results indicate that in *mIkB α^g gld/gld* mice both the reduced proliferation and the increased apoptosis of peripheral T cells in response to TCR signaling contribute to prevent accumulation of abnormal Thy-1.2⁺B220⁺CD4⁻CD8⁻ T cells and prevent splenomegaly, lymphadenopathy, and lymphoproliferation.

3.4.6 Partial rescue of B cell mediated autoimmunity in *mIkB α^g gld/gld* mice.

Hypergammaglobulinemia and high levels of circulating autoantibodies are characteristic features of *gld/gld* mice. The autoimmune syndrome is caused by B lymphocytes, although conflicting data exist on the T cell dependence of the B cell abnormalities in *gld/gld* and *lpr/lpr* mice (97, 129, 131). Therefore, it was analyzed whether the inhibition of NF- κ B in T cells also affected the autoimmune phenotype in *gld/gld* mice. Serum Ig and anti-dsDNA autoantibody levels in wild-type, *gld/gld*, and *mIkB α^g gld/gld* mice were measured by ELISA. As previously described, *gld/gld* mice had increased levels of serum IgM (**Fig. 3.25A**) and IgG (**Fig. 3.25B**) compared to control animals. Anti-dsDNA (**Fig. 3.25C**) autoantibody levels were also markedly increased in *gld/gld* mice. These high levels of serum Ig and anti-dsDNA Ab were clearly reduced in *mIkB α^g gld/gld* mice, but still higher than in wild-type controls (**Fig. 3.25A, B and C**).

The *gld* mutation causes glomerulonephritis due to the deposition of immune complexes in the kidney. Immunohistochemical analysis of kidney sections with anti-Ig Abs revealed strong staining of glomeruli in *gld/gld* mice (**Fig. 3.25D**). Ig deposits in glomeruli from *mIkB α^g gld/gld* mice were clearly reduced compared to non-transgenic *gld/gld* littermates (**Fig. 3.25E**). Thus, the T cell-specific inhibition of NF- κ B partially rescues autoimmune disease in *gld/gld* mice.

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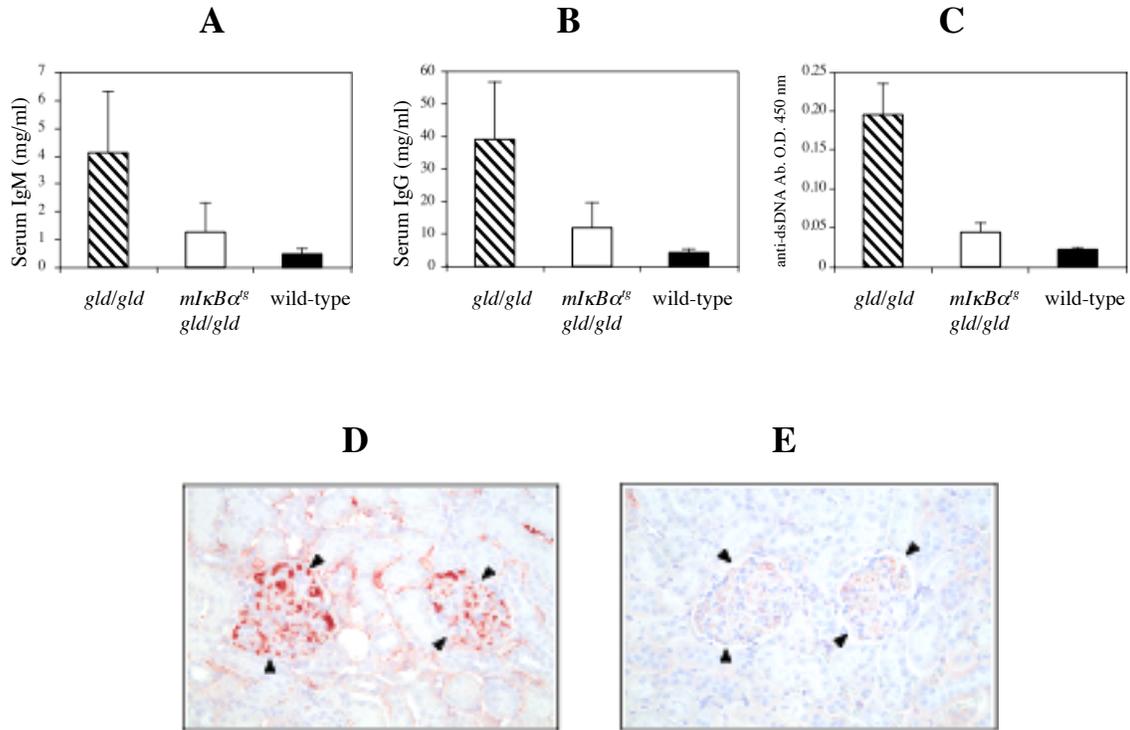


Fig. 3.25: Reduced serum Ig and anti-dsDNA Ab levels in 6 to 8 months old *mIkB α ^{tg}gld/gld* mice.

Serum IgM (A), IgG (B), and anti-dsDNA Ab levels (C) from *gld/gld*, *mIkB α ^{tg}gld/gld*, and wild-type mice were determined by ELISA. Results are shown as mean values \pm SD. Reduced glomerulonephritis in *mIkB α ^{tg}gld/gld* mice. Kidney sections from 8-month-old *gld/gld* (D) and *mIkB α ^{tg}gld/gld* mice (E) were incubated with biotinylated goat anti-mouse IgG and immune complex depositions (red) were visualized. Counterstaining with hematoxylin revealed loss of Bowman's space (arrow heads) in glomeruli from *gld/gld* compared to *mIkB α ^{tg}gld/gld* mice. Original magnification, x40 (Immunohistochemistry of kidney sections was done by Debra Weih).

3.4.7 Rescue of B cell abnormalities in *mIkB α ^{tg}gld/gld* mice is not due to inhibition of NF- κ B in B cells.

The partial correction of B cell abnormalities in *mIkB α ^{tg}gld/gld* mice could be due to leaky expression of the *mIkB α* transgene in B cells, resulting in impaired induction of NF- κ B in B cells. Western blot analysis of whole cell extracts from FACS sorted splenic B cells, T cells, and different thymocyte subpopulations from wild-type as well as *mIkB α ^{tg}* mice revealed that the *mIkB α* transgene is expressed strongly and equally in CD4⁺ and CD8⁺ thymocytes and in peripheral T cells. Interestingly, the *mIkB α* transgene was also weakly expressed in splenic B cells (Fig. 3.26A). Endogenous I κ B α levels were much higher in B cells than in T cells from *mIkB α ^{tg}* mice. The weak expression of

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mIkB α however, could not inhibit the induction of NF- κ B upon LPS stimulation in B cells from *mIkB α ^{tg}gld/gld* mice compared to *gld/gld* mice (Fig. 3.26B). Moreover, proliferative responses of B cells from *mIkB α ^{tg}* mice were normal when compared to wild-type mice (Fig. 3.26C).

