MOLECULAR BIOLOGY IN

Hsiou-Chi Liou

NF-κB/Rel Tra
Factor Family

MOLECULAR BIOLOGY
INTELLIGENCE
UNIT

NF-κB/Rel Transcription Factor Family

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Dedication

This book is dedicated to Dr. David Baltimore
for his pioneer work in the NF-κB field
and leadership in several frontiers of biomedical sciences.

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FOREWORD

The First Few Years of NF-κB

When Hsiou-Chi Liou first asked me to provide a personal view of the "early years of NF-κB research" I hesitated, in large part because retrospection seemed to be of little value at a time when we should be looking forward, not backward. However, after mulling over the idea for some time, I decided to do it because the evolution of NF-κB, from the quest to understand B cell-specific transcription of immunoglobulin (Ig) κ genes to its present industry-sized scope, is a fascinating example of biological multi-tasking by a limited gene family. In this context, I felt that the early development of the project may be of some interest. What follows, thus, is a personal view of the lead-up to the identification of NF-κB and the years immediately thereafter.

Towards the end of my Ph.D. in organic chemistry I became interested in biology and approached David Baltimore for a post-doctoral position. In the early 80s the Baltimore lab had three major research directions: molecular immunology, retrovirology and the biology of poliovirus. My closest encounter with the life sciences prior to this had been graduate courses in biophysical chemistry and biochemistry, which had not prepared me sufficiently to favor one of these topics over the others. Therefore, to apply for post-doctoral fellowships I wrote three short blurbs in each of these areas for David's consideration. He picked one on the control of κ gene recombination by DNA methylation for further elaboration into a proposal. The choice landed me in the immunology sub-group and, when I reached M.I.T. a year later, I started as planned with chromatin structural assays and DNA methylation studies in Abelson virus transformed cell lines. One of the earliest observations we made was that the κ intron enhancer coincided with a DNase 1 hypersensitive site in cell lines that actively recombine Ig κ genes. The altered chromatin structure reflected in the hypersensitive site, with the underlying sequence of the enhancer as its apparent cause, piqued my interest as a chemist. Thus, I initiated studies to "reproduce enhancer function in vitro." David, a biochemist at heart, was enthusiastically supportive.

Phil Sharp's laboratory was next door to ours. Phil, also a Ph.D. in chemistry, was enthusiastic about another chemist's conversion to molecular biology and lent me his copy of *Bacteria and Their Viruses* as a rite of initiation. He put me in touch with Andy Fire when I spoke to him about studying enhancer-dependent transcription in vitro. With Andy's expertise and cooperation, we developed the first in vitro transcription extracts from B lymphocyte cell lines and used them to study the Vk promoter and the κ enhancer. Though the effects of promoter sequences were evident in transcription assays, we did not observe κ enhancer-dependent elevation of transcription in vitro. Months of disappointing results provided the

time and the incentive to think, and rethink, the strategy. While these early studies were in progress I had spoken to the two people at M.I.T who had experience with enhancers. Alex Varshavsky was the first to describe that the SV40 enhancer was located in a nucleosome-free region of the mini-chromosome, and Alex Rich had ideas about enhancers and Z-DNA. We even used anti-Z-DNA antibodies obtained from Rich in some experiments. These conversations forcefully emphasized how little was known about these regulatory sequences and indicated that going for a functional readout in vitro may have been somewhat premature. In retrospect, good examples of promoter/enhancer communication over significant distances remain elusive to this day. Therefore, we decided to step back from the functional approach and began to "simply look" for components of the enhancer.

The electrophoretic mobility shift assay (EMSA) had been developed to study the interactions of purified bacterial DNA binding proteins with their cognate sites. This was very different from the objective we had in mind, which was to identify new DNA binding proteins; however, its intrinsic simplicity strongly recommended it as a method to work with. As a source of putative enhancer binding proteins I settled on a new transcription-competent nuclear extract procedure that had recently been developed in Bob Roeder's laboratory, and set about putting the two together using immunoglobulin-related regulatory sequences as targets. Shortly into this new line of experimentation I was joined by Harinder Singh, then newly arrived in Phil Sharp's laboratory.

Together we made two modifications that proved to be invaluable in making EMSA a widely-used technique in the eukaryotic gene regulation community. First, the use of small, functionally inactive fragments of enhancer DNA allowed greater sensitivity and significantly increased the signal-to-noise ratio in binding gels. Though at the time it was difficult to decide whether a functionally intact enhancer sequence was essential to recruit the necessary proteins to DNA, the advantages noted above encouraged us to continue with small fragments. Indeed, studies with the origin recognition complex since then have shown that intact sequences are sometimes necessary for DNA binding. Second, the use of synthetic nucleic acid polymers with low sequence complexity also increased the sensitivity of the assay. Finally, with additional modifications to co-opt existing DNase 1 footprinting and methylation interference assays for use with EMSA, we generated the first data sets using Vk promoter, κ enhancer and m heavy chain gene enhancer sequences. Amongst the proteins identified in this screen were octamer binding proteins which have since been shown to be important for stem cell biology, immunoglobulin expression and cell-cycle regulated genes, basic helix-loop-helix (bHLH) proteins that play important roles in lymphocyte development, leucine zipper-containing bHLH proteins that are related to c-myc and NF-κB.

NF-κB was identified as a mobility shifted band using the k3 fragment of the κ intron enhancer, and interference assays revealed an 11 base pair binding site towards one end of the fragment; we referred to it as the κB site. The first version of our manuscript continued to refer to the protein(s) that generated the EMSA complex as k3 binding protein(s). Upon reading that draft David suggested we give it a more user-friendly name, which led us to call the protein(s) NF-κB (for nuclear factor that binds the κB site). Unlike all other proteins identified in the initial screen, NF-κB DNA binding activity was detected only in extracts from cells that expressed immunoglobulin light chain genes, including mature B cell lines of human and murine origin as well as plasmacytomas, but not pre-B cell lines that did not express Ig κ or λ. The close correspondence between DNA binding and light chain gene expression suggested that NF-κB was an important κ gene regulatory factor.

To further strengthen the idea, we used the pre-B cell line, 70Z, that had been shown to activate κ gene transcription in response to lipopolysaccharide (LPS) or phorbol esters (PMA). Moreover, κ transcription in response to these agents occurred in the absence of new protein synthesis, providing an especially stringent constraint to test the validity of the hypothesis. We showed that NF-κB induction by these agents indeed occurred in the presence of translation inhibitors cycloheximide and anisomycin. These crucial experiments substantiated the link of NF-κB expression to κ gene transcription and provided the first evidence for a post-translational mechanism of NF-κB activation. Soon thereafter Mike Lenardo and Jackie Pierce mutated the κB site of the κ enhancer and showed that it was essential for enhancer activity.

NF-κB as an important κ enhancer activating protein may have been the end of the story, but for two other observations. First, control cells treated with translation inhibitors in the absence of LPS, or PMA, showed low but consistent activation of NF-κB DNA binding. This led us to postulate the existence of a "short-lived inhibitor" that suppressed NF-κB DNA binding in unactivated cells. Second, the induction of NF-κB in pre-B cells by a general activator such as PMA prompted us to question whether NF-κB activation and function might extend to cell types that had no connection to immunoglobulin expression. In the original studies we found that NF-κB DNA binding was induced in HeLa (epithelial) cells and Jurkat (T lymphocyte) cells by PMA in the absence of protein synthesis. These observations provided the first glimpse that NF-κB may be more than a B lineage-specific κ gene activating protein. Identification of additional tissue-unrestricted NF-κB activating signals soon followed, the earliest ones being double-stranded RNA, TNFα and serum.

A series of biochemical analyses, primarily in David Baltimore's laboratory, fleshed out the characters implicated from this early work. Patrick

Baeuerle showed that the nonDNA-binding form of NF-κB resided in the cell cytosol and could be converted to the DNA binding form in elegant experiments that used a combination of strong anionic detergent followed by its removal in a mixed micelle. The inhibitory activity, which was biochemically separable from NF-κB, was called IκB, and ultimately proved to be the predicted "short-lived inhibitor." Baeuerle and Baltimore also showed that NF-κB is a heterodimer composed of 50 and 65 kD subunits, of which the latter was essential for IκB-mediated inhibition. The detergent release assay greatly aided subsequent purification of NF-κB, first by allowing a simple scan of a variety of nonlymphoid tissue for the most abundant source of NF-κB, and also by providing the means to reveal DNA binding activity for capture on a sequence-specific DNA affinity matrix.

Sankar Ghosh used this strategy to purify NF-κB from rabbit lung extracts. Peptide sequencing and molecular cloning of the 50 kD component revealed the relationship of NF-κB to the avian oncogene *v-rel*, as well as the need for proteolytic processing to convert the precursor p105 to a DNA binding form. Sankar's purification scheme also yielded p65 and IκBα polypeptides, the former of which was cloned by Gary Nolan to reveal another *v-rel* homologous gene. The NF-κB/*v-rel* connection, manifest in a 300 amino acid DNA binding Rel homology domain (RHD), brought those working on *v-rel*, and its cellular counterpart *c-Rel*, into the rapidly expanding NF-κB community. One of the early positive outcomes of this fusion was the identification of v-Rel-associated pp40 protein as the ortholog of IκBα purified from rabbit lung. While these studies were ongoing in the Baltimore lab, Alain Israel and colleagues independently cloned p105 in their search for DNA binding proteins that regulated MHC Class I and b2 microglobulin genes. In parallel, a human cDNA encoding IκBα was isolated by Steve Haskill and Al Baldwin.

As requested by the editor, I have highlighted only the earliest years of NF-κB research here. Obviously, much has happened since then as exemplified by the contributions to this book. Some highlights include the identification of two new Rel family members, the IκB kinases and unique signaling pathways to NF-κB downstream of various cell surface receptors. Additionally, the phenotypes of genetic deletions of NF-κB and NF-κB-associated molecules have revealed the biological scope of this family of proteins. More than 16,000 publications attest to a role for NF-κB in virtually all cell types and all vertebrate species examined to date, with contributions to early embryogenesis, defense against pathogens and the regulation of cell viability. The function of the κ enhancer NF-κB binding site, however, remains enigmatic since the demonstration by Yang Xu and colleagues that its mutation in the endogenous locus does not affect κ gene recombination or expression. Amongst the variety of phenomena that are modulated by NF-κB, its role in inflammatory processes has attracted the greatest attention. Both as a factor that regulates expression of pro-inflammatory cytokines and a factor that is

activated by pro-inflammatory cytokines, NF-κB is a hot target of the pharmaceutical industry. Ironically, the very ubiquitousness of NF-κB that attracted its vast following may also complicate its use as a therapeutic by increasing the likelihood of nonspecific effects. The objective for the future will be to find, or better still, to design, small molecule regulators that maximize positive effects while minimizing the negative.

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PREFACE

Since its discovery by Ranjan Sen and David Baltimore in the late 80s, NF-κB has drawn the attention of experimental biologists, medical professionals, and the biotech/pharmaceutical industry for its broad and diverse role in all aspects of human biology and disease. Inspired by the wealth of knowledge in this field, I have had the privilege of editing this book so that I could share some of these recent and exciting findings.

This book is meant to provide the most current information gathered on the NF-κB transcription factor family. Written by experts of the subject, the book covers such topics as the structural basis and molecular mechanism of NF-κB signal transduction, transcriptional regulation, and target gene expression. Multiple roles for NF-κB are also explored for a growing number of NF-κB dependent biological and pathological processes. In particular, the function of NF-κB in modulating the immune response to pathogenic infection, as well as its involvement in autoimmunity, cancer, and neuronal development, are each emphasized in different chapters. Current strategies to intervene in the NF-κB signaling pathway are further discussed as a potential means of therapy.

Although the book covers many aspects of NF-κB biology, one can anticipate that new functional roles for NF-κB in other biological or medical disciplines will continue to emerge with the increasing availability of new reagents, disease models, and technology. The role of NF-κB in muscle physiology, ischemia, neuronal degenerative diseases, or cardiovascular disease, for instance, remain uncharted areas that warrant investigation based upon a growing body of evidence implicating NF-κB activity in these processes. Certainly the ability to manipulate NF-κB through inactivation, deletion, regulated expression, or gain-of-function mutation, may extend current research beyond inflammation, autoimmunity, and tumorigenesis into novel applications for vaccine development, tumor immunotherapy, and the control of infectious disease.

Finally, I would like to express my appreciation for the significant contribution from each of the authors on the NF-κB field and the book.

Hsiou-Chi Liou, Ph.D.

Chapter 1

Structural Analysis of NF-κB and IκB Proteins

Tom Huxford* and Gourisankar Ghosh

Abstract

By binding specifically to DNA sequences found within their enhancer elements, transcription factors of the NF-κB family activate the expression of genes involved in cellular immunity, inflammation, development, and apoptosis. The maintenance of proper cellular function requires the tight control of NF-κB levels. Regulation of NF-κB is accomplished primarily through its association with members of the IκB family of transcription factor inhibitor proteins. Structural characterization of various NF-κB dimers in complex with target site DNA, IκB inhibitor proteins, and an in vitro selected RNA aptamer reveals how conformational rearrangements of the versatile NF-κB molecule accommodate high affinity binding to different partners. The relative mobility of three structural and functional units permits large conformational changes in NF-κB without changing the DNA binding surfaces. The structures of NF-κB bound to IκBα and IκBβ further illustrate how some of the disordered elements of these proteins become ordered and participate in the formation of these complexes.

Introduction

The NF-κB transcription factor system is notable for the vast array of diverse stimuli that induce its activity, the rapidity with which it converts from an inactive to active state, and the numerous genes whose transcription is directly influenced by NF-κB activity.[1,2] Though originally discovered and characterized as a nuclear factor with binding specificity toward an element within the immunoglobulin kappa light chain gene enhancer of B lymphocytes, transcription factors of the NF-κB family are now recognized as being present in virtually all resting cell types as stable cytoplasmic complexes with a member of the IκB family of inhibitor proteins.

NF-κB Activation Pathway

Inflammatory cytokines (TNF-α, interleukin-1), growth factors and hormones (EGF, insulin), bacterial products (lipopolysaccharide, lipoteichoic acid), viruses (HIV-1, HTLV-1), and their products (double-stranded RNA, Tax protein) initiate signal transduction pathways that converge upon the multisubunit IκB kinase complex (IKK).[3] IKK phosphorylates IκB associated in complex with inactive NF-κB. This leads to the rapid ubiquitinylation and degradation of complex-associated IκB via the 26 S proteasome.[4] Removal of IκB potentiates NF-κB nuclear translocation by unmasking the type I nuclear localization signal (NLS) present within every

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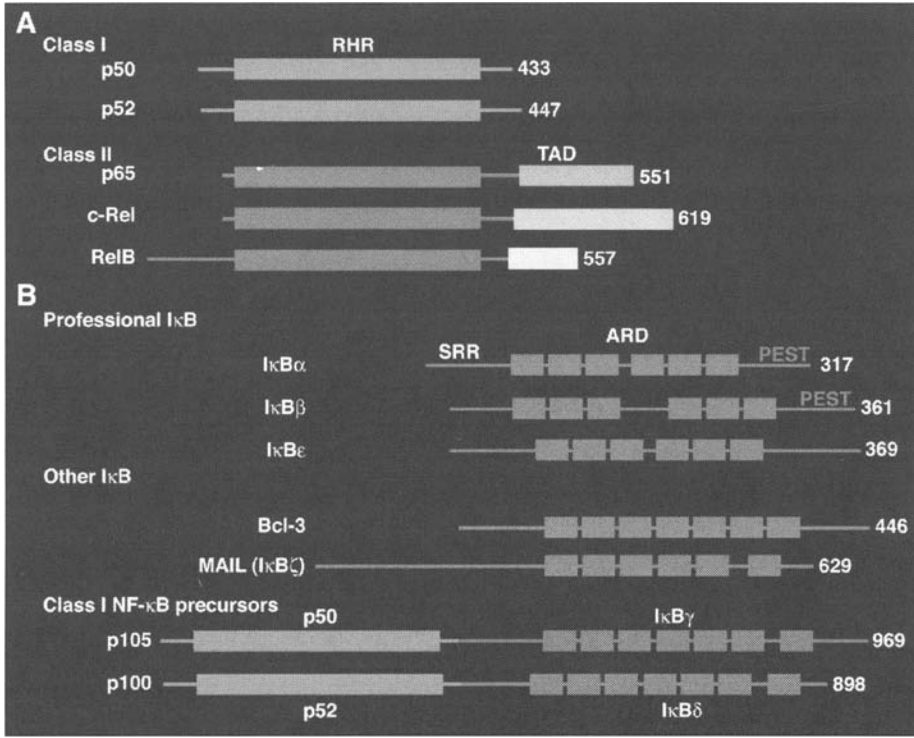

Figure 1. Domain organization of mammalian NF-κB and IκB family proteins. A) Subunits of the mammalian NF-κB transcription factor family all contain the amino-terminal Rel homology region (RHR). The polypeptides are classified based on the absence (class I) or presence (class II) of a carboxy-terminal transcriptional activation domain (TAD). Amino acid numbering corresponds to the size of the human gene products. B) Mammalian IκB family proteins each contain the ankyrin repeat domain (ARD). Additionally, the "professional" IκB proteins (IκBα, IκBβ, and IκBε) contain the amino-terminal signal response region (SRR). IκBα and IκBβ exhibit a carboxy-terminal PEST region. Bcl-3 and MAIL (IκBζ) are classified separately as they function in the nucleus. The p105 and p100 proteins function both as cytosolic IκB proteins an as precursors of the class I NF-κB subunits p50 and p52, respectively.