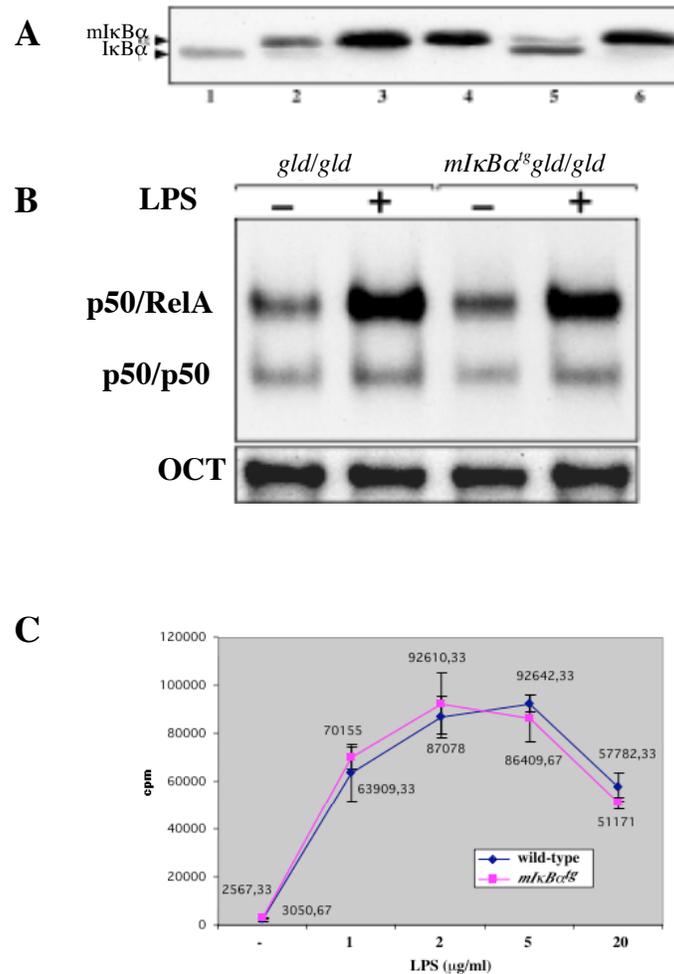


Fig. 3.26: Weak expression of *mIkB α* transgene in B cells of *mIkB α ^{tg}* mice does not effect induction of NF- κ B.

(A) Western blotting analysis using anti-I κ B α Abs to detect endogenous I κ B α and mIkB α levels in whole cell extracts. Extracts from total thymocytes of wild-type (lane 1) and *mIkB α ^{tg}* mice (lane 2); Single-positive FACS-sorted CD4⁺CD8⁺ (lane 3) and CD4⁺CD8⁻ (lane 4) thymocytes from *mIkB α ^{tg}* mice; FACS-sorted B220⁺ (lane 5) and Thy-1.2⁺ (lane 6) splenocytes from *mIkB α ^{tg}* mice. mIkB α migrates slower because of the N-terminal T7 tag. Note the weak expression of mIkB α in B cells of *mIkB α ^{tg}* mice. (B) Normal induction of NF- κ B in B cells from *mIkB α ^{tg}gld/gld* mice. Splenic B cells from *gld/gld* and *mIkB α ^{tg}gld/gld* mice were purified by magnetic separation after staining with anti-B220 magnetic beads and purity was checked by flowcytometry. Nuclear extracts prepared from purified B cells after treating with 5 μ g/ml of LPS for one hour were analyzed by EMSA for the induction of NF- κ B. The integrity of the nuclear extracts was checked by EMSA using an octamer oligonucleotide probe. (C) Normal proliferation of B cells from *mIkB α ^{tg}* mice upon activation by LPS. Splenic B cells were purified from wild-type and *mIkB α ^{tg}* mice as described for panel B and were stimulated with the indicated amounts of LPS.

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Proliferation was measured by the amount of (³H)Thymidine incorporation, which was plotted against the concentration of LPS. Mean values \pm SD of triplicates are shown.

These results indicate that despite the weak expression of the *mIkB α* transgene NF- κ B induction in B cells of *mIkB α ^{tg}gld/gld* mice is normal and that the partial correction of B cell abnormalities in these mice is not due to inhibition of NF- κ B in the B cells. Collectively, the results presented here indicate a T cell specific requirement of NF- κ B function for the development of the *gld/gld* phenotype.

4 DISCUSSION

4.1 Role of Rel/NF- κ B family members in NKT cell development.

Compared to the development of mainstream T cells, NKT cell development is less well understood in terms of cellular interactions, molecular requirements, signaling pathways and transcription factors. Although significant progress has been made in the understanding of NKT cell development by identifying different developmental intermediates (62, 63), the molecules that regulate different stages of NKT cell development are still largely unknown.

Previous reports have revealed a requirement of signaling through Fyn and Lck kinases for NKT cell development (54, 55). Fyn has been shown to be required within NKT precursors for their development, but the mechanism by which Fyn regulates NKT cell development and the transcription factors that are activated by Fyn within NKT cells is not clear. So far the only transcription factor that was shown to be required for NKT cell development is Ets-1 (56). However, the precise role of Ets-1 in terms of developmental stage, signaling pathway and cell type in which Ets-1 is required during the NKT cell development has not been addressed.

The findings from this study revealed distinct non-redundant roles for the NF- κ B family of transcription factors p50/NF- κ B1, p52/NF- κ B2, and RelB in both hematopoietic and non-hematopoietic cells for the development of thymic NKT cells. While p50/NF- κ B1 is required in hematopoietic cells, RelB is required in the non-hematopoietic compartment. Similar to RelB, p52/NF- κ B2 is also required in non-hematopoietic cells. Moreover classical I κ B α -regulated NF- κ B function is required in a cell intrinsic manner within NKT precursors for their development. Interestingly, lack of RelB in radiation-resistant stromal cells results in a more severe decrease in NKT cell numbers than blocking total NF- κ B function via the non-degradable mI κ B α inhibitor in NKT precursors. Reduced numbers of NKT cells in NF- κ B-deficient mice is not simply due to the lack of expression of the NK1.1 marker because both NKT cell specific TCR, V α 14-J α 18 expression and CD1d-tetramer⁺TCR β ⁺ cells also are reduced in NF- κ B-deficient mice.