NF-κB subunit.[5,6] Free NF-κB dimers rapidly accumulate in the nucleus where they bind with specificity to 10 base pair DNA elements present within the promoters of target genes. These elements, collectively referred to as κB DNA, share the consensus sequence 5'-GGGRNNYYCC-3' (where R, N, and Y represent purine, any, and pyrimidine nucleotide bases, respectively).[7] Active nuclear NF-κB upregulates the expression of hundreds of genes including cytokines (interleukins-1, -2, and -6), immunoreceptors (immunoglobin kappa light chain, MHC class I, T-cell receptor β chain), cellular adhesion molecules (ICAM-1, ELAM-1), and many others.[3] Among these is included the gene that encodes IκBα. Within minutes of NF-κB induction, newly translated IκBα appears in the nucleus where it is capable of removing NF-κB from κB DNA and facilitating transport of the inactive complex back to the cytoplasm thus re-establishing the preinduction state.[8-10]

Mammalian NF-κB Family Proteins

The mammalian NF-κB family of inducible, dimeric transcription factors is composed of five subunits, p50, p52, p65 (RelA), c-Rel, and RelB (Fig. 1A). These associate one with another to form active homo- and heterodimers. The five polypeptide subunits are related through

a highly conserved amino-terminal sequence of approximately 300 amino acids in length known as the Rel homology region (RHR). Contained within the RHR are all of the amino acid sequences necessary for dimerization, site specific DNA binding, binding to IκB inhibitor proteins, and nuclear localization.

The type I NF-κB subunits p50 and p52 are generated by proteolytic processing of the precursor proteins p105 and p100, respectively. Each of the p105 and p100 precursors contains an IκB-like ankyrin repeat domain within its carboxy-terminal half. These ankyrin repeat domains are sometimes referred to as IκBγ and IκBδ, respectively. As a result of this domain organization, p105 and p100 function in the cell as IκB family inhibitors of NF-κB activity. The proteolytic removal of the carboxy-terminal ankyrin repeat domains occurs either in a constitutive co-translational manner in the case of p50 or by a stimulus-dependent mechanism in p52.[11-13] Mature p50 and p52 subunits are integral components of strong transactivating NF-κB dimers, most notably the p50/p65 NF-κB heterodimer. They lack inherent transcriptional potential, however, and NF-κB dimers consisting only of p50 and p52 subunits fail to activate transcription of reporter genes.[14]

The p65, c-Rel, and RelB subunits each contain unique transcriptional activation domains carboxy-terminal to their respective RHR. Consequently, NF-κB dimers that contain at least one of these subunits, classified as type II, act as strong activators of transcription.

Mammalian IκB Family Proteins

The mammalian IκB family of transcription factor inhibitor proteins consists of IκBα, IκBβ, IκBε, Bcl-3, and MAIL (IκBζ), as well as the aforementioned p105 and p100 NF-κB precursors (Fig. 1B).[15-17] The primary distinguishing characteristic of the IκB family proteins is a centrally-located ankyrin repeat domain (ARD) consisting of six or seven ankyrin repeats. Ankyrin repeats are helical tandem repeating units of approximately 33 amino acids in length related in structure to leucine rich repeats (LRR), HEAT repeats, armadillo repeats (ARM), and tetratricopeptide repeats.[18,19] Tandem repeats stack sequentially to form an elongated domain devoid of a classical hydrophobic core. Several hundred ARD-containing proteins have been identified exhibiting as many as twenty-four consecutive ankyrin repeats. The function of ankyrin repeat proteins is varied though many participate in protein-protein interactions.

Of the IκB family proteins, IκBα, IκBβ, and IκBε serve to bind and sequester NF-κB dimers in the cytosol and respond to NF-κB inducing stimuli by releasing NF-κB. These three inhibitors are sometimes referred to as the "professional" IκBs. In contrast, Bcl-3 functions within the nucleus where it binds to NF-κB on DNA and alters the transcriptional signal, while p105 and p100 serve as inactive NF-κB subunit precursors. The precise function of MAIL (IκBζ) remains unknown, though it appears to reside primarily in the nucleus, prefers binding NF-κB p50 subunits, and is involved in interleukin-6 production.

Amino-terminal to the ARD in the "professional" IκB proteins is a region that contains the conserved serine sites of phosphorylation by IKK and lysine sites of polyubiquitinylation. Both of these features are required for stimulus-dependent removal of IκB and NF-κB activation. This amino-terminal element, known as the signal response region (SRR), lacks ordered three-dimensional structure in solution. Carboxy-terminal to the ARD of IκBα and IκBβ lies a polypeptide region rich in the amino acids proline, glutamic acid, serine, and threonine. This appropriately named PEST region is common to many proteins that, like IκBα, show a high rate of turnover in resting cells.[20,21] It has been shown that the PEST region of IκBα also functions in binding NF-κB and inhibiting DNA binding.[22,23]

NF-κB Structural Biology

Structural biology attempts to address cell biological questions by determining structures of key molecules or molecular complexes. Successful elucidation of the structure will often suggest mechanisms of molecular action that could not otherwise be addressed. The study of

NF-κB signaling has benefited in recent years from a number of structural studies. In this chapter, we will review these structural studies of NF-κB and IκB proteins within the context of the above outlined NF-κB activation pathway.

NF-κB Structure

The RHR structure from various NF-κB homo- and heterodimers has been determined by X-ray crystallography bound to different κB DNA sequences.[24-32] These structures provided the first atomic resolution views of NF-κB. They reveal the subunit domain organization and they suggest mechanisms for dimerization selectivity, and DNA binding specificity.

The NF-κB RHR is composed of three structural elements, which can be separated by treatment of the molecules with the appropriate proteases. Beginning from the amino-terminus, these are the amino-terminal domain, the dimerization domain, and the NLS polypeptide (Fig. 2).

Amino-Terminal Domain Structure

The NF-κB amino-terminal domain exhibits a variation of the immunoglobulin (Ig) fold. This domain, present in numerous proteins, is especially prevalent among immune receptors. The secondary structure of the protein fold is entirely beta strand in nature. It is formed as two antiparallel beta sheets of three and four strands sandwich one upon the other. The NF-κB amino-terminal Ig-like domain contains a noncanonical insertion prior to its final two beta strands. This insertion is largest in p50 where it forms two long alpha helices separated by an extended coil. In p65 the insertion consists of only one alpha helix.

The amino-terminal domain is sometimes referred to as the DNA binding domain. It earns this nickname because all of the amino acids involved in direct contact and readout of target DNA bases originate from this domain. The majority of these contacts are mediated by amino acids on the loop linking the first and second beta strands. This loop, referred to as L1, is highly conserved in both sequence and structure among all NF-κB subunits.

Dimerization Domain Structure

The NF-κB dimerization domain, or carboxy-terminal domain as it is sometimes referred to, also assumes an Ig-like fold with two stacked antiparallel beta sheets of three and four strands each. Amino acid side chains from the first, second, and fifth beta strands of two NF-κB dimerization domains contact each other symmetrically to form the dimer interface. Each subunit contributes 14 dimer forming amino acid side chains. The dimer interface is mainly hydrophobic in nature and buries approximately 1500 $Å^2$ solvent accessible surface area.

The RelB homodimer is unique among NF-κB family dimers. It is not observed in vivo and fails to form heterodimers with either p65 or c-Rel. The recently determined X-ray crystal structure of the RelB dimerization domain in the absence of binding partner shows that it does form at high concentration. Interestingly, the structure reveals that two RelB subunits contact one another with 8 Å greater separation and a rotation of 30° relative to the dimer interface of other NF-κB dimers. Furthermore, the individual subunits cross over after the fourth beta strand so that the resulting protein is an intertwined dimer. This result is surprising as relatively few amino acid substitutions exist between RelB and the classical p50 homodimer (Vu and Huang, unpublished result). Currently, efforts are underway to determine the source of this significant structural difference and its possible biological implications.

Prior to the last beta strand of the NF-κB dimerization domain resides an amino acid sequence containing a consensus serine phosphorylation site target for cyclic-AMP-dependent protein kinase. Phosphorylation at this p65 subunit serine residue number 276 has been shown to be a requirement for maximum activation of gene transcription.[33]

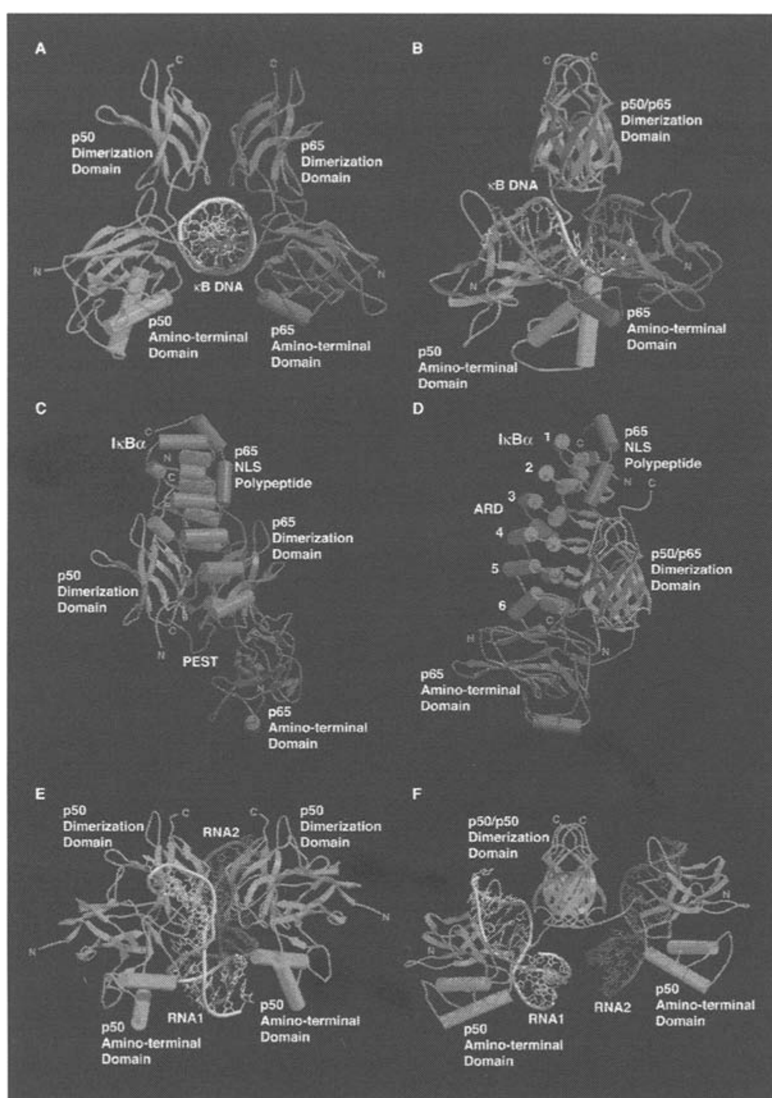

Figure 2. Ribbon diagram representations of NF-κB RHR bound to κB DNA, IκBα, and an RNA aptamer. A) The NF-κB p50/p65 heterodimer bound to κB DNA. The p50 subunit is shown in green, the p65 subunit is red, and the DNA double helix is depicted in two-tone grey. The structure is viewed down the pseudosymmetrical dimer interface and the separable structural elements of the complex are labeled. Note that the NLS polypeptide segment is present but not visible in the NF-κB heterodimer bound to DNA. B) Same complex rotated 90° with respect to the vertical axis in A). C) The NF-κB p50/p65 heterodimer in complex with IκBa. NF-κB subunits are oriented and colored as in A). IκBα is colored purple. D) NF-κB/IκBα complex rotated 90° with respect to C). Ankyrin repeat domain (ARD) is labeled and individual ankyrin repeats are numbered. Note the difference in the position of the NF-κB p65 subunit amino-terminal domain relative to it DNA bound conformation. E) NF-κB p50 homomdimer bound to a selected RNA aptamer and aligned along the dimerization domain symmetry axis as in A) and C). The p50 subunits are shown in green and the two RNA molecules are depicted in two shades of grey. F) The p50 homodimer/RNA complex rotated 90° about the vertical axis. Again, note the drastic change in the position of the amino-terminal domains in complex with RNA.

NLS Polypeptide Structure

A small segment of approximately 30 amino acids carboxy-terminal to the dimerization domain constitutes the third structurally independent component of the NF-κB RHR. This region, within which is contained the type I nuclear localization sequence, is present but unobserved in a number of the NF-κB/DNA complex crystal structures. It is also absent from the high resolution structures of the NF-κB dimerization domains solved in the absence of DNA.[34,35] The systematic lack of observed electron density in maps calculated from X-ray diffraction data is indicative of disordered regions within the protein. Furthermore, it has been shown that the removal of this segment leads to improved crystal quality and extended X-ray diffraction resolution.[36] These observations suggest that, in its DNA bound conformation, this NF-κB NLS-containing polypeptide represents a flexible segment that lacks an ordered structure. As discussed later in this chapter, such is not the case when NF-κB binds to IκB inhibitor proteins.

Structurally Related Proteins

Since the original determination of the NF-κB RHR structure two additional transcription factor families, NFAT and STAT, have been found to use a similar mode of binding to DNA with Ig-like domains. The nuclear factor of activated T-cells or NFAT proteins share modest sequence homology with the NF-κB RHR. NFAT1-4 function as monomers and require additional regulators of transcription for stable DNA complex formation.[37] NFAT5, on the other hand, is dimeric both in solution and bound to DNA. The recently determined X-ray crystal structure of an NFAT1 bound to a pseudopalindromic target DNA sequence reveals how two monomeric NFAT proteins can cooperate in binding DNA. The structure shows striking similarity to the NF-κB/DNA complex crystal structures, though the two proteins contact one another asymmetrically as opposed to the symmetrical interactions displayed by the NF-κB dimerization domain.[38] The signal transducer and activator of transcription or STAT proteins bind DNA through an amino-terminal domain with structural similarity to the amino-terminal Ig-like domain of NF-κB.[39,40] Like NF-κB, NFAT and STAT are involved in lymphocyte activation and the immune response.

NF-κB/DNA Complex

In complex with κB DNA the NF-κB RHR resembles the wings of a butterfly (Fig. 2A). The NF-κB RHR contacts the DNA major groove along one entire turn of the double helix (Fig. 2B). The most striking observation from NF-κB/DNA complex structural determination is that it does so entirely through ordered loops. This is a stark contrast to the canonical sequence specific DNA binding domains that employ secondary structure elements to "read" the DNA sequence. The NF-κB DNA contacting loops connect the beta strands of the amino-terminal and dimerization domains and are, therefore, held in place due to the domain stability. In all, ten loops make DNA contacts. Five of these are contributed by the amino-terminal domain while the five others emanate from the dimerization domain. This arrangement serves to explain, in part, why such a large protein is required for binding to a relatively small DNA half site of five base pairs.

In general, DNA binding specificity is derived from DNA base-contacting amino acid side chains. Nearly all of the base-specific contacts in NF-κB/DNA complexes are mediated by amino acids within the first loop of the amino-terminal domain, the so-called loop L1. The p50 subunit contributes side chains Arg54, Arg56, and Glu60 that contact the second and third guanine bases within the κB consensus DNA sequence. His64, also from the loop L1 of the p50 amino-terminal domain, contacts the first guanine. A similar set of side chains are contributed by the homologous loop from the NF-κB p65 subunit, with the notable exception that the His64 of p50 is replaced by Ala43 in p65. As a consequence, a 5'-G is not required within the κB DNA half sites contacted by p65 subunits.