It is unlikely that the reduced numbers of thymic NKT cells is due to increased emigration from thymus because CD4⁺ NKT cells, which are thymus-dependent (47, 48) are also

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reduced in spleen and BM of *nfkb1*^{-/-}, *relB*^{-/-}, and *mIκBα*^{tg} mice. The selective loss of these cells is accompanied by an increase in CD8⁺ NKT cells in spleen of *nfkb1*^{-/-} but not *relB*^{-/-} mice. One possible explanation for this observation is that p50/NF-κB1 prevents the expansion of CD8⁺ NKT cells. Alternatively, the lack of p50/NF-κB1 may result in the up-regulation of the NK1.1 marker on conventional CD8⁺ T cells (132). Due to the very low frequency, it is difficult to analyze CD8⁺ NKT cells and the specific increase of this subpopulation in *nfkb1*^{-/-} mice may help to study the function and origin of CD8⁺ NKT cells in more detail. In the case of *mIκBα*^{tg} mice both CD4⁺ and CD8⁺ NKT cells are equally reduced in the periphery, presumably due to the defective survival of T cells in the periphery of these mice (33, 35, 84). *nfkb2*^{-/-} mice were not included in this analysis due to the mild defect in the thymic NKT cell development in these mice. Taken together, these findings indicate a specific requirement of distinct Rel/NF-κB members in different cell types for the proper development of thymic Vα14i NKT cells, but not for all the NKT cells.

4.1.1 Lymphotoxin signaling in NKT cell development.

NKT cells are derived from CD4⁺CD8⁺ DP thymic precursors (60, see also introduction) suggesting that NKT cell development is similar to conventional T cells until the DP stage and that at this stage the interaction of CD1d with the Vα14-Jα18 TCR triggers both positive selection and NKT lineage commitment. Moreover, analysis of transgenic mice overexpressing Vα14-Jα18 TCR revealed increased numbers of thymic NKT cells (133), further strengthening the CD1d/Vα14-Jα18 TCR driven instructive model, rather than development from a pre-committed precursor. Since a CD1d/Vα14-Jα18 TCR-specific signal regulating positive selection and/or commitment to the NKT lineage has not been identified yet (41) it is important to identify key factors within signaling pathways, which specifically regulate the development of NKT cells. Fyn and Lck kinases deliver TCR proximal signals in conventional T cells (134, 135). Recent reports indicate a cell-intrinsic requirement of Fyn kinase within NKT precursors for their development, suggesting that similar to the variant TCR of conventional T cells, the invariant Vα14-Jα18 TCR of NKT cells also activates Fyn. Since TCR activation results in NF-κB activation, it is likely that activation of the Vα14-Jα18 TCR also results in the activation of classical IκBα-

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regulated NF- κ B. In line with this notion, analysis of *mIkB α ^{tg}* mice in this study revealed that (i) similar to Fyn and kinase, NF- κ B is also required in a cell-intrinsic manner within NKT precursors for their efficient maturation and that (ii) activation of V α 14-J α 18 TCR with α -GalCer results in the upregulation of LT α and LT β in an NF- κ B dependent manner. Moreover, the data presented here indicate a subunit-specific requirement of NF- κ B for inducible LT α and LT β gene expression. Whereas p50/NF- κ B1 is required for the induction of LT α , but not of LT β , both p52/NF- κ B2 and RelB are dispensable for LT α and LT β expression. Analysis of *mIkB α ^{tg}* mice revealed that, NF- κ B subunits other than RelB, p50/NF- κ B1, or p52/NF- κ B2 are required for the inducible expression of LT β . Future experiments have to show whether the lack of RelA and/or c-Rel results in impaired inducibility of LT β and/or LT α gene expression. Collectively, these data suggest that NF- κ B regulates LT expression in both mainstream T and NKT cells and that similar to conventional TCR activation of V α -14-J α 18 TCR by glycolipids presented by CD1d also induces classical I κ B α -regulated NF- κ B.

In the light of previous reports indicating a requirement of signaling by membrane bound LT for proper development of NKT cells (57, 58), it was surprising to see no severe defect in NKT cell development in the absence of LT signaling (Table 3.1). The significance of α -GalCer induced NF- κ B dependent expression of LT α and LT β is not clear since the observations from this study clearly demonstrated that both LT α and LT β and their cognate receptor LT β R are not essential for NKT cell development. Moreover it remains to be studied whether NF- κ B downstream of CD1d/V α 14-J α 18 TCR activates signaling pathways other than LT pathway, that are involved in NKT cell development.

It is not clear why LT-deficient mice generated by different groups behave differently with respect to NKT cell development. Similar contradictory observations were made for cathepsin-S^{-/-} mice. While one report suggested a role for cathepsin-S in NKT cell development, the other report suggested that it is dispensable (136, 137). One possibility is that the genetic background of these knock-out mice from different groups is different and that NKT cell development could be sensitive to the lack of LT signaling on one but not on another genetic background. Alternatively, since the LT belongs to the TNF superfamily of cytokines, a possible redundancy in their function may result in the compensation for the lack of LT by other members of this family. However analysis of

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the mice deficient for LT α , LT β , TNF, LIGHT as well as mice doubly deficient for TNF and LT α revealed no significant defect in NKT cell development, with the exception of *light*^{-/-} mice which revealed a 3-4 fold reduction in the thymic NKT cell percentage (Table 3.1). It is important to note that these mice except *light*^{-/-} mice are on a pure B6 background. Moreover only one *light*^{-/-} mouse was analyzed and this needs to be studied further. LIGHT signals through its receptor, HVEM, and also through LT β R. Analysis of mice lacking HVEM or LT β R revealed no defect in NKT cell development. Interestingly, similar to *light*^{-/-} mice, *ltbr*^{-/-}*hvem*^{-/-} double-deficient mice had 3-fold reduced thymic NKT cell percentages (Table 3.1). Thus redundancy among ligands and receptors of TNF-LT system (83) may compensate for the lack of individual family members during the development of NKT cells. Therefore, it may be necessary to study NKT cell development in mice which completely lack the possibility of signaling through the TNF-LT system. *lta*^{-/-}*tnf*^{-/-}*light*^{-/-} triple-knock-out mice are being generated for this purpose and will be analyzed in future.