The modular organization of the NF-κB RHR allows for significant movement of the amino-terminal domains relative to the dimerization domains. When the first NF-κB p65 homodimer/DNA complex was determined, it was noted that one subunit contacts its κB DNA half site in a manner similar to that observed in the p50/p65 heterodimer while the second p65 subunit amino-terminal domain rotates 18° in order to maximize nonspecific contacts with the DNA backbone.[27] This rearrangement was later determined to result from the choice of a pseudopalindromic κB DNA target with one too many A:T base pairs. When two additional κB DNA targets were used for crystallization, it was found that by varying amino-terminal domain placement, the NF-κB p65 homodimer could accommodate binding to diverse κB DNA sites without sacrificing overall binding affinity.[30] The discovery that the amino-terminal domain is capable of significant global movement relative to the dimerization domain in order to accommodate various binding partners has become a recurring theme in many of the subsequently determined NF-κB complex crystal structures.

NF-κB/IκB Complex

The determination of two different NF-κB p50/p65 heterodimer/IκBα complex cocrystal structures allows for analysis of the structure of IκBα as well as comparison of the NF-κB RHR with its DNA-bound conformation (Fig. 2C,D).[41,42] Comparison of this structure with that of IκBβ, solved in complex with the NF-κB p65 homodimer dimerization domain, and Bcl-3, determined as a free ARD, allow for general and specific rules of IκB structure and point to their differences in function.[43,44]

IκB Ankyrin Repeat Structure

As revealed in the NF-κB/IκB complex structures, the ankyrin repeats of IκBα and IκBβ exhibit the typical fold. This consists of a closely packed helix-turn-helix motif followed by a loop that extends perpendicularly from the end of the second helix and closes in a tight beta-turn. Consecutive repeats stack with roughly 11 Å spacing. The conservation of small amino acid side chains at the turn in the helix pair allows for a slight curvature in the ARD. Both IκBα and IκBβ contain nonconsensus insertions between ankyrin repeats 3 and 4. The insert is only six amino acids in IκBα. IκBβ, however, contains a sequence of 47 amino acids that bears little homology to any known proteins. The placement of the inserts is on the back of the ARD at some distance from the NF-κB binding partner, and all but ten of the IκBβ insert residues are disordered in the NF-κB p65 homodimer/IκBβ complex structure.[43] Therefore, the insert region does not appear to affect NF-κB binding directly, though the large insert between ankyrin repeats 3 and 4 of IκBβ has been shown to mediate interaction with the small Ras-like GTPase κB-Ras.[45]

One interesting aspect of the IκB ankyrin repeat domains is their instability relative to other ARD-containing proteins (C.A. Hughes, unpublished results). One way in which this is manifest is that the IκBα ARD has resisted crystallization in the absence of NF-κB binding partner despite considerable efforts on the part of one of the authors. By contrast, the structures of a number of proteins containing either actual or idealized ARDs have been determined by X-ray crystallography.[44,46-49] One of these is the IκB family member Bcl-3, which contains seven ankyrin repeats.[44] The relative instability of IκBα and IκBβ are indicative of their role as substrates for rapid proteolysis by the 26 S proteasome.

IκB PEST Region

The length of the IκBα PEST region included within the crystal differs for the two NF-κB/IκBα complex crystal structures. The structure that contains the longer PEST reveals that it forms a sigmoidal polypeptide loop devoid of secondary structure. The poorer quality of the electron density maps and higher temperature factors associated with the amino acids in this region indicate that a significant amount of thermal motion is exhibited by the IκBα PEST

region in the NF-κB/IκBα complex crystals. The corresponding region is present but not observed in the crystal structure of the NF-κB p65 homodimer/IκBβ complex.

NF-κB/IκB Complex Interface

As previously mentioned, the two NF-κB/IκBα complex crystal structures contain unique elements by virtue of the design of protein constructs employed in the preparation of the complex crystals.[50,51] As a result of these differences, one structure reveals a protein-protein interaction surface of 4300 $Å^2$ while the second contains 3800 $Å^2$ of buried surface area. When the portions common to the two structures are superimposed, the resulting complex buries 4800 $Å^2$ solvent accessible surface area. A comparison of the elements common to both structures reveals a nearly identical contact area, however, indicating the correctness of the two structures and the precision of the experimental technique. Both structures reveal that NF-κB and IκBα contact one another through a discontinuous interface. The contribution of three discrete, discontinuous patches to the overall protein-protein interface explains its tight binding affinity with dissociation constants measured in solution on the order of 1 nM.

The NF-κB p65 subunit NLS polypeptide makes extensive contacts with ankyrin repeats 1 and 2 of IκBα. Within this interaction, the type I NLS of the p65 subunit adopts an alpha helical structure. In this conformation, amino acids Lys301, Arg302, and Arg304 participate in direct ion pairing interactions with amino acid side chains in IκBα. Additional hydrophobic interactions tether amino acids carboxy-terminal to the basic NLS sequence and the top of the first ankyrin repeat of IκBα. As mentioned previously, this same NLS polypeptide region is disordered in several NF-κB/DNA structures. When bound by its cognate receptor, importin-α, the type I NLS mediates translocation of NF-κB from the cytoplasm to the nucleus. X-ray crystallographic studies on ARM repeat containing importin-α proteins bound to various NLS peptides have revealed that these bind in an extended polypeptide conformation.[52,53] Taken together, these results indicate that the NLS polypeptide element of the NF-κB p65 subunit is capable of making a transition from alpha helical structure in its IκB bound state to extended beta-like structure upon binding to importin-α and, finally, to unstructured polypeptide upon binding DNA.

Next within the discontinuous NF-κB/IκBα binding interface is the interaction of IκBα ankyrin repeats 3, 4, and 5 with the dimerization domains of the NF-κB p50/p65 heterodimer. One surprising observation is that the p50 subunit dimerization domain mediates the vast majority of contacts within this interaction surface. This is counterintuitive in light of the cellular and biochemical data that clearly show a preference of IκBα toward binding NF-κB dimers containing at least one p65 subunit.[54] A closer inspection of the contact surfaces of IκBα and NF-κB throughout this region of the complex interface reveals few specific side chain interactions. It has been shown that, although this binding surface contributes significantly to overall NF-κB/IκBα binding affinity, the p65 NLS polypeptide is chiefly responsible for directing the specificity of IκBα toward p65-containing NF-κB homo- and heterodimers.[35]

The third and final component of the discontinuous NF-κB/IκBα complex interface involves the amino-terminal domain of the NF-κB p65 subunit and the carboxy-terminal PEST region of IκBα. This portion of the structure exhibits significantly higher thermal motion when compared to the rest of both NF-κB/IκBα complex X-ray crystal structures. In the complex, the acidic IκBα PEST juxtaposes with an acidic patch unique to the bottom of the NF-κB p65 dimerization domain. The result is the formation of an extended composite acidic surface measuring some 40 Å by 20 Å. Relative to its DNA bound conformation, the p65 amino-terminal domain rotates by 170° and translates nearly 40 Å. Aside from its new position, the p65 subunit amino-terminal domain remains virtually unchanged. The consequence of this *en bloc* movement is to place the basic DNA contacting surface of p65 in a position to oppose the newly formed acidic surface. This long-range electrostatic interaction is thought to play a major role in the DNA-inhibitory binding activity of IκBα. In support of this hypothesis, increasing the strength of the acidic surface through hyperphosphorylation of the IκBα

PEST by protein kinase CK2 (casein kinase II) in vitro has been shown to increase NF-κB binding affinity and DNA-inhibitory binding activity of IκBα.[54]

NF-κB p50 Homodimer/RNA Aptamer Complex

The recent determination of the X-ray crystal structure of the NF-κB p50 homodimer bound to an RNA aptamer reveals a new binding conformation available to the NF-κB RHR.[55] Whereas one NF-κB dimer binds to one decameric κB DNA sequence with each subunit binding symmetrically to one five base pair half site, an RNA aptamer selected both in vitro and in vivo in yeast for high affinity binding to NF-κB p50 binds only one subunit of the homodimer.[56-58] The complex crystal structure reveals how this is possible (Fig. 2E, F).

The RNA aptamer forms a hairpin structure that is bound by the DNA contacting amino acid residues on the p50 amino-terminal domain loop L1. As in the case of the NF-κB p65 subunit in complex with IκBα, the NF-κB p50 subunit amino-terminal domain changes its relative orientation in order to accommodate binding the RNA aptamer. Relative to its DNA bound conformation, the domain swings 40° and translates by 10 Å to bind one RNA molecule away from the dimer interface and classical location of DNA binding. What is surprising about this domain movement is that it results in an orientation in the opposite extreme position relative to the dimerization domain platform from that exhibited in the NF-κB/IκBα complex structures. Even more interesting, the DNA binding loop L1 and its DNA-contacting amino acid side chains remain virtually unchanged from their positions in any of the other NF-κB structures. The high affinity binding exhibited by the p50 homodimer/RNA complex results from the evolution of an arrangement of chemical groups on the selected RNA aptamer that perfectly complements the DNA-binding properties of the loop L1 side chains and, therefore, mimics the natural κB DNA target site.[59]

Conclusions

The NF-κB RHR is a versatile binding factor by virtue of the relative flexibility of its three structural components: amino-terminal domain, dimerization domain, and NLS polypeptide. The observation that all of the DNA binding loops of NF-κB are preformed and most of the DNA-contacting amino acid side chains approximate their DNA bound conformations even in the absence of any binding partner indicates that the hinge points that join the three structured elements dictate the NF-κB binding conformations (C.B. Phelps, unpublished data). In particular, a flexible linker of approximately 10 amino acids in length joining the amino-terminal and dimerization domains is chiefly responsible for allowing the relative motion of the amino-terminal domain. Other multidomain transcription factors, STAT for example, lack this flexible linker. One manifestation of this difference is the recent successful crystallization and X-ray crystal structure determination of *Dictyostelium discoideum* STATa homodimer in the absence of DNA.[60] By contrast, the dynamic NF-κB RHR has continually resisted crystallization and structural determination in the absence of some binding partner.

The IκBα and IκBβ proteins represent a subset of ARD-containing proteins that display a low degree of folding stability. It is not hard to imagine that the selective pressure exerted by nature to engineer an NF-κB binding partner that responds to stimuli by being rapidly degraded would result in a molecule with the characteristics of the IκB proteins. It is interesting, however, that both NF-κB and IκB proteins exhibit such flexibility and yet bind with such high affinity and stability.

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CHAPTER 2

NF-κB Signal Transduction by IKK Complexes

Zhi-Wei Li* and Michael Karin

Abstract

Transcription factor NF-κB plays a major role in many physiological and pathological processes while its regulation is best understood in inflammatory and immune system. The central event in NF-κB signaling pathway is the activation of IKK complex, the convergent point of diverse NF-κB activation signaling. This review addresses the cell signaling of IKK and NF-κB activation in response to various immune and inflammatory stimuli as revealed by the analysis of mice and cells lacking specific signaling transducers.

NF-κB, IκB, IKK and the Canonical Pathway for NF-κB Activation

NF-κB is a master transcription factor that plays a major role in inflammatory and immune response.[1] It was originally found in nuclei of B cells and named for its ability binding the κ-chain enhancer of immunoglobulin in B cells. NF-κB was later found in the cytoplasm of all cell types, where it enters the nucleus upon stimulation. NF-κB transcription factors are evolutionarily conserved from insects to mammals. In mammals, the NF-κB family consists of five members (p65/RelA, RelB, c-Rel, p50/NF-κB1 and p52/NF-κB2). These proteins share an N-terminal domain of about 300 amino acids, which bears homology to the product of the *v-rel* oncogene, the Rel homology domain (RHD), and includes regions for DNA binding, dimerization and nuclear translocation (Fig. 1). DNA binding by NF-κB requires dimerization and most members of this family form both homo- and heterodimers except for RelB, which forms only heterodimers with p50 or p52. Mammalian NF-κB proteins can be classified into two groups; the first group, consisting of p65 (RelA), RelB and c-Rel, are expressed as mature proteins and possess a transcriptional activation domain at their C-termini. NF-κB dimers containing any one of these subunits can activate target gene transcription upon induction by certain stimuli. The second group consists of p50 (NF-κB1) and p52 (NF-κB2), which are first expressed as large precursors p105 and p100, respectively. NF-κB1 precursor p105 is constitutively processed to produce p50, whereas p52 is proteolytically released from p100 only upon stimulation. Both p50 and p52 lack a potent transcriptional activation domain and therefore cannot activate transcription as homodimers, or as p50/p52 heterodimers. In fact, p50/p52 dimers may suppress expression of NF-κB target genes.

The C-termini of p105 and p100 contain multiple ankyrin repeats, which are required for association with NF-κB and are the distinguishing structural feature of the IκBs, the specific inhibitors of NF-κB (Fig. 1). Therefore, p105 and p100 can serve an IκB-like function by

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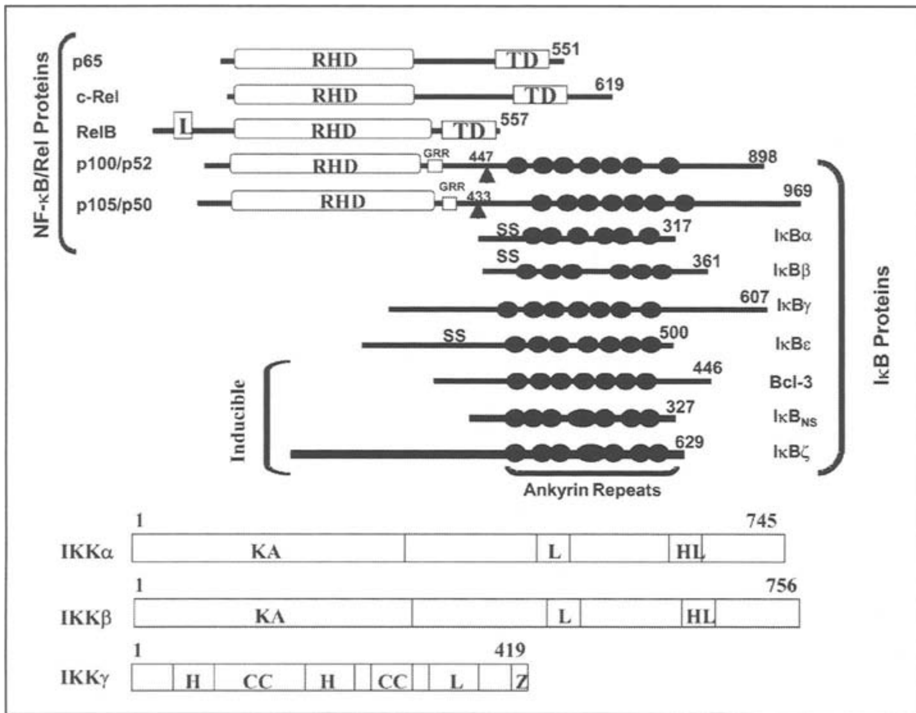

Figure 1. Mammalian NF-κB, IκB and IKK proteins. CC: coiled coil; GRR: glycine rich repeat; H: α-helix; HLH: helix-loop-helix; L: leucine zipper; RHD: Rel homology domain; SS: serine phosphorylation sites; TD: transactivation domain; Z: zinc finger. Modified from Li ZW, Rickert RC and Karin M. Genetic dissection of antigen receptor induced-NF-kappaB activation. Mol Immunol 2004; 41(6-7):701-714.

retaining RelA, RelB or c-Rel in the cytoplasm. Three major mammalian IκB proteins, IκBα, IκBβ and IκBε, have been identified.[1] These IκBs have overlapping yet distinct inhibitory specificity and thus can differentially inhibit NF-κB dimers. In addition, the C-terminal portion of p105 can be expressed as an independent transcript that encodes IκBγ, which is expressed only in the lymphoid cells. Another mammalian IκB family member is the nuclear protein Bcl-3.[1] Although it contains ankyrin repeats, Bcl-3 functions as a transcriptional activator with p50 or p52 homodimers, rather than an inhibitor of NF-κB. This activity may be caused by Bcl-3-mediated displacement of p50 or p52 homodimers from NF-κB binding site to allow binding of NF-κB molecules with transactivation domains, such as p65, c-Rel and RelB. Alternatively, Bcl-3 may also activate gene transcription by its own transactivation domain.[2] Bcl-3 production is inducible and is required for humoral immune response. Other two inducible IκB family members are IκBζ (also called MAIL or INAP) and $I\kappa B_{NS}$.[3-6] IκBζ is required for Toll-like receptor (TLR) and interleukin 1 (IL-1) receptor (IL-1R) activation induced production of IL-6,[7] and $I\kappa B_{NS}$ is induced by TCR (T cell receptor) activation,[6] suggesting that other inducible IκBs may exist and respond to diverse stimulation. However, how these inducible IκBs function has yet to be determined.