The only observed NKT cell defect in the absence of LT β R signaling, is the impaired migration of NKT cells to the liver (see Fig. 3.16). It has previously been shown that migration of thymic NKT cells to the liver depends on the LFA-1 expression by NK cells (125). Since *ltbr*^{-/-} mice have a severe defect in NK cell development (124), impaired migration of NKT cells to the liver in these mice could simply be due to the lack of NK cells. Interestingly *relB*^{-/-} mice also have significantly reduced numbers of liver NK cells, although their development appears to be not significantly effected (data not shown). Therefore it remains to be studied whether signaling through LT β R and RelB may regulate the migration of NK cells to the liver.

4.1.2 NIK-mediated regulation of RelB: Implications in NKT cell development.

Functional NIK in non-hematopoietic cells is essential for NKT cell development (72). This is consistent with the lack of constitutive RelB DNA-binding in thymus of *aly/aly* mice and the stromal requirement of RelB for NKT cell development as shown here. Both *relB*^{-/-} and *aly/aly* mice show similar structural disorganization of the thymus in terms of highly reduced number of medullary epithelial cells and disorganized cortico-medullary junctions (77-79). The marked defect in RelB DNA-binding in *aly/aly* thymus offers an

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explanation for the similarity of this phenotype. In contrast to *aly/aly* mice, thymic DNA-binding of RelB is reduced but not severely impaired in *ltbr*^{-/-} and normal in *tnfr1*^{-/-} mice, indicating that another receptor associated with NIK may be involved in RelB activation, NKT cell development, and the establishment of proper thymic architecture. Constitutive RelB DNA-binding in spleen also is severely impaired in *aly/aly* mice, only mildly reduced in *ltbr*^{-/-} and normal in *tnfr1*^{-/-} mice. Interestingly, in contrast to thymus, disturbed splenic architecture observed in *aly/aly*, *ltbr*^{-/-} and *relB*^{-/-} mice is strikingly similar (19). Moreover, *ltbr*^{-/-}, *aly/aly* and *relB*^{-/-} mice lack PP and LN (19), suggesting that regulation of RelB in LTβR-expressing cells via NIK is important to maintain proper splenic architecture as well as for the development of PP and LN. Recently, NIK was shown to be required for the processing of the NF-κB2 p100 precursor to p52 (122). Since the C-terminal domain of p100 was shown to specifically inhibit RelB function (121), lack of RelB DNA-binding in the absence of functional NIK most likely is due to defective processing of the inhibitory p100 precursor. Similar to RelB, p52/NF-κB2 is also required in stromal cells for normal NKT cell development. This observation correlates with a stromal requirement of both RelB and p52/NF-κB2 for proper spleen (138, 139) and PP development (123). Thus p52/RelB rather than p50/RelB heterodimers in the thymic stroma are involved in the regulation of NKT cell development, although one cannot rule out that p50/RelB complexes also contribute. Collectively, these data indicate that the alternative NIK/p100/RelB pathway in thymic stroma downstream of a receptor distinct from LTβR regulates NKT cell development and establishment of proper thymic architecture.

4.1.3 Stage Specific Requirement of distinct NF-κB complexes during NKT cell development.

Recent reports indicate that CD1d-mediated positive selection of CD4⁺CD8⁺ DP NKT precursors results in the generation of NK1.1⁻ precursors that are committed to the NKT lineage. They proliferate and differentiate through CD44^{lo}NK1.1⁻ and CD44^{hi}NK1.1⁻ stages into mature CD44^{hi}CD4⁺/DN NK1.1⁺ NKT cells (60, 62, 63). There was no significant reduction in the frequency of CD1d-tetramer⁺ DP^{hi} NKT precursors in 12-day-old *relB*^{-/-} and *mIkBa*^{t8} mice (data not shown), indicating that NF-κB is not required for the rearrangement of the Vα14-Jα18 TCR. Since expression of CD1d is normal on

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thymocytes of *relB*^{-/-} and *mIkbα*^{tg} mice, it is unlikely that initial positive selection is impaired in these mice. The analysis of young mice revealed, however, that RelB function in the thymic stromal cells is required for the generation of normal numbers of CD1d-tetramer⁺ NK1.1⁻ precursors, which are the immediate products of positive selection of DP NKT precursors by CD1d. This indicates that RelB in stromal cells regulates maturation/expansion of positively selected NKT precursors. Alternatively, positive selection initiated by CD1d expressed on DP thymocytes may result in the interaction of DP NKT precursors with thymic epithelial cells to trigger signaling through NIK and RelB. NIK-mediated activation of RelB in epithelial cells, may finally complete the positive selection event. This model may also explain why the immediate products of positive selection (NK1.1⁻ NKT precursors) are severely reduced in *relB*^{-/-} mice. In addition, *relB*^{-/-} mice have a defect in the progression from the CD44^{lo}NK-1.1⁻ to the CD44^{hi}NK-1.1⁻ stage, while further differentiation into mature CD44^{hi}NK-1.1⁺ NKT cells is not impaired (see also Fig. 3.10). Interestingly, although thymic expression of many genes that are important for NKT cell development is not affected in *relB*^{-/-} and *mIkbα*^{tg} mice, a reproducible reduction of IL-15 mRNA levels was observed in thymus from *relB*^{-/-} mice. This finding is consistent with a stromal function of RelB since IL-15 produced by thymic stromal cells has been shown to be essential for early steps during NKT cell development and homeostasis (140, 141). Although an NF-κB binding site has been reported in the IL-15 promoter (142), it remains to be shown whether RelB directly or indirectly regulates expression of IL-15. Thus, reduced IL-15 expression in *relB*^{-/-} thymus may contribute to the reduced NKT cell numbers in RelB-deficient mice. Together, these data indicate an essential role of RelB in thymic stromal cells at an early step of Vα14i NKT cell development.

Analysis of *mIkbα*^{tg} mice, on the other hand, revealed that classical IκBα-regulated NF-κB function within NKT precursors is dispensable for the generation of early tetramer⁺ NK1.1⁻ cells, whereas it is required at a later step of NKT cell development. In particular, the progression from CD44^{hi}NK1.1⁻ to the mature CD44^{hi}NK1.1⁺ stage is impaired, whereas the earlier CD44^{lo}NK1.1⁻ to CD44^{hi}NK1.1⁻ transition is only mildly affected (Fig. 4.1). Previous reports have shown that signaling through the common-γ chain is required for the transition from NK1.1⁻ to NK1.1⁺ stage of NKT cell development (49). Therefore, it remains to be studied whether signaling through common-γ chain depends on NF-κ

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within NKT precursors, to regulate the NK1.1⁻ to NK1.1⁺ transition of NKT cells. However unlike *mIkBα^{tg}* mice, there was no defect in the number of NKT cells in mice deficient for common-γ chain. This indicates that in addition to the transition from NK1.1⁻ to NK1.1⁺ stage NF-κB is required to maintain the normal numbers of NKT cells. One possible mechanism by which NF-κB within NKT precursors regulates the maintenance of normal numbers of NKT cells is by controlling the proliferative expansion of NK1.1⁻ precursors (see Fig. 4.1).