In addition to the ankyrin repeats, the C-terminal acidic region of IκBs is necessary for their inhibitory activity. The PEST motif in the C-terminal acidic region of IκB is the target site of IκB phosphorylation that is responsible for the basal turnover of these proteins and their induced degradation in response to UV irradiation.[8] The IκBs inhibit NF-κB activity by masking the nuclear localization signal (NLS) of NF-κB, thereby retaining NF-κB in the cytoplasm

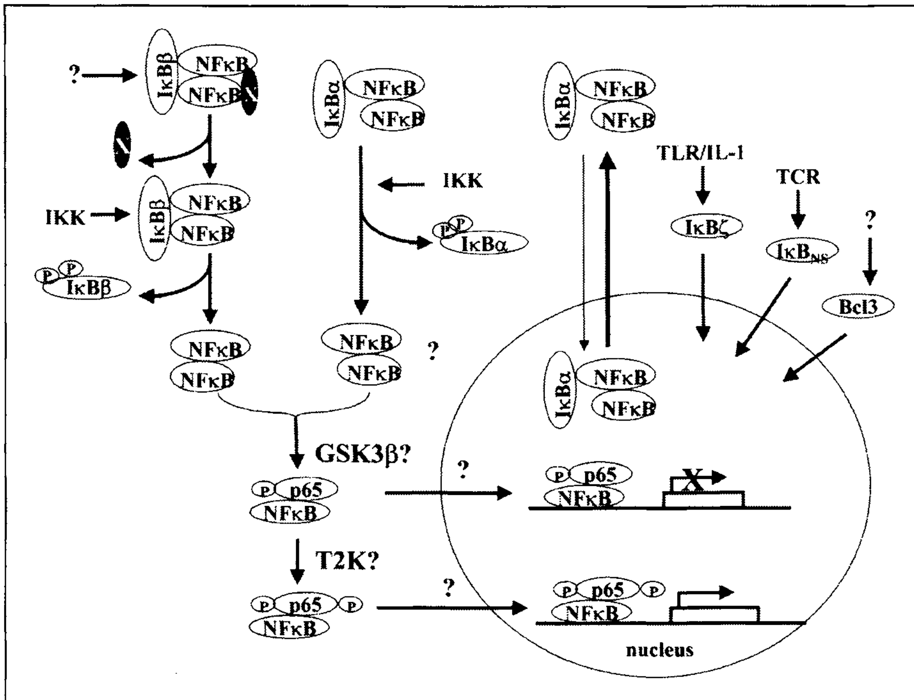

Figure 2. Regulation of NF-κB activity by IκBs and protein kinases. IκBα:NFκB complex shuttles between the cytoplasm and nucleus although majority of the complex is in the cytoplasm. The IκBβ:NF-κB complex is retained in cytoplasm by IκBβ and protein X such as κB-Ras. Upon stimulation, unidentified molecule removes X from IκBβ:NF-κB complex. IKK phosphorylation of IκBs leads to the ubiquitination and degradation. Released NF-κB is phosphorylated and then induces gene expression. Nuclear import of inducible IκBs (Bcl3, IκBζ, $I\kappa B_{NS}$) occurs only upon certain stimuli. For p65 containing NF-κB, GSK3β phosphorylation might enhance DNA binding, and further T2K phosphorylation might lead to gene transcription. Question markers indicate unidentified or unconfirmed molecule(s). Modified from Li ZW, Rickert RC and Karin M. Genetic dissection of antigen receptor induced-NF-kappaB activation. Mol Immunol 2004; 41(6-7):701-714.

(Fig. 2). There are two variations of this model.[9] One mechanism is used by IκBβ and causes cytoplasmic retention of NF-κB due to the masking of two NLSs on NF-κB dimers. Interaction between the NF-κB:IκBβ complex and the small guanosine triphosphatases κB-Ras-1, -2 also contribute to NF-κB activation. When binding to κB-Ras, IκBβ cannot be phosphorylated by IKK, thus blocking the NF-κB activation signal from IKK.[10] IκBα and IκBε, which both mask one NLS of NF-κB, utilize the other mechanism in which the 2nd NLS of NF-κB and the nuclear export signal (NES) of IκBα or IκBε causes the IκB:NF-κB complex to shuttle between the nucleus and cytoplasm. Recently, Moorthy et al suggested that IκBs, including IκBγ (the c-terminus of p105), utilize the same binding mechanism, and the localization of IκB:NF-κB complex might be controlled by other protein(s).[11] It was suggested that κB-Ras is a protein that interacts only with IκBβ but not other IκB family members. The interaction of κB-Ras with the NF-κB:IκBβ complex causes the binding of two NF-κB NLS motifs by IκBβ. Removal of κB-Ras release one NLS and leads to nuclear import of the NF-κB:IκBβ complex.[12] If this is true, free κB-Ras should be detected upon NF-κB activation stimuli. The differential control between IκBα and IκBβ may lead to biphasic activation of NF-κB. As a target gene of NF-κB, IκBα is promptly upregulated upon NF-κB activation and therefore

controls the fast transient activation of NF-κB, whereas IκBβ, whose transcription is not controlled by NF-κB, controls the persistent activation of NF-κB.[13] Two MAP3Ks, MEKK3 and MEKK2 were suggested to regulate the biphasic activation of NF-κB upon TNFα (tumor necrosis factor alpha) and IL-1α by participating in assembling of IκBα:NF-κB/IKK and IκBβ:NF-κB/IKK complex, respectively.[14]

In response to extracellular stimuli, the IκBs are rapidly phosphorylated due to activation of a protein kinase complex called the IκB kinase (IKK).[15] This phosphorylation event targets the IκBs for polyubiquitination and degradation by the 26S proteasome and thereafter the release of NF-κB dimers that translocate to the nucleus to regulate gene transcription. IKK (Fig. 1) contains two closely related catalytic subunits, IKKα and IKKβ, which contain a protein kinase domain at their N-terminal portion, whereas their C-terminal portion contains protein interaction motifs such as a leucine zipper (LZ) and a helix-loop-helix (HLH) domain. IKKα and IKKβ can both directly phosphorylate IκBs and their activity depends on dimerization through their leucine zipper motifs. In addition, IKK activity also depends on the HLH motif that may act as an intramolecular activator of the kinase domain. While the major native IKK complex is based on IKKα:IKKβ heterodimers, in vitro, both IKKα and IKKβ can also form functional homodimers. IKKβ is the major kinase controlling canonical pathway of NF-κB activation, in which phosphorylation of IκB by IKK release NF-κB to enter nuclear and regulate gene expression. The native IKK complex also contains a regulatory subunit, IKKγ that can form homodimers and is necessary for assembly of the IKK complex and recruitment of upstream activators to the IKK complex. The C-terminal domain of IKKγ is essential for IKK kinase activity,[16] while the N-terminus is required for binding of IKKγ to the catalytic subunits and therefore is also important for IKK activity.[17] Recently, it was found that the C-terminal oligomerization domain of IKKγ is required for dimerization whereas tetramerization, which enhances IKK kinase activity, needs the N-terminal domain.[18] Consistent with this finding, Weil et al found that the IKKγ N-terminus is sufficient for IKK and NF-κB activation when recruited to the plasma membrane.[19] Although reports identifying signaling events that only require IKKα or IKKβ are beginning to emerge, the similar phenotypes of IKKβ-null and IKKγ-null mice strongly suggest that IKKγ is required for activation of IKKβ.[20] IKKα$^{-/-}$, IKKβ$^{-/-}$ double knockout mice showed the same phenotype as IKKγ knockout mice, suggesting that IKKγ may also control IKKα activation in the context of the tri-subunit IKK complex. IKKγ-deficient mice die earlier than IKKα- or IKKβ- deficient mice, making it difficult to precisely determine the function of IKKγ in a variety of physiological processes. Hopefully, the generation of conditional IKKγ knockout mice will circumvent this difficulty. Most recently, another protein, ELKS (for the relative abundance of its constitutive amino acids: glutamic acid (E), leucine (L), lysine (K), and serine (S)), was suggested to be an IKK regulatory subunit.[21] Knocking down ELKS by RNA interfering leads to defect in NF-κB activation, including reduction in IKK kinase activity, IκB phosphorylation and degradation, NF-κB DNA binding activity, NF-κB targeting gene expression and the protection of cell death induced by TNFα. It was suggested that ELKS functions probably by recruiting IκBα to the IKK complex. However, the physiological function of ELKS remains to be explored.

IKKα and Noncanonical Pathway for NF-κB Activation

In addition to the canonical NF-κB activation pathway that is mostly dependent upon IKKγ-regulated IKKβ activation, IκB phosphorylation and degradation, and then NF-κB activation,[15] there are at least two situations in which NF-κB activation was reported to depend only on IKKα (Fig. 3). One is IKKα-dependent p100 processing, which is believed to be the mechanism for LTβ (lymphotoxin beta) and Blys (B lymphocyte stimulator, also called BAFF, TALL-1, zTNF4 or THANK) induced NF-κB activation.[9] Ligation of the LTβR (LTβ receptor) or Blys receptor (BR3) leads to NIK (NF-κB-inducing kinase) activation which in turn phosphorylates IKKα and thereby activates NF-κB by phosphorylating p100 and causing the release of p50:RelB dimers. Indeed, NIK-deficient mice are defective in LTβ induced NF-κB

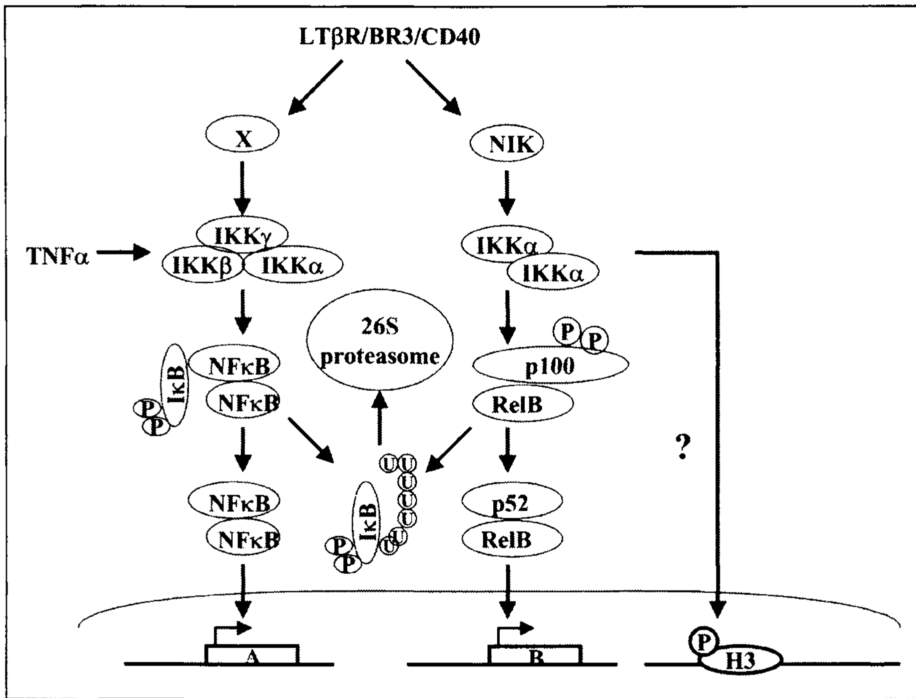

Figure 3. NF-κB activation is differentially regulated by IKKα and IKKβ. Upon LTβ/Blys/CD40L stimulation, their receptor LTβR/BR3/CD40 activates NIK and thereafter IKKα homodimers, which in turn phosphorylate p100 and lead to p100 processing, and release p52/RelB heterodimers. An unidentified X signal pathway (it contains TRAF6 for CD40 pathway) activates the IKK holoenzyme composed of all three subunits. In most cases, the activity of this complex depends on IKKβ. Modified from Li ZW, Rickert RC and Karin M. Genetic dissection of antigen receptor induced-NF-kappaB activation. Mol Immunol 2004; 41(6-7):701-714.

activation.[22] IKKα-deficient mice show defective p100 processing in B cells and this might be due to a defect in the phosphorylation of p100 by IKKα.[23] In the case of Blys signaling, in vivo data also support this NIK-IKKα-p100 model. Blys knockout mice showed reduced generation of mature follicular B cells.[24] This phenotype is also observed in IKKα$^{-/-}$ fetal liver transplanted mice and in NF-κB2$^{-/-}$ mice.[23,25-27] Blys/BR3 signaling promotes p100 processing and thereafter prevents apoptotic B cell death. A BR3-Fc fusion protein, which blocks BR3 signaling, inhibits p100 processing and attenuates the development of autoimmune disease in a mouse model.[28] Blys-induced p100 processing and NF-κB activation are impaired in NIK mutant B cells, and p100 processing is independent of IKKγ, the regulator of the canonical pathway of NF-κB activation.[29] The involvement of IKKα in Blys signaling, however, has not been determined using IKKα-deficient B cells. In NF-κB2$^{-/-}$ B cells, other NF-κBs still respond to Blys stimulation.[29] This seems to be not simply due to the compensation of other NF-κB components since Blys-mediated cell survival is impaired in IKKβ-deficient B cells (Z.W. Li, unpublished result). Together, these findings suggested that IKKα is not the only signaling molecule that regulates Blys-mediated B cell survival. With respect to LTβR stimulation, induction of some NF-κB target genes requires NIK and IKKα, whereas expression of other genes depends only on IKKβ and, presumably, IKKγ. RelB upregulation and p100 processing as well as translocation of p52:RelB dimers into the nucleus depend only on NIK and IKKα. However, p100 expression is controlled by RelA and IKKβ.[30] Consistent with these

findings, LTβR stimulation leads to IκBα degradation and a shift of DNA binding molecules from RelA- to RelB-containing NF-κB dimers. Prior activation of the IKKβ-dependent pathways results in upregulation of p100:RelB dimers available for IKKα-induced processing.[31] Saccani et al also found that upon proinflammatory stimulation, NF-κB mediated gene transcription in monocyte-derived dendritic cells is fine-tuned by exchange of NF-κB dimmers.[32] However, the mechanism controlling this dimer exchange is still unclear. CD40L is another NF-κB activator that utilizes noncanonical pathway,[33] although it also utilizes canonical pathway.[34] CD40 was known to recruit TRAF6 (TNF receptor associated factor 6) for NF-κB activation as justified by analysis of TRAF6 knockout mice.[35] It would be interesting to determine how TRAF6 differentially affects both canonical and noncanonical pathways that activate NF-κB through recruitment of IKKβ and IKKα, respectively.

The second mechanism proposed for IKKα-regulated NF-κB activation is the phosphorylation of histone H3 residue serine 10 by IKKα.[36,37] Although both groups suggested that IKKα and not IKKβ, is the kinase phosphorylating this residue in vitro, more work is needed to demonstrate this in vivo and resolve a number of questions raised from the discrepancy between the in vitro evidence for H3 phosphorylation by IKKα and contrasting in vivo findings.[38] Most prominently, it is well established that IKKα-deficiency in mice results in postnatal death but normal TNFα signaling, whereas IKKβ-deficiency in mice results in liver apoptosis due to defective TNFα-depended NF-κB activation.[9] A crucial question therefore is why the phenotype of $IKK\alpha^{-/-}$ mice is so different from that of $IKK\beta^{-/-}$ mice if IKKα phosphorylation of histone H3 is critical for TNFα induced NF-κB activation. It is most likely that IKKα-dependent H3 phosphorylation is of little physiological relevance in vivo. Recently, it was found that the skeletal morphological defect in IKKα null mice was attributed to failed epidermal differentiation that is regulated by kinase-independent functions of IKKα.[39] Although this defect was related to increased FGF8 (fibroblast growth factor 8) expression, how IKKα inhibits FGF8 expression is still a myth.[39] Interestingly, nuclear translocation of IKKα is required by this inhibitory function. This finding suggested that IKKα may do play a role by functioning in nucleus.