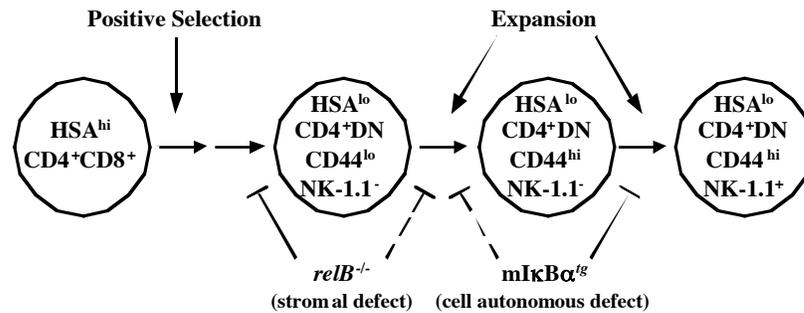


Fig. 4.1: Model of NF-κB-regulated Vα14i NKT cell development in thymus.

Lack of RelB in stromal cells results in impaired generation of CD44^{lo}NK1.1⁻ precursors which are the immediate products of positive selection. Lack of RelB in stromal cells also results in defective transition from CD44^{lo}NK1.1⁻ to CD44^{hi}NK1.1⁻ stage. Blocking NF-κB in NKT precursors predominantly blocks the NK1.1⁻ to NK1.1⁺ transition and to a lesser extent blocks CD44^{lo}NK1.1⁻ to CD44^{hi}NK1.1⁻ transition. NF-κB in NKT precursors is most likely required for the proliferative expansion of these cells, because initial generation of CD44^{lo}NK1.1⁻ precursors is normal *mIkBα^{tg}* mice.

Taken together, these results demonstrate that distinct NF-κB subunits regulate different stages of NKT cell development, with an important role of the classical IκBα-regulated NF-κB pathway within NKT precursor cells, while the alternative NIK/NF-κB2/RelB pathway operates in the thymic stroma. The identification of stromal cell-derived factor(s) regulated by RelB and hematopoietic cell-derived factor(s) regulated by classical NF-κB would help to better define the role of NF-κB in the regulation of different stages of NKT cell development. It is also important to identify the receptor(s) distinct from LTβR, which is required for NKT cell development by activating the NIK/NF-κB2/RelB alternative pathway.

4.2 Regulation of NF- κ B activation by LT β R and TNFR.

Within the thymus and spleen NIK and to a lesser extent LT β R appear to be regulating the DNA-binding of RelB complexes. However, it is not clear whether these effects are direct or indirect. It has been previously shown that signaling through LT β R needs functional NIK (74, 75) whereas conflicting data exists for the role of NIK downstream of TNFR (74, 75, 120). Since both NIK and RelB are required in thymic stromal cells for NKT cell development, it is important to understand whether in addition to constitutive RelB DNA-binding, signal-induced RelB DNA-binding also is regulated by NIK. The observation from this study that LT β R is not essential for NKT cell development obscures the certainty as to downstream of which receptor NIK and RelB regulate NKT cell development. However, keeping the possible redundancy among the TNFR family members, which may compensate for the lack of LT β R during the NKT cell development, it is important to understand whether activation of TNFR family members requires NIK to induce NF- κ B in stromal cells. In addition to its possible role in NKT cell development, LT signaling through NIK has been shown to be essential for development and architectural maintenance of lymphoid organs (19, 81). Signaling through TNFR also has been shown to play critical role in the maintenance of proper architecture but not for development of secondary lymphoid organs (19). Similar to *aly/aly* mice and mice with defective LT signaling, mice deficient for NF- κ B family members RelB, RelA and NF- κ B2 have defective lymphoid organ development (19). These observations indicate that signaling through TNF-LT system may regulate NF- κ B downstream of NIK.

To gain a better insight into the regulation of NF- κ B by NIK in stromal cells, signaling through LT β R and TNFR was studied in primary MEFs as a model system. The results obtained in this study demonstrate that TNF almost exclusively induces RelA heterodimers independently of NIK. Interestingly, signaling through LT β R results in NIK-dependent and independent induction of qualitatively and quantitatively different complexes of NF- κ B with distinct kinetics. Whereas DNA-binding of RelA heterodimers is induced independently of NIK, induction of DNA-binding of RelB heterodimers by LT β R is strictly dependent on NIK. Moreover, induction of RelB DNA-binding by LT β R occurs late after the initial induction of RelA. Since I κ B α degradation appears to be normal downstream of LT β R in the absence of NIK (75), the defective

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induction of RelB complexes by LT β R in *aly/aly* MEFs must be due to another inhibitor or to regulation of RelB by a different mechanism. Previous reports have shown that the c-terminus of p100 molecule can inhibit RelB function (121) and NIK has been shown to be required for the phosphorylation and proteolytic processing of inhibitory p100 to p52 (122). However, no direct evidence for the NIK-mediated processing of p100 under physiological conditions exists. This study demonstrates that activation of LT β R results in the processing of inhibitory p100 to p52 in a NIK-dependent manner with kinetics similar to the induction of DNA-binding of RelB. In addition to NIK, IKK α also has been shown to be required for both signal-dependent processing of p100 to p52 and RelB DNA-binding (123, 143). Moreover, NIK has been shown to phosphorylate and activate IKK α (144). Thus, similar to classical I κ B α -mediated regulation of NF- κ B complexes, NIK/IKK α -dependent processing of inhibitory p100 to p52 followed by nuclear translocation and DNA-binding of RelB complexes defines an alternative NF- κ B activation pathway (**Fig. 4.2**). It is important to note that in lymphoid organs such as thymus and spleen, absence of LT β R does not result in complete lack of constitutive RelB DNA-binding, whereas lack of functional NIK results in severely impaired constitutive RelB DNA-binding. This could be due to the requirement of NIK downstream of other receptors distinct from LT β R. Indeed, in B cells NIK has been shown to be required for the activation of the so-called alternative pathway and RelB induction downstream of CD40 and BAFF-R (145, 146), suggesting that the NIK-regulated alternative pathway is perhaps a global regulator of RelB activity in many cell types. In contrast to activation of LT β R, activation of TNFR does not result in the processing of p100 and results in the accumulation of this inhibitor (123). This may probably explain why TNF signaling does not result in a significant DNA-binding of RelB.