Modification of NF-κB and Its Signaling Molecules

Phosphorylation of NF-κB subunits also contributes to the regulation of NF-κB activation by facilitate the recruitment of various transcription cofactors.[9,40] Several kinases, including PKAc (catalytic subunit of protein kinase A), MSK1 (mitogen- and stress-activated kinase-1), RSK1 (ribosomal subunit kinase-1), PI3K/AKT, CKII (casein kinase II), GSK3β (glucose synthase kinase 3β), T2K (TRAF2-associated kinase, also called TBK or NAK), IKK, PKCζ (protein kinase C) and NIK, were suggested to phosphorylate NF-κB subunits,[9,40] and GSK3β, T2K, IKK, PKCζ and NIK were confirmed by gene targeting to be essential for NF-κB-regulated gene transcription by particular stimuli.[20] While additional evidence suggests that IKK, PKCζ and NIK act upstream of IκB, GSK3β and T2K are the more likely candidates to act downstream of IKK and phosphorylate p65.[9] In response to a variety of stimuli, cells deficient in either GSK3β or T2K exhibit normal IκB degradation and NF-κB nuclear translocation, but are defective in NF-κB target gene transcription. Furthermore, either GSK3β or T2K knockout mice die during mid-gestation due to massive liver apoptosis,[41,42] which is the same phenotype observed in $IKK\beta^{-/-}$ or $p65^{-/-}$ mice.[43-46] However, NF-κB DNA binding activity is only affected in the GS3Kβ knockout, and not in the T2K knockout (Fig. 2), suggesting that these kinases differentially control p65 activity downstream of IκB degradation. To provide further evidence for the phosphorylation of p65 by GSK3β or T2K, it will be important to compare p65 phosphorylation in wild type and GSK3β- or T2K-deficient cells in response to particular stimuli. Further in vivo data to support this hypothesis could be generated by attempting to rescue the GSK3β- or T2K-deficiencies by overexpression of constitutively activated p65.

Acetylation of RelA is also reported to affect NF-κB activation, perhaps by regulating the interaction between newly synthesized IκB and RelA, This acetylation is reversible and

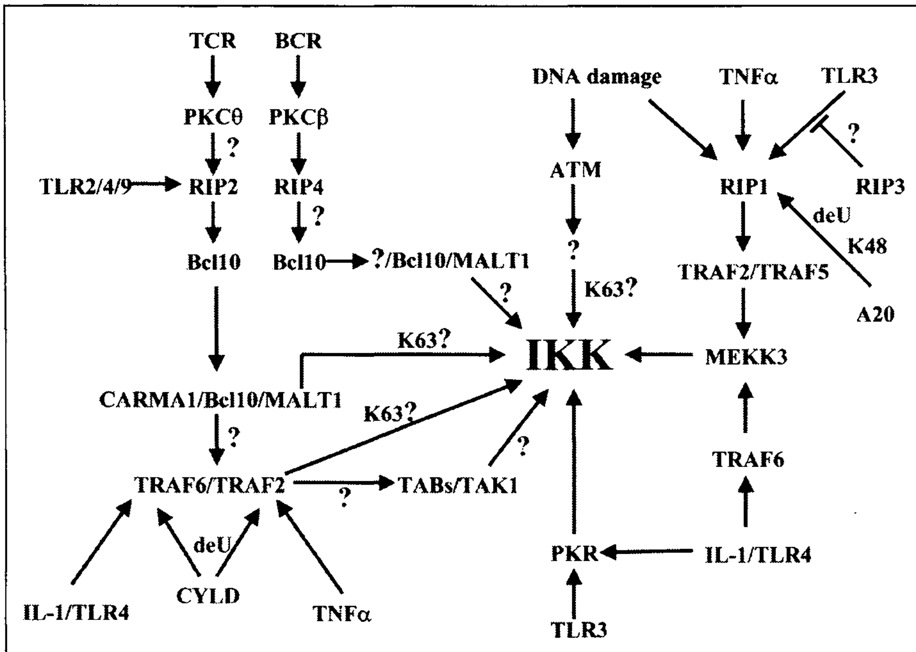

Figure 4. Activation of IKK complex. Different isoforms of PKCs and RIPs are utilized by different signaling pathway to activate IKK, whereas TAK1 and MEKK3 are involved in several IKK activation pathway. K48, K63 or deU represent ubiquitination that target recipient for degradation, activation, or de-ubiquitination, respectively. The K63 ubiquitination target in IKK complex is IKKγ. TRAF2/TRAF5 was confirmed to be involved only in TNFα, not in DNA damage and TLR3 induced IKK activation. PKR is involved in TLR3, not TLR4 induced IKK activation. Question markers indicate unidentified molecule(s) or unconfirmed pathway(s).

therefore controls the strength and duration of NF-κB activation.[47] The acetyltransferases and deacetylase in this reversible event were suggested to be the p300/CBP (cAMP-responsive element-binding protein) and p300/CBP-associated factor, and HDAC3 (histone deacetylase 3), respectively.[47,48] In addition to RelA acetylation, it was also reported that p50 acetylation upon stimulation affects NF-κB activation, perhaps by enhancing the DNA binding activity of p50.[47,48] The enzymes responsible for p50 acetylation/deacetylation have not been described, neither the mechanism that coordinates acetylation/deacetylation and activation signaling pathways. Several questions should be answered to justify the in vivo role of NF-κB acetylation, the most important one is whether NF-κB acetylation/decetylation is a physiologically relevant event. This could be done by analyzing NF-κB activation in mice or cells deficient in p300/CBP or other putative NF-κB modification enzymes.

Ubiquitination might be the hottest field in NF-κB community recently. IκB Ubiquitination plays an essential role in IκB degradation and NF-κB activation induced by various extracellular stimuli.[15] Recent works suggested that several other NF-κB signaling molecules could also been ubiquitylated and the ubiquitination of these molecules is required for transducing NF-κB activation signal (Fig. 4). The type of ubiquitin linkage controls the fate of an ubiquitylated protein. K48-linked polyubiquitination generally targets proteins for degradation in the proteasome, whereas K63-linked ubiquitination can regulate protein function.[49] TRAF6 is the first NF-κB signal transducer reported to be an ubiquitin ligase to mediate IKK activation.[50,51] Later, TRAF2 was also found to be an ubiquitin E3 ligase,[52] although its role as an ubiquitin

ligase in NF-κB activation has yet to be explored. Another NF-κB signaling regulator A20 was reported to be a de-ubiquitination/polyubiquitination enzyme downregulating NF-κB signaling by de-ubiquitinating RIP (receptor interacting protein) and target it for degradation by polyubination.[53] The most interesting topic might be the ubiquitination of IKKγ. Upon nuclear export inhibitor Leptomycin B treatment, it was found that IKKγ competes with p65 and IKKα for binding to the N terminus of CBP and therefore leads to transcriptional repression of the NF-κB pathway.[54] Whether this is a truly physiological situation remains to be clarified since IKKγ-deficient mice show the phenotype similar to IKKβ- or p65-deficient mice, although it is possible that IKKγ has other function in addition to the activation of IKKβ and p65 upon TNFα stimulation during liver development. The existence of IKK-unbound free IKKγ has been reported by others.[55] These free IKKγ molecules shuttle between cytoplasma and nucleus. Upon genotoxic stress, free IKKγ is sumoylated in an ATM (ataxia telangiectasia mutant) independent manner. The sumoylation leads to nuclear localization of IKKγ. Although IKKγ is later desumoylated, this modification is required by the sequential ubiquitylation in an ATM-dependent manner and ultimately activation of IKK in the cytoplasma as evidenced by the ATM RNA interfering knocking down analysis. This work proposed a novel mechanism for NF-κB activation upon genotoxic stress. The reality of this novel mechanism remains to be testified by analyzing the sumoylation modification enzymes.

Inhibition of the family cylindromatosis tumor suppressor gene (CYLD) enhances the activation of NF-κB. CYLD binds to IKKγ and regulate IKK activity by de-ubiquitination of TRAF2, and to a less extent, of TRAF6.[56-58] CYLD may function through binding to TRIP (TRAF-interacting protein) and stabilize TRIP by removing the ubiquitins and thereby blocking NF-κB activation (Fig. 4).[59] The ubiquitination of IKKγ was also suggested to be regulated by c-IAP1 in TNFα activation of NF-κB.[60] Using purified protein and a cell-free system, it was found that Bcl-10 activates NF-κB through the intrinsic ubiquitin ligase activity of paracaspase MALT1 (mucosa associated lymphoid tissue),[61] or through MALT1 to induce oligomerization and activation of TRAF6 (Fig. 4).[62] TAK1 (transforming growth factor β-activated kinase 1) is further required to phosphorylate IKKβ in response to TCR activation.[62] Although different groups proposed unidentical model regarding IKKγ ubiquitination in the same Bcl-10 signaling pathway, the above work established signal flow chart from Bcl-10, MALT1, TRAF6, TAK1 and IKK to NF-κB activation, and emphasized the significance of IKKγ ubiquitination. Since the essential role of TRAF6 in TCR activation of NF-κB does not match the phenotype of TRAF6 knockout T cells, any significant functional defect of that has not been reported, there might be other missing piece(s) in this NF-κB activation signaling pathway, or compensation from other molecule such as TRAF2 as suggested.[62] Analysis of NF-κB activation in TRAF2-/-, TRAF6-/- double knockout T cells would be very helpful to elucidate this signaling pathway. It is also interesting to see how TRAF2 and TRAF6 function in TCR activation of NF-κB without affecting TNFα and IL-1 signaling wherein these two molecules were required for NF-κB activation.

Molecules Involved in Multiple NF-κB Activation Signaling Pathway

NF-κB activation signaling pathway is best understood in the immune and inflammatory system. Genetics dissection of antigen receptor induced-NF-κB activation was recently reviewed.[63] Receptors and adaptors for NF-κB were also reviewed in this book (see Chapter 3). Recent studies suggested that certain signaling molecules could be utilized by different pathways to activate IKK (Fig. 4). Following receptor-proximal signaling events upon TCR or BCR activation, different PKC isoforms are utilized to induce NF-κB activation during T cell and B cell development. In pro-B cells, preBCR activation of NF-κB is regulated by PKCλ through both IKKα and IKKβ.[64] In mature B cells, BCR activation of NF-κB may be regulated by PKCβ through IKKα.[65,66] It is important to note, however, that an additional IKKα-mediated mechanism other than increased NF-κB DNA binding activity must account for the PKCβ-associated defect since DNA binding by NF-κB is not the major consequence of PKCβ

inactivation.[64] This is also different from PKCθ-regulated NF-κB activation in T cells, which utilizes IKKβ instead of IKKα.[67]

Downstream signaling from PKC to NF-κB activation is believed to be mediated by CARMA1 (CARD carrying member of the MAGUK family proteins 1), Bcl10 and MALT1,[68] although how PKC activates CARMA1 and how MALT1 activates IKK remain to be determined in vivo. Of notice, TNFα- and IL-1- mediated NF-κB activation is not affected by CARMA1-, Bcl10- or MALT1-knockout, suggesting that these three signaling molecules are unique for a PKC-dependent pathway. Interestingly, although all of these three molecules are required for NF-κB activation induced by antigen receptor ligation, their functions in lymphocyte development are different. Only B cell and not T cell development is defective in mice deficient in CARMA1.[69] However, both B and T cell development are defective in Bcl10 and MALT1 knockout mice.[70-72] These findings suggest that in addition to CARMA1, other molecules might be involved downstream of PKC in T cells, which may also recruit Bcl10 and MALT1 to induce NF-κB activation. Ruland et al also suggested that except for MALT1, other molecule might exist in B cells to relay BCR activation signal to NF-κB activation since the B cell development is only affected moderately in MALT1 deficient mice.[72]

Recent progress suggested that some known NF-κB signaling transducers might be involved in several signaling pathway, although further in vivo studies are needed to verify it. The essential role of RIP for NF-κB activation has been established long time ago. RIP1, the NF-κB activator in TNFα signaling pathway,[73] was recently reported to be essential for TLR3-mediated NF-κB activation.[74] It would be interesting to determine how the RIP1 downstream signaling molecules function in TLR3 signaling without affecting TNFα signaling pathway. It was also reported that RIP1 is essential for DNA-damage-induced NF-κB activation by inducing IκBα degradation, suggesting that this activation may go through IKK.[75] Indeed, upon DNA damage, it was confirmed that RIP forms a complex with IKK, and IκBα degradation requires IKKβ. Interestingly, the kinase activity of RIP is not required and IKK activation was not confirmed by kinase assay. Although RIP is involved in both TNFα and DNA damage-induced NF-κB activation, other signaling molecules, such as TNFR1 (TNF receptor 1), TRAF2, TRAF5 and FADD (Fas-associated death domain protein) are involved only in TNFα induced NF-κB activation. Although ATM was found to be required for the formation of RIP-IKK complex upon DNA damage, how does RIP activate IKK remains to be solved.[75]

RIP2, another RIP family protein, is involved in TLR-mediated NF-κB activation.[76,77] RIP2$^{-/-}$ cells and mice exhibit impaired responses, including defective NF-κB activation, cytokine production and are resistant to endotoxic shock in response to LPS, dsRNA and peptidoglycan stimulation. It was suggested that RIP2 plays a role in signaling by TLR2/4/9 and Nod1.[76,77] RIP2 is also involved in TCR signaling to NF-κB activation. RIP2-deficient T cells show severely reduced NF-κB activation upon TCR engagement, as well as some other defect in TCR signaling, T cell differentiation and function,[76,77] whereas RIP1 is important for TNFR2 signaling in thymocyte development and apoptosis, but is not required for thymocyte proliferation.[78] How RIP1 and RIP2 are involved in TCR signaling differentially is not yet clear. Ruefli-Brasse et al reported that upon TCR activation, RIP2 associates and phosphorylates Bcl-10 and therefore involved in TCR mediated NF-κB activation.[79]

Different from other RIP family members, RIP3 is dispensable for NF-κB activation by several NF-κB activators, including the engagement of TCR, BCR, TNFR1, TLR2 and TLR4.[80] In contrast, RIP3 negatively regulates the RIP1-induced NF-κB activation in TLR3 signaling pathway.[74] Analysis of transgenic mice expressing a kinase dead version of another RIP family member, RIP4, suggested that RIP4 may be required for BCR signaling to NF-κB.[81] RIP4 was cloned based on its association with PKCβ,[82] suggesting that the involvement of RIP1 or RIP2 in TCR signaling may allow for the interaction with other PKC isoforms. Biochemical experiments suggested that RIP4 is involved in PKC activation of NF-κB that is independent of Bcl10.[83] However, additional in vivo data is needed to confirm the existence of this

Bcl10-independent signaling pathway in NF-κB activation. A detailed understanding of RIP function awaits further investigation.

Downstream of RIP, TRAF2 and TRAF5 are the mediators of IKK activation in TNFα induced NF-κB activation signaling pathway. TRAF2 regulates TNFα induced NF-κB activation in cooperation with TRAF5. TRAF2 and TRAF5 single-knockouts show a mild phenotype, but TRAF2/TRAF5 double knockout are severely impaired in TNFα-induced NF-κB activation, and are therefore very sensitive to TNFα-induced apoptosis.[84-86] How TRAF2/5 and RIP activate IKK and NF-κB is still controversial.[9] It was reported that MEKK3 is involved in IKK activation downstream of TRAF2 and RIP in response to TNFα stimulation.[87] Interestingly, MEKK3 appears to be a multiple edge sword, it is also required for IL-1 and LPS induced activation of NF-κB, as well as JNK and p38, but not ERK,[88] perhaps due to the existence of TRAF6, another member of TRAF family in the IL-1 and TLR signaling pathway.

NIK, which is required for activation of the noncanonical NF-κB pathway by Blys in B cells as discussed above, also plays a role in TCR induced NF-κB activation, which is distinct from the PKC mediated signaling pathway.[89] Since NF-κB activation is only slightly attenuated in NIK mutant thymocytes as well as mature T cells, it appears that NIK may not play a major role in NF-κB activation in T cells. PKR, the sensor kinase for virus infection and perhaps also involved in LPS stimulated NF-κB activation,[90] was reported to directly activate IKK by protein-protein interaction.[91,92] Using Bone marrow or fetal liver derived macrophages of various knockout mice, Hsu et al validated the TLR 4 pathway in macrophage.[93] Consistent with the previous finding, in addition to the role in antivirus infection, they found that PKR is required for macrophage apoptosis after activation of TLR4. However, perhaps due to cell type specificity, PKR is not required for the activation of MAPK p38, JNK, ERK or activation of IKK in response to LPS stimulation in macrophage. This is different from that in embryonic fibroblast where PKR is required for p38 activation in response to LPS and other proinflammatory stimuli.[90] Hence, the full physiological function of PKR in NF-κB activation remains to be determined in a variety of cell types.