The existence of classical I κ B α -regulated and alternative p100-regulated pathways, makes NF- κ B signaling quite complex. Both I κ B α and p100 consist of ankyrin repeats which binds to the RHD of NF- κ B. Therefore I κ B α may also be able to bind and inhibit RelB similar to other members of the Rel/NF- κ B family. It is therefore possible that inhibition by p100 is not the sole mechanism by which RelB nuclear translocation is regulated. If the alternative pathway of NF- κ B activation regulates RelB nuclear translocation via p100 processing, in the absence of p100 processing, RelB should be

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exclusively cytoplasmic. This notion is true for the inducible nuclear translocation of RelB downstream of LT β R (see Fig. 3.15). However, although TNF signaling leads to the accumulation rather than processing of p100, RelB translocates to the nucleus downstream of TNFR. Moreover inducible nuclear translocation of RelB downstream of TNFR occurs even in the absence of NIK. Thus these results clearly demonstrate that (i) signaling through LT β R regulates RelB DNA-binding via NIK/p100 mediated alternate pathway and (ii) nuclear translocation of RelB is regulated by the alternate pathway in a signal-dependent manner and that RelB nuclear translocation is not exclusively dependent on p100 processing and/or NIK. It is possible that two independent pools of RelB exist, one that is predominantly bound by p100 and the other by I κ B α or other unknown inhibitor. This has to be tested however by further experimentation.

Collectively, these observations provide insight into the mechanism of NF- κ B activation by a previously unknown alternate pathway and its importance in the development of NKT cells, thymic medullary epithelial cells, and secondary lymphoid organs.

4.3 I κ B α -mediated inhibition of RelB DNA-binding and function of RelB in T cells.

In order to address the question whether I κ B α in addition to p100 molecule can inhibit the DNA-binding of RelB, thymocytes were chosen as a model system since I κ B α is the predominant regulator of NF- κ B activation in this cell type. Transgenic expression of a mutant non-phosphorylatable and non-degradable form of I κ B α (mI κ B α) has been shown to inhibit almost all NF- κ B DNA-binding in thymocytes (see Fig. 3.2). To test whether mI κ B α can also inhibit DNA-binding of RelB heterodimers, *mI κ B α '^{tg}* and *relB*^{tg} mice were crossed to generate *relB*^{tg}*mI κ B α '^{tg}* double-transgenic mice that co-express high levels of both mI κ B α and RelB in thymocytes. The data obtained from the analysis of these double-transgenic thymocytes revealed that I κ B α indeed can significantly inhibit DNA-binding of RelB. Previous reports have suggested that p52/RelB complexes are less susceptible for inhibition by I κ B α (147). The results presented here however, demonstrate that I κ B α can inhibit DNA-binding of p52/RelB complexes as well (see Fig. 3.17). Thus, these results demonstrate that regulation of RelB nuclear translocation and DNA-binding is a quite complex process and is mediated by both classical I κ B α and alternative p100 pathways in a signal- and cell-type dependent manner (see **Fig. 4.2**).

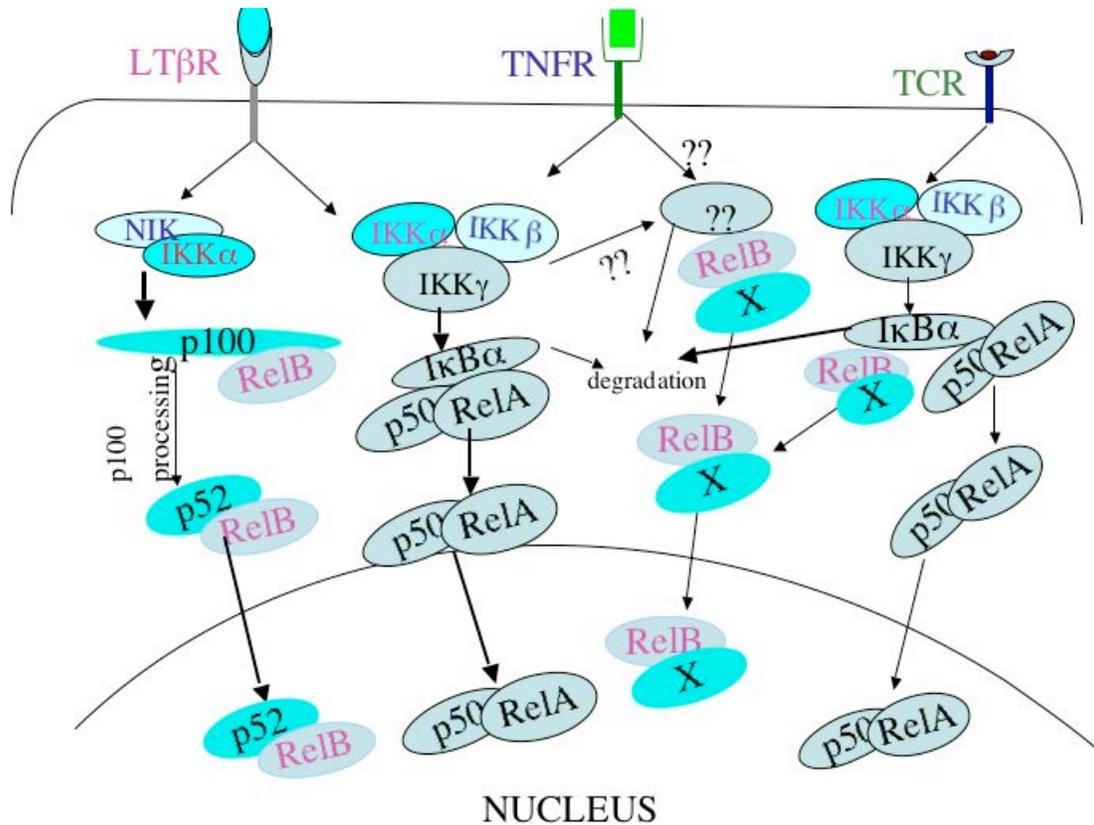


Fig. 4.2: Model for the regulation of nuclear translocation of RelB and classical p50/RelA complexes: Regulation of RelB by both classical and alternative pathways.