TAK1 might be the most potential candidate as IKK kinase in multiple signaling pathways, perhaps due to its interaction with TRAF6. Interaction of TAK1 and TRAF6 involves TAK1 binding proteins TAB1 and TAB2. Association of TAK1 with TRAF6 leads to activation of TAK1, and activated TAK1 in turn activates IKK.[50] However, TAB1 was suggested to be important for heart development.[94] Analysis of TAB2 knockout mice indicated that the TAK1:TAB complex is not essential for IL-1 and TNFα signaling, but is required for preventing liver apoptosis,[95] suggesting the existence of other signaling molecules that link TRAF6 and IKK. One candidate of these signaling molecules is TAB3.[96] TAB3 and TAB2 bind cooperatively, but not competitively, to TRAF6, and TAB3 associates with both TRAF2 and TRAF6 and therefore links them with TAK1. RNA interfering experiment demonstrated that TAB2 and TAB3 play a redundant but critical role in the IL-1- and TNF-induced activation of TAK1.[96] These finding explained the involvement of TAK1 in both IL-1 and TNF signaling pathway.[97] If this were the truly physiological situation, it would be interesting to explore the differential role of TAK1 and MEKK3, another MAP3K involved in both IL-1 and TNF induced NF-κB activation.[87,88] Much convincible data should be the analysis of TAK1 knockout mice and cells.

The critical role of IKK and many of its upstream signaling molecules in NF-κB activation has been confirmed by gene targeting experiments. IKK upstream signaling molecules are diverse. A better understanding of NF-κB activation may provide helpful information for the design of drugs targeting NF-κB in various diseases such as cancer, inflammation, autoimmune diseases and infectious disease. However, due to the general involvement of NF-κB in developmental and functional aspects of various cells, cell type-specific inhibition of NF-κB is needed for meaningful NF-κB targeted therapies. Attractive targets for such drugs include specific inhibitors for PKCθ in T lymphocytes, PKCβ in B lymphocytes, or Bcl10 in T and B cells. On the other hand, transcriptional targets of NF-κB could be more specific target, and might also represent interesting modalities for drug intervention.

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CHAPTER 3

Receptors and Adaptors for NF-κB Signaling

Shao-Cong Sun* and Edward W. Harhaj

Cells communicate with their environment via various surface receptors that recognize specific molecules (ligands) present in the extracellular environment. Upon binding to a specific ligand, the receptors transduce signals into the cell, triggering multiple intracellular signaling cascades that lead to gene expression and other biochemical events involved in specific cellular functions. The signaling pathway that leads to activation of the transcription factor NF-κB has drawn much attention, since it regulates diverse biological processes such as immune and inflammatory responses, cell growth and survival, and tumorigenesis.[1-4] In this chapter, we summarize the receptors and their intracellular signaling molecules that target NF-κB activation. Consistent with its pleiotropic biological functions, the NF-κB signaling pathway responds to signals initiated by a large variety of receptors (Table 1). The focus of this chapter will be on three major families of the NF-κB-inducing receptors: the antigen receptors that mediate antigen recognition by lymphocytes, the toll-like receptors (TLR) involved in innate immune responses and the connection between innate and adaptive immune responses, and members of the tumor necrosis factor receptor (TNFR) superfamily that mediate diverse biological functions.

Antigen Receptors

Lymphocytes of the adaptive immune system have evolved a mechanism to recognize a great diversity of antigens in order to detect and combat a wide range of pathogens. This unique function of lymphocytes is mediated by their surface antigen receptors, the B-cell receptors (BCR) and T-cell receptors (TCR), multiprotein complexes composed of clonally variable antigen-binding subunits and invariant signaling chains.[5] TCR recognizes antigenic peptides bound to major histocompatibility complex (MHC) molecules on antigen presenting cells (APC), whereas BCR recognizes epitopes associated intact antigens. However, the intracellular signaling network leading to NF-κB activation is highly similar for these two types of antigen receptors. In both cases, the network involves receptor-proximal signaling events characterized by the activation of protein tyrosine kinases (PTKs), signal amplification involving phosphorylation and membrane-recruitment of adaptor molecules and other factors, and activation of the NF-κB effector kinase, IKK (IκB kinase), by a distal signaling complex composed of several recently characterized adaptor proteins.[6]

Receptor-Proximal Signaling Events

Following antigen binding, both BCR and TCR form clusters and are recruited to membrane compartments, enriched in cholesterol and sphingolipid, known as lipid rafts.[7] Many other signaling factors, including Src family of PTKs, the protein tyrosine phosphatase CD45,

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NF-κB/Rel Transcription Factor Family, edited by Hsiou-Chi Liou.
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Table 1. NF-κB-inducing receptors

Receptors	Major Function
Antigen receptors	
BCR	B-cell development and activation
TCR	T-cell development and activation
Pattern recognition receptors	
TLR1-TLR11	Activation of macrophages, neutrophils, DCs, B cells, etc
NOD1 and 2	Intracellular pathogen recognition
Scavenger receptors	Activation of phagocytes
TNFR superfamily	
TNFR 1 and 2	Activation and apoptosis of diverse cell types
4-1BB	T-cell costimulation
Baff-R	B-cell maturation
CD27	T-cell costimulation
CD30	T-cell activation, apoptosis
CD40	B-cell differentiation, DC maturation
Fas	Cell death, lymphocyte homeostasis
DR4, 5	Apoptosis of transformed cells, thymocytes
EDAR	Ectodermal differentiation
XEDAR	Skeletal muscle homeostasis?
LTβR	Secondary lymphoid tissue development
OX-40	T-cell memory
RANK	Osteoclast and DC maturation
RELT	T-cell costimulation?
TIR domain-containing cytokine receptors	
IL-1R	Host defense and inflammation
IL-18R	T helper 1 (T_H1) cell development and function
Cell adhesion molecules	
α5β1 integrin	Cell adhesion and angiogenesis
α5β3 integrin	Survival of endothelial cells
α6β4 integrin	Survival of mammary epithelial cells
β2 integrins	Activation of neutrophils and monocytes
G protein-coupled receptors	
KSHV-GPCR	Transformation of vascular endothelial cells
C3a, C5a receptors	Proinflammatory in monocytes
CXCR1, 2, 6	Chemoattractant and proinflammatory
Bradykinin receptor (B2)	Proinflammatory peptide
Proteinase-activated receptors	Proinflammatory in endothelial cells and microglia
Lysophosphatidic acid receptors	Growth factor for fibroblasts and endothelial cells
Growth factor receptors	
GM-CSF-R	Proliferation and differentiation of hematopoetic cells
NGF-R (p75, TrkA)	Survival of neurons
PDGF-R	Survival, proliferation and migration of mesenchymal cells
EGF receptors	Proliferation, differentiation and survival

and adaptor molecules, are also concentrated in the lipid rafts. As a result of their aggregation within the lipid rafts, several Src family of PTKs are activated, which constitutes the initial step of intracellular signaling mediated by the antigen receptors. The primary members of the Src PTKs include Lck and Fyn in T cells, and Fyn, Lyn, and Blk in B cells. Upon activation, these kinases phosphorylate specific tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the cytoplasmic tails of BCR and TCR invariant chains. Phosphorylated ITAMs serve as docking sites for recruiting PTKs of the Syk family, which includes Syk in B cells and ZAP-70 in T cells. Once activated in the receptor complex, Syk and ZAP-70 amplify the receptor signals by phosphorylating various downstream targets (Fig. 1).

Adaptors Are Central Components Involved in Signal Amplification by ZAP-70 and Syk

Among the targets of ZAP70 and Syk are a number of adaptor molecules that play a central role in transducing the receptor-proximal signal to downstream signaling cascades.[8,9] One such adaptor that primarily functions in T cells is LAT (linker of activation in T cells), which is identified as a protein associated with the plasma membrane via palmitoylated cysteine residues. Upon phosphorylation by ZAP-70, LAT interacts with phospholipase C-γ (PLC-γ) and recruits this lipid enzyme to the inner face of the plasma membrane, where it is activated via phosphorylation by PTKs from the Src, Syk, and Tec families. Activated PLC-γ digests the membrane phospholipids phosphotidylinositol bisphosphate (PIP_2) into two important second messengers, diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which mediate protein kinase C (PKC) activation and calcium mobilization, respectively (Fig. 1). In B cells, a functional homolog of LAT, termed BLNK (B-cell linker, also named SLP-65) plays a similar role. BLNK is a cytoplasmic protein, but can be recruited to the membrane and phosphorylated by Syk upon BCR stimulation. The phosphorylated BLNK interacts with PLC-γ2 and a Tec PTK, Bruton's tyrosine kinase (Btk), and the complex formation allows PLC-γ2 to be phosphorylated and activated, resulting in generation of DAG and IP3.

Another adaptor protein phosphorylated by ZAP-70 in T cells is SLP-76. Like the B-cell adaptor BLNK, SLP-76 is a cytoplasmic protein but is recruited to the membrane upon phosphorylation by ZAP-70. This adaptor interacts with a guanine nucleotide exchange factor (GEF), Vav, and serves to recruit Vav to the plasma membrane for activation. This activation process requires the cooperative action of the TCR signal and a costimulatory signal, which in naïve T cells is primarily mediated by the CD28 molecule. As a result, both SLP-76 and Vav are required for NF-κB activation by the TCR/CD28 signals.[10,11] Vav appears to exert its NF-κB-inducing function in different ways. For example, Vav regulates the recruitment of PKCθ to lipid rafts,[12] an essential step in the activation of this key signaling component of the NF-κB pathway.[13] This function of Vav also requires its canonical target, the small GTP-binding protein Rac. Additionally, Vav has been suggested to function in a more upstream signaling step involving activation of PLC-γ.[14] More recently, Vav has been shown to directly interact with a component of IKK, IKKα, which is required for Vav-induced NF-κB activation.[15]

The adaptor molecule SHC (SH2 domain-containing transforming protein) is another factor that connects the receptor-proximal signal to downstream pathways leading to NF-κB activation. Upon TCR ligation, SHC is rapidly recruited to the TCR complex, where it is phosphorylated by ZAP-70 and the Src family of PTKs.[16] Interestingly, SHC is required for activation of a specific member of the NF-κB family, c-Rel, that plays a critical role in the production of the T-cell growth factor interleukin-2 (IL-2).[17] How SHC regulates this specific axis of NF-κB signaling is not clear.

Connecting the Antigen Receptor Signals to NF-κB Activation by Carma/Bcl10/MALT1

Activation of PKC isoforms is an important consequence of the receptor-proximal signal transduction and signal amplification in B and T cells. In T cells, PKCθ plays a critical role in

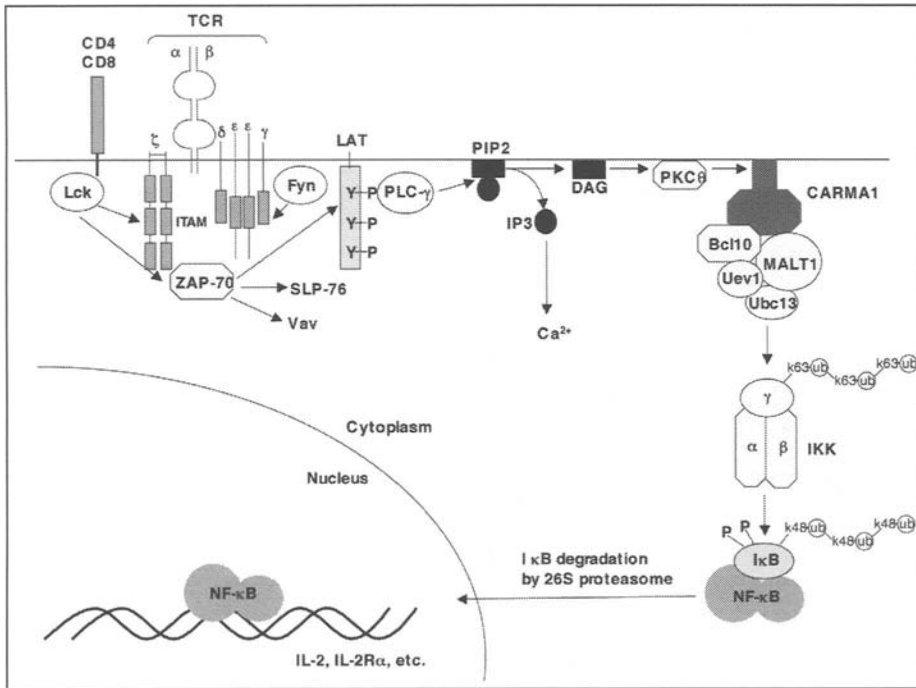

Figure 1. NF-κB signaling pathway initiated through the TCR. TCR ligation triggers the activation of Src PTKs, Lck and Fyn, which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domains of the TCR invariant chains. The phosphorylated ITAMs serve as an anchor to recruit ZAP-70 to the TCR complex, where it is activated through phosphorylation by Lck. The activated ZAP-70 amplifies the TCR signal by phosphorylating a number of target proteins, including LAT, SLP-76, and Vav, all of which are involved in NF-κB activation. Upon membrane recruitment, LAT activates PLC-γ, which in turn cleaves the membrane lipid PIP2 to DAG and IP3, leading to activation PKC and calcium mobilization, respectively. PKCθ induces the formation of an IKK-activating signalsome that activates IKK through a mechanism that involves Lys63-linked IKKγ ubiquitination.

NF-κB activation by the TCR signal.[18] PKCθ-deficient T cells are defective in the activation of both NF-κB and AP-1, which is associated with a blockade in IL-2 production and cell proliferation in response to TCR ligation. A functional homologue of PKCθ in B cells is a conventional PKC isoform, PKCβ, which is required for NF-κB activation by the BCR signal.[19,20]

Significant progress has been made recently to understand the signaling events that link PKCs to NF-κB activation. Central to these events is the coordinated action of a protein complex composed of CARMA1, Bcl10, and MALT1[18] (Fig. 1). Genetic deficiency in any one of these molecules abolishes NF-κB activation by antigen receptor ligation.[18] CARMA1 (also known as CARD11 and Bimp3) is a member of the CARD-containing membrane-associated guanylate kinase (MAGUK) family and is predominantly expressed in lymphoid tissues. CARMA1 interacts with Bcl10 via the CARD domain present in both proteins. Additionally, CARMA1 may also interact with MALT1,[21] a member of the paracaspase family, although MALT1 is generally thought to associate with the CARMA1/Bcl10 complex through binding to Bcl10.[18] Within this trimeric complex, CARMA1 provides a functional link with the upstream signals. CARMA1 is normally distributed diffusely in the plasma membrane but is rapidly recruited into the lipid rafts upon TCR crosslinking;[22] this relocalization appears to require PKCθ, although the underlying mechanism remains unclear. Via physical interaction with CARMA1, both Bcl10 and

MALT1 are also recruited to the lipid rafts, where they undergo oligomerization and trigger the assembly of a ubiquitin-dependent IKK-activating signalsome.[23,24]

According to two recent studies,[23,24] Bcl10 and MALT1 associate with two ubiquitin-conjugating enzymes, Ubc13 and Uev1A (also known as MMS2), and catalyze Lys63-linked ubiquitination of the IKK regulatory subunit, IKKγ (also named NEMO), which in turn is required for IKK activation by the TCR signal (Fig. 1). What remains controversial is the identity of the E3 ubiquitin ligase. Although one study suggests that MALT1 may function as a ubiquitin ligase,[24] the other study challenges this idea and provides in vitro evidence for the involvement of a known ubiquitin ligase, TNF receptor-associated factor 6 (TRAF6), in the ubiquitination of IKKγ and activation of IKK downstream of TCR.[23] Notwithstanding, both studies establish IKKγ ubiquitination as a mechanism of IKK activation by the Bcl10/MALT1 complex.

Toll-Like Receptors

Toll-like receptors (TLRs) form a major family of pattern recognition receptors (PRRs) that mediate detection of microbes based on general structural features, known as pathogen-associated molecular patterns (PAMPs).[25] TLRs not only play a critical role in the early-phase host defense against infections but also regulate the nature and magnitude of the adaptive immune response.[26,27] To date, eleven TLRs have been identified in mammals, which recognize a broad range of microbial components, such as peptidoglycans, double-stranded RNA, lipopolysaccharide, and CpG DNA motifs of bacterial origin.[28] The TLRs contain an extracellular domain with leucine-rich repeats (LRR) and a cytoplasmic region sharing homology with the IL-1 receptor (IL-1R), termed Toll/IL-1R homology (TIR) domain. The LRR mediates ligand recognition, while the TIR domain is required for initiating intracellular signaling. Largely through activation of NF-κB and MAP kinases (MAPKs), TLRs induce the expression of various proinflammatory cytokines, including TNF-α, IL-1, IL-6, and IL-12. Additionally, certain TLR members, TLR3 and TLR4, also induce expression of interferon-responsive genes through activation of both NF-κB and transcription factors belonging to the interferon-regulated factor (IRF) family (Fig. 2).