Triggering of LT β R results in the initial activation of classical I κ B α -regulated pathway resulting in the nuclear translocation of p50/RelA complexes. This process is independent of NIK and is under the control of classical IKK complex consisting of IKK α , β and γ . With delayed kinetics LT β R triggering also results in the strong induction of nuclear translocation of RelB complexes via an alternative pathway which is dependent on proteolytic processing of p100. This process is regulated by an alternative kinase complex consisting of NIK and IKK α which phosphorylates p100 resulting in its processing. Activation of TNFR on the other hand predominantly regulates the classical I κ B α -regulated pathway resulting in the nuclear translocation of p50/RelA complexes and does not depend on NIK. TNFR activation also results in the nuclear translocation of RelB independently of p100 processing and NIK (see text for details) presumably via the classical pathway. In addition TNFR activation causes cytoplasmic accumulation of RelB complexes (Yilmaz et al., 2003. also see text for details). Activation of TCR results in the nuclear translocation of both RelA and RelB complexes via the classical I κ B α -regulated pathway. X can be any heterodimeric partner of RelB.

Inhibition of RelB complexes by mI κ B α was however incomplete in *relB^{tg}mI κ B α ^{tg}* double-transgenic mice, which resulted in the DNA-binding of RelB complexes alone in the thymocytes from these mice. This allowed to study the precise role of RelB within T cells.

All the NF- κ B family members including RelB are expressed in T cells and it has been unclear whether RelB has any function within T cells, since *relB^{-/-}* T cells develop

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normally. Lack of individual NF- κ B family members does not effect the T cell development. However, complete inhibition of NF- κ B in T cells results in the highly reduced numbers of peripheral T cells with defective survival and proliferative responses (33, 35, 84), indicating a significant redundancy among NF- κ B family members in regulating the development and function of mainstream T cells. NF- κ B family members also have distinct functions in the regulation of some of the genes critical for the proliferation of T cell. For instance inducible expression of IL-2 gene in peripheral T cells has been shown to be exclusively dependent on c-rel (18, 25) and this study shows that in thymocytes induction of LT α but not LT β is exclusively dependent on p50/NF- κ B1.

It is of importance to understand the extent of such redundancy and distinctness in the function NF- κ B family members, which would provide further insight into their role in T cell development and function. Although there is some insight into the distinct and redundant functions among c-rel and RelA (21, 22), it is not clear whether RelB has any function in T cells. Studies based on the *relB*^{-/-} T cells, obscures the certainty of RelB functions due to redundancy and compensation by other NF- κ B members. It is therefore necessary to completely inhibit all the NF- κ B complexes except ReB complexes to study its precise role in T cells. *relB*^{tg}*mIkB α* ^{tg} double-transgenic mice provided such a model system to study the functions that RelB complexes alone can do in T cells, since the NF- κ B complexes other than RelB were almost completely inhibited by *mIkB α* .

RT-PCR analysis revealed that inducible expression of genes encoding NF- κ B2 but not NF- κ B1, LT α or LT β can be driven by RelB complexes alone (see **Fig. 3.18**). Moreover, expression of NF- κ B2 is normal in the absence of RelB suggesting that RelB shares significant redundancy in the regulation of transcription of *nfkb2* gene and that this redundancy in the transcription activation function of RelB is gene specific. Such specificity can be due to two distinct mechanisms; (i) RelB can bind to the κ B elements present in the promoters of genes encoding NF- κ B2, but not NF- κ B1, LT α and LT β (ii) RelB can bind to the κ B elements in the promoters of genes encoding NF- κ B1, LT α and LT β , but cannot interact with other transcription factors and/or co-activators to initiate the transcription of these genes. These issues have to be studied by future experiments.

Overexpression of RelB in *mIkB α* ^{tg} mice rescued the numbers of peripheral CD8⁺ T cells but not thymic NKT cell defect (see **Fig. 3.19**). The reason for the reduced peripheral T

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cells in *mIκBα^{tg}* mice appears to be defective survival as well as impaired proliferation. Therefore, it remains to be studied whether RelB complexes in *relB^{tg}mIκBα^{tg}* double-transgenic mice rescue the survival and/or proliferative response of T cells. Since peripheral CD8⁺ T cell percentages are normal in *relB^{-/-}* mice, these observations collectively indicate that RelB has redundant role in the expression of *nfkb2* gene, maintenance of normal numbers of CD8⁺ T cells in the periphery.

Interestingly, overexpression of RelB in thymocytes of *relB^{tg}* mice impaired the NKT cell development, resulting in a 2 to 3-fold reduced percentage of thymic NKT cells. Moreover, thymocytes from *relB^{tg}* mice revealed reduced DNA binding of p50/RelA complexes upon PMA + PHA treatment (Ingmar sholl and also see Fig. 3.17). It is therefore possible that similar to *mIκBα^{tg}* mice, the reduction in the NKT cell population in *relB^{tg}* mice is also due to a defective transition from the NK1.1⁻ to NK1.1⁺ stage. Collectively, these results provide insight into the function of RelB complexes in T cells; (i) functional redundancy among RelB and other Rel/NF-κB family members in the maintenance of normal numbers of peripheral T cells and (ii) a previously unknown, inhibitory role of RelB in NKT cell development. Infact RelB has been shown to inhibit the function of other NF-κB members by forming transcriptionally inactive complexes (148) but the physiological significance of such inhibition was not clear. The impaired development of NKT cells in *relB^{tg}* mice could be due to inhibition of other NF-κB complexes by RelB, which perhaps provides insight into physiological significance of the inhibitory role of RelB.

4.4 Inhibition of NF-κB in T cells protects from developing generalized lymphoproliferative disorder.

In this study, transgenic *gld/gld* mice that express a non-degradable form of IκBα under the control of the mouse *lck* promoter and the human CD2 3'-LCR have been analyzed. EMSA results show that expression of the *mIκBα* transgene blocks activation of NF-κB in both thymocytes and peripheral T cells. Very low levels of mIκBα expression were detected in splenic B cells. However, NF-κB activation in B cells of *mIκBα^{tg}* mice was not impaired most likely due to still high levels of endogenous IκBα. Moreover, stimulation of B cells by LPS did not reveal significant differences in proliferation between transgenic and non-transgenic mice (see Fig. 3.26).

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Inhibition of NF- κ B in T cells prevented the accumulation of Thy-1⁺B220⁺CD4⁻CD8⁻ abnormal T cells in both spleen and LNs eliminating splenomegaly and lymphadenopathy. The reason for the rescue of the T cell abnormalities in *mIkB α ^{tg}gld/gld* mice could be the result of NF- κ B inhibition either in thymus or in peripheral T cells. Whereas no significant differences in thymocyte subpopulations were detected between wild-type and *mIkB α ^{tg}gld/gld* mice, an overall 2-fold reduction in the percentage of T lymphocytes in spleen and LNs was observed. Although CD8⁺ T cells were more affected than CD4⁺ T cells, both subpopulations were clearly present in the periphery of *mIkB α ^{tg}gld/gld* mice. This suggests that the inhibition of NF- κ B in both CD8⁺ and CD4⁺ T cells, corrects the T cell abnormalities observed in *gld/gld* mice. This is also supported by earlier reports showing that both CD4⁺ and CD8⁺ T cells are required for the accumulation of DN T cells and development of the lymphadenopathy in *gld/gld* and *lpr/lpr* mice (97, 98). However, the exact source for the Thy-1⁺B220⁺CD4⁻CD8⁻ abnormal T cells is still not clear.

Peripheral T cells from *mIkB α ^{tg}gld/gld* mice showed severely reduced proliferation and \approx 3-fold increased apoptosis upon CD3/CD28 stimulation compared to non-transgenic *gld/gld* mice, indicating that both mechanisms may contribute to the correction of the T cell abnormalities in *mIkB α ^{tg}gld/gld* mice. Other reports also showed that the inhibition of NF- κ B renders T lymphocytes more susceptible to apoptosis (33, 35). In particular, lack of NF- κ B sensitizes peripheral T cells to TNF, TRAIL and TCR-mediated death of T cells (33, 35, 84, 149). It is important to note that lack of NF- κ B increases the sensitivity of T cells predominantly to death pathways other than Fas pathway. Increased apoptosis in *mIkB α* -transgenic T cells could for instance, result from decreased expression of anti-apoptotic NF- κ B target genes, such as cIAP, TRAF, Bfl1, Bcl-2, Bcl-XL or A20 (104). Also, it has been shown that *gld/gld* mice have increased levels of serum TNF (150) and *gld* T cells produce high amounts of TNF upon activation (151). The *mIkB α* transgene had no effect on TNF production by activated T cells despite κ B binding sites in the TNF promoter (data not shown). Therefore, it is possible that activated T cells from *mIkB α ^{tg}gld/gld* mice become more susceptible to TNF-mediated apoptosis. This is supported by the finding that TNF accounts for all TCR-induced death in *lpr* or *gld* T cells (152).

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Together, the results obtained from this study suggest that inhibition of NF- κ B in T cells compensates for the loss of FasL/Fas-mediated apoptosis due to the activation of other cell death pathways upon TCR triggering, thereby preventing the accumulation of Thy-1⁺B220⁺CD4⁻CD8⁻ abnormal T cells and correction of T cell abnormalities in *gld/gld* mice.

Serum Ig and autoantibody levels were reduced in *mIkB α ^{tg}gld/gld* compared to non-transgenic *gld/gld* mice but still higher than in wild-type controls. These findings indicate that NF- κ B function in T cells is required for the complete development of the B cell abnormalities. The constitutive expression of the nuclear orphan receptor Nur77 in transgenic T cells also resulted in the complete elimination of the *gld* T cell abnormalities and a partial reduction of serum Ig and anti-dsDNA autoantibody levels (128). It is still unclear whether eliminating Thy-1⁺B220⁺CD4⁻CD8⁻ abnormal T cells and associated T cell abnormalities is sufficient to correct the B cell-mediated autoimmunity in *gld/gld* and *lpr/lpr* mice. Several reports indicate that the correction of the T cell phenotype does not rescue the B cell abnormalities in *gld/gld* and *lpr/lpr* mice and that the B cells of *lpr/lpr* mice have an intrinsic defect (97, 129). Conversely, it has been shown that abnormal DN T cells are required for the development of autoimmunity in *gld/gld* and *lpr/lpr* mice and that the correction of the T cell phenotype eliminates the B cell abnormalities (153, 154).

The findings from this study that elimination of Thy-1⁺B220⁺CD4⁻CD8⁻ abnormal T cells, lymphadenopathy and splenomegaly in *mIkB α ^{tg}gld/gld* mice does not completely correct the autoimmune disease is in agreement with an intrinsic B cell defect, which results from impaired elimination of autoreactive B cells via FasL/Fas-mediated apoptosis (90, 155, 156). The partial correction of the B cell abnormalities in *mIkB α ^{tg}gld/gld* mice could be attributed to some extent to the reduced numbers of peripheral T cells, limiting T cell/B cell contacts required for clonal expansion of Ag-specific B cells. In addition, the reduced proliferation and increased apoptosis of CD4⁺ T cells from *mIkB α ^{tg}gld/gld* mice, indicates that T cell help, which is required for full development of the B cell phenotype, is severely impaired in *mIkB α ^{tg}gld/gld* mice. This notion is supported by the markedly reduced production of IL-4 and IFN- γ by *mIkB α ^{tg}* T cells after CD3/CD28 stimulation (33, 35 and data not shown).

Collectively, this study provides evidence that inhibition of NF- κ B within T cells protects *gld* and possibly also *lpr*, mice from the accumulation of Thy-1⁺B220⁺CD4⁻CD8⁻

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abnormal T cells and thereby eliminates splenomegaly, lymphadenopathy and partially rescues B cell abnormalities.

4.5 OUTLOOK.

Overall this study provides insight into the role of individual NF- κ B members in the development/function of distinct T cell subtypes including, NKT, mainstream T and the abnormal T cells in *gld/gld* mice. Moreover, this study revealed an alternative pathway of NF- κ B activation involving NIK/p100/RelB in vivo in thymus and spleen, as well as in vitro downstream of LT β R. Requirement of distinct NF- κ B complexes regulated by classical I κ B α -regulated pathway within NKT precursors and the alternate NIK/p100/RelB pathway in thymic stromal cells during different stages of NKT cell development has been revealed. The downstream target genes of both classical and alternate pathways that are involved in NKT cell development have to be identified by future experiments to gain further insight into the biology of NKT cells.

This study further shows that while activation of p50/RelA complexes is independent of NIK, nuclear translocation of RelB is regulated by both classical and alternate pathways in a signal and cell type-dependent manner. Moreover RelB complexes are sufficient to maintain the normal numbers of peripheral CD8⁺ T cells, revealing redundancy in the function of RelB and other NF- κ B family members. Such redundancy in the function of Rel/NF- κ B family members and activation of distinct NF- κ B complexes by classical and alternate pathways perhaps fine tunes the NF- κ B response to regulate the development and function of distinct T cell subtypes.

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