Adaptors Involved in TLR-Proximal Signaling

Initiation of intracellular signaling by TLRs requires a family of TIR domain-containing adaptor proteins; these include myeloid differentiation protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP, also named MAL for MyD88-adaptor like), TRIF (also named TICAM-1), and TRAM.[28] Upon ligand binding, TLRs recruit the adaptors to their cytoplasmic tails via TIR/TIR interactions. Although most of the adaptors are differentially used by the different TLRs, MyD88 serves as a common adaptor for all TLR members. In addition to the TIR domain, MyD88 contains a death domain that mediates interaction with a family of death domain-containing serine/threonine kinases, IRAKs (interleukin-1-receptor-associated kinases). MyD88 functions to recruit IRAKs to the TLR complex for their activation, which in turn is required for initiation of downstream signaling events, including activation of IKK and its target NF-κB. Germline inactivation of the MyD88 gene abolishes NF-κB activation by all the TLRs, except for TLR3 and TLR4 that retain partial and delayed NF-κB signaling activity.[28] Moreover, induction of proinflammatory cytokine genes by all the TLRs is blocked in MyD88-deficient mice, although the ability of TLR3 and TLR4 to induce interferon-responsive genes is retained. Thus, TLR3 and TLR4 have both MyD88-dependent and independent signaling pathways, whereas the signaling function of the other TLR members is completely dependent on MyD88.

In contrast to the universal role of MyD88, TIRAP is selectively involved in the signaling function of TLR2 and TLR4 (Fig. 2). Gene targeting studies suggest that TIRAP functions in the MyD88-dependent signaling pathway but is not involved in the MyD88-independent TLR signaling.[29,30] The MyD88-independent signaling function of TLR3 and TLR4 is me-

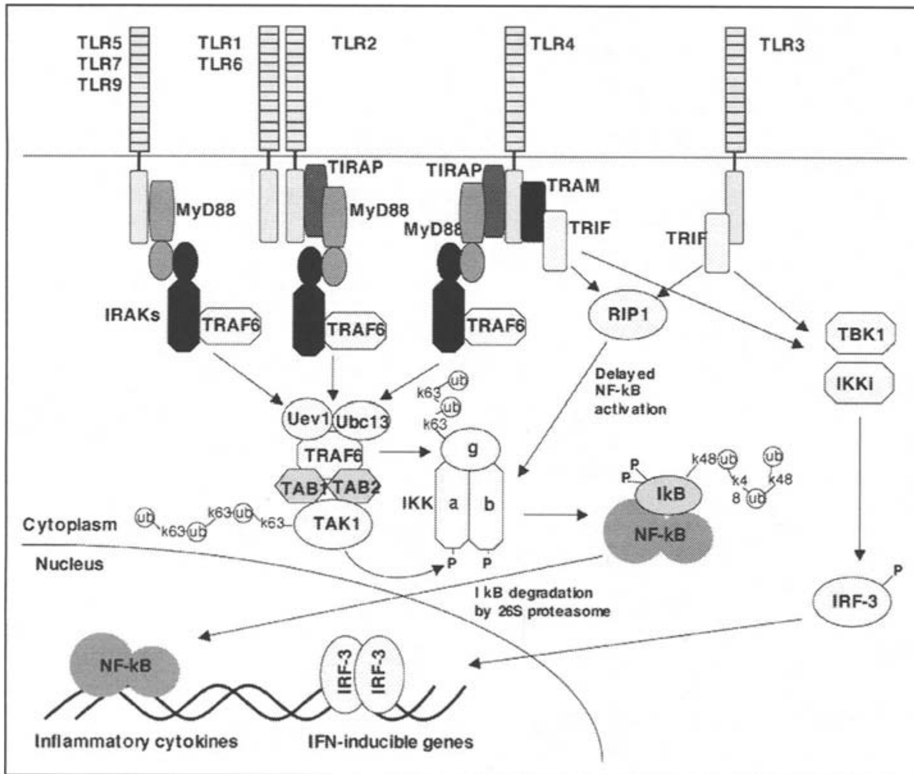

Figure 2. NF-κB signaling pathway initiated through TLRs. TLRs interact with microbial components via their extracellular leucine repeat domain and intracellular signaling adaptors via their cytoplasmic TIR domain. A common TLR adaptor is MyD88, which is involved in NF-κB activation by all the TLRs. MyD88 recruits IRAKs and TRAF6 to the receptor complex, triggering the assembly of a signalsome composed of TRAF6 and several other proteins. TRAF6 functions as a ubiquitin ligase inducing the Lys63-linked ubiquitination of both IKKγ and an IKK-activating kinase TAK1. Both IKKγ ubiquitination and TAK1-mediated IKK phosphorylation may contribute to IKK activation. The adaptor TRIF mediates delayed activation of NF-κB via the RIP1 kinase. Additionally, TRIF is required for activation of the IRF-3 by TLR3 and TLR4.

diated by the more recently identified adaptor TRIF. TRIF deficiency blocks the delayed activation of NF-κB and the induction of interferon-inducible genes by TLR3 and TLR4, although it has no effect on NF-κB activation by other TLRs or the MyD88-dependent early-phase NF-κB activation by TLR4.[31,32] TRIF directly interacts with TLR3 and binds to TLR4 via another adaptor, TRAM.[33,34] As a result, TRAM is specifically required for the TRIF-dependent and MyD88-independent signaling function of TLR4.[28]

Connecting the TLR Signals to NF-κB Activation by IRAKs and TRAF6

IRAK family of kinases plays a critical role in transducing the MyD88-dependent TLR signals. Gene targeting studies demonstrate that IRAK-4 is a key member of this family that is essential for NF-κB activation by both TLRs and IL-1R.[35] IRAK-4 appears to function as an activator of another IRAK member, IRAK-1. Upon ligand binding, both IRAK4 and IRAK1 are recruited to the receptor complex, where IRAK-1 is phosphorylated, likely by IRAK-4.[36] IRAK-1 in turn recruits the adaptor molecule TRAF6 for activation. The IRAK-1/TRAF6

complex is then released to the cytoplasm, resulting in the formation of a signalsome composed of TRAF6, an IKK-activating kinase-TGF-β activating kinase (TAK1), two adaptor proteins, TAB1 and TAB2, as well as the ubiquitin-conjugating enzymes Ubc13 and Uev1A[37-40] (Fig. 2). Within this protein complex, TRAF6 functions as an E3 ubiquitin ligase to catalyze Lys63-linked polyubiquitination, which appears to trigger the activation of TAK1 and subsequent activation of IKK.[40] Recent studies suggest that the TLR-mediated NF-κB activation in macrophages and certain subset of B cells also requires Bcl10,[41] an adaptor known to be involved in IKK activation downstream of antigen receptors. Interestingly, as discussed above, Bcl10 is a component of the TRAF6 signaling complex in T cells.[23] It is thus conceivable that Bcl10 may also participate in TRAF6-induced IKK activation in the TLR signaling pathway.

MyD88-Independent Pathway of NF-κB Activation

As noted above, both TLR3 and TLR4 induce delayed NF-κB activation in MyD88-deficient cells, and this MyD88-independent signaling pathway is mediated by the adaptor TRIF (Fig. 2). Unlike the MyD88-dependent signaling pathway, the TRIF-dependent pathway of NF-κB activation does not seem to go through IRAKs but instead involves direct interactions with downstream signaling factors. A recent study suggests that TRIF directly interacts with TRAF6 and TAK1, resulting in activation of both NF-κB and IRF-3.[42] The N-terminal region of TRIF contains three TRAF6-binding motifs that are required for the TRIF/TRAF6 interaction. Interestingly, the C-terminal region of TRIF induces NF-κB signaling via another mechanism that involves interaction with a serine/threonine kinase termed receptor-interacting protein 1 (RIP1).[43] This region of TRIF contains a RIP homotypic interaction motif (RHIM) that mediates the interaction of TRIF with RIP1. The critical role of RIP1 in TRIF-mediated NF-κB activation is supported by studies using RIP1-deficient mouse embryonic fibroblasts.[43]

IKK, a Kinase Controlling Two Signaling Pathways Downstream of TLR4

Recent studies revealed a surprising function of IKK downstream of the TLR4. In addition to its role in NF-κB activation, the canonical IKK plays an essential role in the activation of Tpl2 (also named Cot),[44] a MAPK kinase kinase (MAP3K) specifically regulating activation of ERK MAPK by the TLR4 ligand LPS.[45] This novel function of IKK is mediated through phosphorylation of the nfκb1 gene product p105,[44] which functions as both a stabilizer and inhibitor of Tpl2.[46,47] In all the cell types so far analyzed, Tpl2 and p105 exist as a stable complex,[46-48] which appears to contain at least one other protein, ABIN-2.[49] In NFκB1-deficient macrophages and other cell types, Tpl2 is rapidly degraded due to the lack of its binding protein p105, which is associated with a defect in LPS-stimulated activation of ERK.[46] Interestingly, activation of the Tpl2 complex requires phosphorylation and degradation of p105, a process that requires the action of IKKβ and IKKγ.[44] By regulating the activation of Tpl2, IKK targets another TLR-mediated signaling pathway involved in ERK MAPK activation.

TNF Receptor Superfamily

The tumor necrosis factor receptor (TNFR) superfamily consists of at least 30 members, which regulate cell proliferation, apoptosis, and/or differentiation. TNFRs contain cytoplasmic regions of varying lengths, some of which contain death domains that dictate the specificity of the desired signal response. All of the receptors appear to utilize common mechanisms to activate the transcription factors NF-κB and AP-1. Upon ligand binding, TRAF family members are recruited to the receptor and coordinate signaling cascades consisting of adaptor proteins, kinases, and other enzymatic components. Recent studies reveal an emerging theme of complexity in TNFR signaling mechanisms, including usage of multiple receptors by single ligands, cross-talk between NF-κB and AP-1 signaling networks, and ubiquitination of signaling proteins as regulatory mechanisms.

TNFR1/TNFR2

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine and a key regulator of cell death, proliferation, inflammation, and immunity. TNF-α binds to two distinct receptors, TNFR1 and TNFR2. TNFR1 binds to soluble TNF-α whereas TNFR2 binds to membrane-bound TNF-α.[50] Genetic deletion of either receptor ablates the majority of TNF signaling, suggesting cooperativity between the two receptors.[51] A trimeric form of TNF-α binds to TNFR1 and activates NF-κB and AP-1 transcription factors, which in turn regulate the expression of genes involved in apoptosis and inflammation.[52] Inhibition of NF-κB signaling, or treatment of cells with protein synthesis inhibitors, sensitizes cells to TNF-mediated apoptosis, indicating a critical role for NF-κB in the transcription of genes involved in the protection from cell death.[50]

TNFR1 ligation elicits a signaling cascade that leads to activation of NF-κB and c-jun NH_2-terminal kinase (JNK). Binding of TNF-α to TNFR1 triggers the trimerization and translocation of TNFR1 to lipid rafts. TNFR1 recruits the adaptor protein TRADD that serves as a platform for the binding of other signaling proteins, including RIP1 and TRAF2, known to mediate activation of IKK and NF-κB.[53] In the absence of TRAF2, TNF-α still activates NF-κB, however the combined deletion of TRAF2 and TRAF5 abrogates TNF-mediated NF-κB activation.[54] In addition to the TNFR1, TRADD, RIP1, and TRAF complex in the plasma membrane, a distinct complex comprised of TRADD, RIP1, FADD, and caspase-8 assembles in the cytoplasm and mediates cell death.[55] However, simultaneous activation of NF-κB protects against cell death by inducing the expression of the caspase-8 inhibitor, $FLIP_L$, which assembles into the cytoplasmic death-inducing complex.[55] TNFR2 does not directly trigger cell death, since FADD is not recruited to TNFR2. However, TNFR2 may influence cell death by regulating the activity of JNK which may be pro-apoptotic.[56] Thus, the relative levels of TNFR1 and TNFR2 expression, as well as IKK and JNK activation, are important factors for TNF-mediated cell death.

There is considerable evidence for extensive cross-talk between the NF-κB and AP-1 pathways during TNF-α signaling. Murine embryonic fibroblasts (MEFs) lacking IKKβ or the NF-κB subunit RelA/p65 exhibit prolonged TNF-α-mediated JNK activation and increased apoptosis.[57,58] It has been proposed that NF-κB-induced genes such as GADD45β or X-linked-inhibitor of apoptosis (XIAP) may inhibit cell death by suppressing JNK activation.[57,58] GADD45β directly binds and inhibits the catalytic activity of MKK7, a MAP kinase kinase that is upstream of JNK, as a mechanism to inhibit TNF-α-mediated JNK activation.[59]

IKK is recruited to the TNFR via a direct interaction of the IKK regulatory subunit IKKγ with RIP1.[60] In addition, the chaperone proteins, Cdc37 and Hsp90, interact with IKK and mediate their shuttling from the cytoplasm to the membrane.[61] TNF-α is a potent activator of IKKβ, a central component of the canonical NF-κB pathway. IKKβ phosphorylates IκBs leading to their ubiquitination and degradation by the proteasome, thus allowing the liberation and nuclear translocation of NF-κB heterodimers (see Fig. 3). In contrast, TNF-α does not stimulate p100 processing to p52 in the noncanonical NF-κB pathway that is dependent on the MAP3K, NF-κB inducing kinase (NIK), and IKKα.[62,63]

The mechanism of IKK activation within the TNFR complex in lipid rafts has remained elusive. Since ubiquitination of signaling proteins by the assembly of Lys63 (K63)-linked ubiquitin chains is critical for the activation of IKK by T cell receptor stimulation of T cells,[23] it is possible that a similar mechanism exists for TNFR1 signaling. Indeed, a recent study suggests that A20, a negative regulator of TNF-mediated NF-κB activation, inactivates RIP1 by a unique mechanism involving deubiquitination of K63-linked ubiquitin chains as well as the coordinated catalysis of K48-linked ubiquitin chains, leading to RIP1 inactivation and subsequent degradation by the proteasome.[64] Moreover, IKKγ is also a target of ubiquitination by TNF-α stimulation.[65] Thus, it is tempting to speculate that ubiquitination of signaling proteins plays an important regulatory role for the activation of IKK by TNFR superfamily members.

The role of MAP3Ks in activation of NF-κB by TNF-α has been extensively studied. Although many MAP3Ks, such as NIK or MEKK1, activate NF-κB when overexpressed, genetic studies have revealed that only MEKK3 and TAK1 are critical for TNF-mediated NF-κB activation. MEKK3 interacts with RIP, and links RIP to activation of IKK by directly phosphorylat-

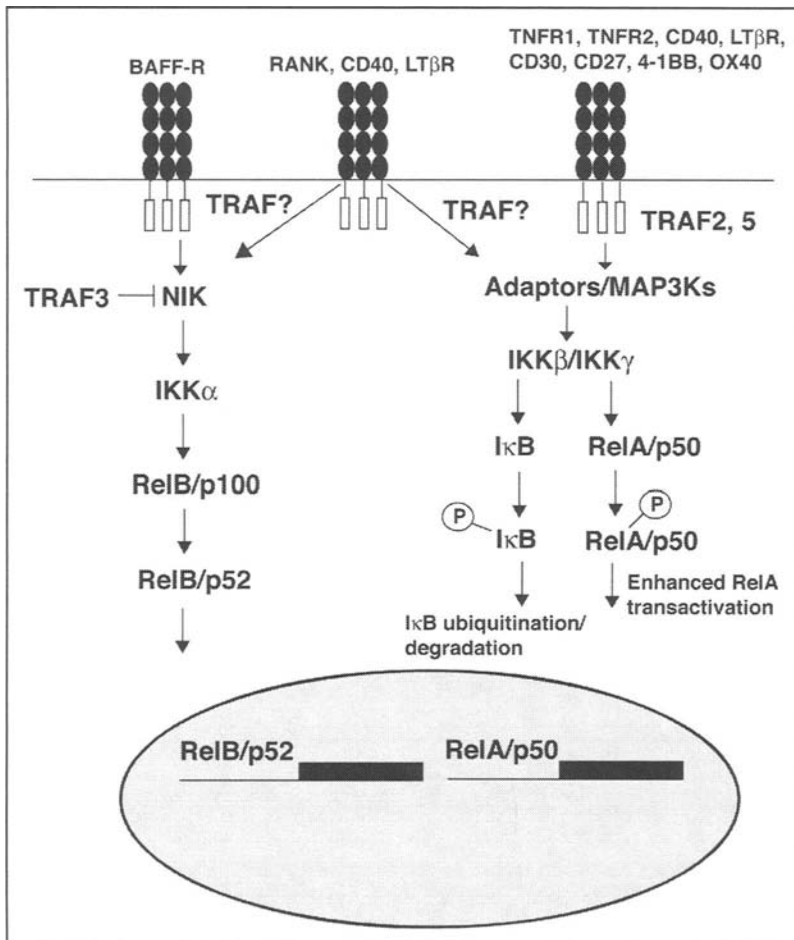

Figure 3. NF-κB signaling pathways activated by TNFR family members. The majority of TNFR proteins activate the canonical NF-κB pathway that consists of IκB degradation and nuclear translocation of NF-κB heterodimers. TNF-α, and possibly other TNF family members, mediates the phosphorylation of RelA that potentiates RelA-mediated transactivation. A single TNFR family member, BAFF-R, activates only the noncanonical NF-κB pathway that results in processing of p100 to p52. A subset of TNFR proteins, RANK, CD40 and LTβR, activate both the canonical and noncanonical pathways.

ing IKKβ.[66] Knockdown of TAK1 expression by siRNA transfection reduced TNF-α-mediated NF-κB activation, suggesting that TAK1 may be a critical component of TNF signaling.[67]

TNF signaling also enhances NF-κB activation by mediating site-specific phosphorylation of multiple serine residues within RelA, all of which appear to enhance NF-κB transactivation. For example, casein kinase II mediates the phosphorylation of RelA on serine 529 in response to TNF.[68] In addition, serine 536 of RelA is phosphorylated by IKK during TNF-α stimulation.[69] TNF-α stimulation also induces RelA phosphorylation on serine 311 that is dependent on the atypical protein kinase C family member, PKCζ.[70] Finally, RelA is phosphorylated on serine 276 by the mitogen- and stress-activated protein kinase-1 (MSK1) within the nucleus in response to TNF-α stimulation.[71] It is unclear which of the identified phosphoacceptor sites within RelA are critical for TNF-α-mediated NF-κB transactivation. Reconstitution of $RelA^{-/-}$ MEFs with RelA point mutants revealed that only mutation of serine 276 abrogated TNF-α-mediated NF-κB

activation.[72] However, different approaches, such as the generation of RelA mutant knock-in mice, may be required to appreciate the role of specific RelA serines in response to TNF-α signaling.

CD40

CD40 is a member of the TNFR superfamily and is expressed in a wide variety of cell types such as B cells, dendritic cells (DCs), macrophages, endothelial and epithelial cells, and neurons.[73] The ligand for CD40, CD154 (also known as CD40L), is mainly expressed in activated T cells, but it may also be expressed in monocytes. Binding of CD40L to CD40 regulates cell survival, proliferation, germinal center formation, and immunoglobulin class switching in B cells as well as antigen presentation, maturation, and survival of dendritic cells.[73] Binding of CD40 to CD40L triggers clustering of CD40 within lipid rafts where signaling complexes are assembled. The intracellular domain of CD40 contains two binding sites for TRAF proteins and has been shown to interact with TRAF 1, 2, 3, 5, and 6.[74] The TRAFs then couple CD40 signaling to the activation of NF-κB, phosphoinositide 3-kinase (PI3K), and Map kinases ERK, p38, and JNK.[74]

CD40 activates the canonical NF-κB pathway in B cells and dendritic cells.[75] CD40 ligation of B cells also triggers the noncanonical NF-κB pathway resulting in NIK/IKKα-dependent p100 processing and p52/RelB nuclear translocation.[76] The contributions of the canonical and noncanonical NF-κB pathways to the CD40 gene program have been recently evaluated. CD40 signaling through the canonical NF-κB pathway regulates the proliferation of B cells whereas both pathways are required for B cell aggregation.[77] Furthermore, the gene program elicited by CD40 in B cells requires specific contributions from both the canonical and noncanonical NF-κB pathways.[77]

The signaling intermediates downstream of CD40 that mediate IKK activation have not been well characterized. Act1 (also known as CIKS) was identified as an IKKγ binding protein that is recruited to CD40 in response to stimulation with CD40L.[78] In addition, Act1 interacts with TRAF3 and TRAF6, and may play a role in CD40-mediated NF-κB activation.[79] It has been difficult to ascertain the relative contributions of individual TRAF proteins in CD40-mediated NF-κB activation due to issues of redundancy. A recent study using a B cell line lacking TRAF2 revealed that both TRAF2 and TRAF6 are required for CD40-mediated NF-κB activation.[80] Conversely, TRAF3 may play a negative regulatory role in NF-κB activation. Specifically, TRAF3 may be a negative regulator of CD40-mediated p100 processing by inducing the degradation of NIK.[81] Future studies will require the use of cells deficient in multiple TRAF family members to circumvent issues with redundancy.

Lymphotoxin Beta Receptor

The lymphotoxin beta receptor (LT-βR) is critical for the development and organization of lymphoid tissue. LTβ is expressed in stromal tissue and binds to LTα1β2 and the closely related ligand LIGHT that is expressed in lymphocytes.[82] The LTβR utilizes TRAF proteins to propagate signals leading to the activation of NF-κB and AP-1. The cytoplasmic domain of LTβR binds to TRAF2, 3, and 5, however only TRAF2 and TRAF5 are required for LTβR-mediated activation of NF-κB.[83] LTβR signaling activates both the canonical and noncanonical NF-κB pathways. LTβR mediates the processing of p100, resulting in nuclear translocation of p52/RelB dimmers.[84] A distinct panel of genes can be attributed to the canonical and noncanonical NF-κB pathways induced by LTβR-mediated NF-κB activation. Whereas the expression of the adhesion molecule VCAM-1 was dependent on the canonical pathway, the expression of chemokine and cytokine genes such as SLC, ELC, BLC, SDF-1-α, and BAFF were regulated by the noncanonical pathway.[84]

BAFF Receptor

B cell activating factor (BAFF) plays an important role in the development and survival of peripheral B cells.[85] BAFF binds to three distinct receptors: BAFF-R (also known as BR-3), B cell maturation antigen (BCMA), and transmembrane activator and CAML interactor (TACI).

However, only BAFF-R is specific for BAFF since BCMA and TACI also interact with the related ligand APRIL. BAFF-R is a potent activator of the noncanonical NF-κB pathway that involves NIK-dependent p100 processing in B cells.[86,87] BAFF-mediated NF-κB activation appears to selectively activate the noncanonical pathway since IκBα degradation was not observed after BAFF stimulation of B cells.[86] BAFF activation of p100 processing contributes to the survival of splenic transitional B cells, likely through the induction of anti-apoptotic factors bcl-2 and bcl-x.[86] BAFF-mediated p100 processing requires de novo protein synthesis, which is also common to CD40- and LTβ-mediated p100 processing. It is not clear if this reflects the synthesis of an unknown protein required for p100 processing or continued synthesis of NIK and/or p100.Since induction of p100 processing is associated with NIK accumulation,[81] it is likely that the de novo synthesis of NIK is an important step in noncanonical NF-κB signaling. The intracellular signaling proteins downstream of BAFF-R that lead to NIK activation are unknown, although it has been reported that TRAF3 binds to BAFF-R and negatively regulates NF-κB activation.[88]

RANK

RANK and its ligand, RANKL, are critical regulators of bone remodeling, differentiation of osteoclasts, dendritic cell survival, and lymph node formation.[89] RANK is expressed in osteoclast precursors, myeloid precursors, activated B cells, and dendritic cells. RANK signaling promotes cell survival and differentiation. The RANK protein has a long cytoplasmic region (383 amino acids) which binds to TRAF 1, 2, 3, 5 and 6 and mediates activation of NF-κB, JNK, and AKT.[89] RANK signaling activates the canonical NF-κB pathway in osteoclasts and dendritic cells.[90] RANKL also triggers NIK-dependent p100 processing and promotes osteoclastogenesis.[91] In addition to its roles in osteoclastogenesis and immune regulation, RANK is also important for mammary gland development. RANK signaling requires IKKα for regulation of the expression of cyclin D1, a gene that is critical for mammary epithelial cell proliferation.[92] However, it is unclear if both the canonical and noncanonical NF-κB pathways are required for RANK-mediated mammary epithelial cell proliferation.

OX40, CD27, CD30, and 4-1BB

A host of other TNFR superfamily members such as OX40, CD27, CD30 and 4-1BB activate NF-κB signaling, however little is known regarding the mechanisms of NF-κB activation by these receptors.[51] Each of the receptors has been reported to activate the canonical NF-κB signaling pathway via binding to specific TRAFs. OX40 has been shown to bind to TRAF2, 3, and 5 with TRAF2 and 5 playing positive regulatory roles.[93] CD27 binds to TRAF2 and 3, and while TRAF2 appears to be an activator, TRAF3 may inhibit NF-κB activation.[94] CD30 interacts with TRAF2 and 5.[95] 4-1BB binds to TRAF1, 2, and 3, although only a dominant negative TRAF2 inhibits 4-1BB-mediated NF-κB activation.[96] It must be noted that many of the conclusions derived from these studies relied on overexpression of dominant negative TRAF mutants. Thus, it is important to confirm these findings with cells lacking individual TRAF proteins.

Other Receptors Mediating NF-κB Activation

In addition to the three families of immune receptors discussed above, a large number of other receptors are known to induce NF-κB activation (Table 1). IL-1R and IL-18R contain the TIR domain and activate NF-κB using the MyD88-dependent pathway of the TLRs.[97] Both IL-1R and IL-18R play an important role in mediating inflammatory signals.[98] G-protein-coupled receptors (GPCRs) form a large family of cell surface receptors that signal through the heterotrimeric G proteins.[99] The GPCRs are involved in diverse biological functions, such as leukocyte chemotaxis and activation, platelet aggregation, and hormone action. One of the major downstream signaling events of the GPCRs is activation of NF-κB. Although the pathways leading to NF-κB activation by the different GPCRs are not well defined and appear to be divergent, it seems clear that they converge to the activation of IKK.[99] An-

other type of NF-κB-inducing receptors are the integrins, a family of cell adhesion molecules involved in cell-cell interaction, adhesion, angiogenesis, cell trafficking and survival.[100] A number of integrins, such as the β2, α5β1, α5β3 and α6β4 integrins, also induce NF-κB signaling in multiple cell types, although the pathways have not been well defined.[101] In addition, growth factor receptors, including those for epidermal growth factor (EGF) and nerve growth factor (NGF), mediate activation of NF-κB in numerous cell types. Many growth factors act as potent cellular mitogens and have diverse roles in normal biological processes and pathological states such as cancer.[102] Little is known regarding the mechanisms of NF-κB activation by growth factors, although a recent report indicates that EGF stimulation requires IKKα and RelA for optimal induction of the *c-fos* gene.[103] In addition to cell surface receptors, certain NF-κB-inducing receptors are located within cells. One example is NOD2, a CARD domain-containing protein that specifically recognizes peptidoglycans, and acts as a cytoplasmic sensor for microorganisms.[104]

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Chapter 4

Cellular Dynamics of NF-κB Associated Proteins

Daliya Banerjee and Ranjan Sen*

Introduction

NF-κB DNA binding activity, and thus NF-κB-dependent gene expression, is regulated by the IκB family of inhibitory proteins. In unstimulated cells NF-κB proteins are complexed to IκB proteins and do not bind DNA. The observation that NF-κB/IκB complexes are located in the cytoplasm led to the model that the IκB proteins serve as cytoplasmic "tethers" that keep NF-κB out of the nucleus.[1,2] This was proposed to occur by masking the nuclear localization sequence (NLS) of the p65/RelA component of NF-κB.[3] Cell stimulation leads to IκB degradation and release of DNA binding NF-κB that activates gene transcription. However, even the earliest studies with IκB proteins indicated a level of complexity beyond a simple sequestration model. For example, IκBα was shown to efficiently disrupt NF-κB/DNA complexes.[4] Because such complexes only formed in the nucleus, this in vitro property was not easily reconciled with its function as a cytoplasmic tether. Furthermore, the model did not satisfactorily explain why the NLS of p50, which did not interact with IκBα,[3] did not direct nuclear translocation of the p50/p65/IκB heterotrimer even if the p65/NLS was blocked by IκB. Indeed, when the X-ray crystal structures of NF-κB/ IκB complexes became available, it was also apparent that the p50 NLS was "exposed".[5,6] This chapter summarizes developments in the past five years that lead to a more dynamic view of NF-κB cell biology.

Nucleocytoplasmic Shuttling of IκB Proteins

The family of small IκB proteins includes IkBα, β and ε (Fig. 1); in addition, the C-termini of the precursors p100 and p105 regulate sub-cellular distribution and DNA binding of Rel proteins. Finally, the ankyrin domain-containing proteins Bcl-3 and IκBζ resemble IκBs, but are functionally quite different because they do not inhibit NF-κB DNA binding. The properties of even the inhibitory IκBs differ considerably between each other. For example, unlike IκBα, IκBβ binds NF-κB/DNA complexes without disrupting them[7] and also interacts with a unique cytosolic component κB-Ras.[8] Early indications that IκBα was not "simply" a cytoplasmic tether came from the detection of this protein in nuclear extracts. Kerr et al first showed that anti-IκBα antibody super-shifted a small proportion of the NF-κB/DNA complex detected by electrophoretic mobility shift assays using WEHI 231 (B cell) nuclear extracts.[9] Thereafter, IκBα was transiently detected in nuclear extracts derived from HeLa cells treated with TNFα.[10] These authors proposed that newly synthesized IκBα migrated to the nucleus to inhibit NF-κB activity and terminate the NF-κB response. The ability of IκBα to restore

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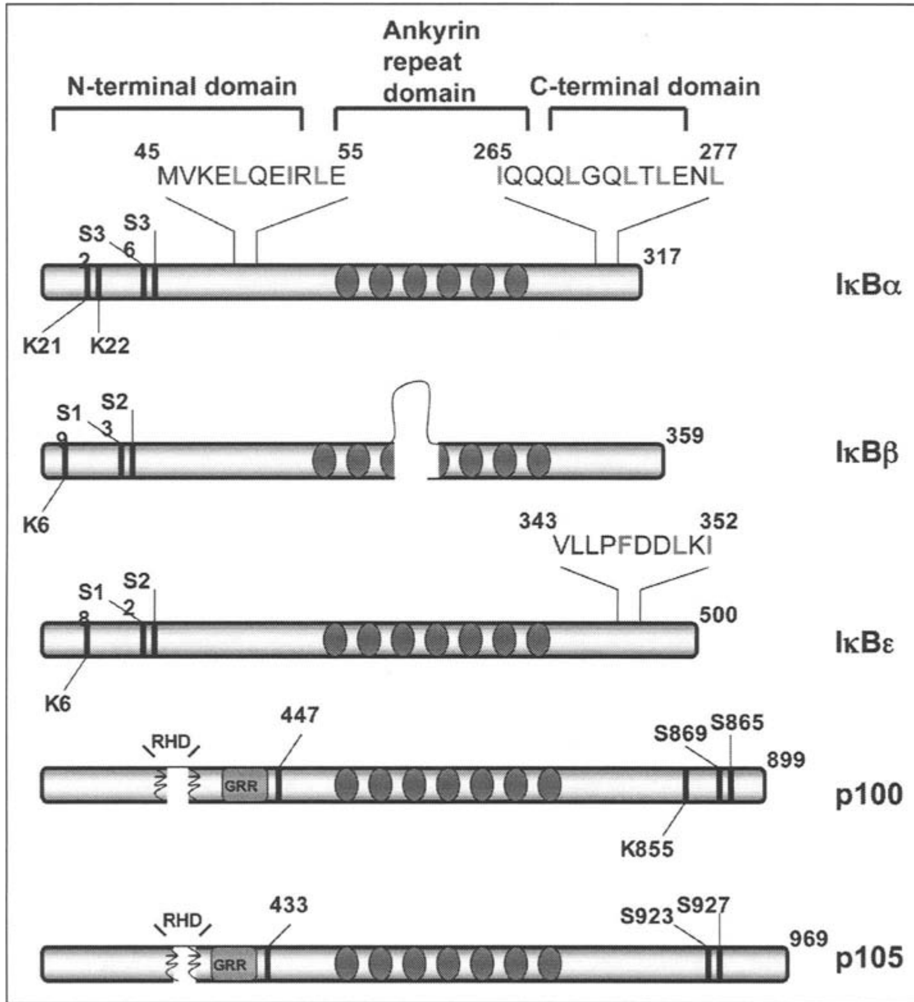

Figure 1. The family of IκB proteins. Schematic of IκB family members highlighting features discussed in this chapter. The ankyrin repeats are designated as red ovals, with the third repeat of IκBβ shown as interrupted by a loop. The amino acid sequences shown in single letter code represent the nuclear export sequences (NES) identified in IκBα and IκBε; the hydrophobic residues that are important for function are indicated in red. Serine residues indicated in the N-terminal domains of IκBα, β and ε are the ones that are phosphorylated by IκB kinases and recognized by β-TRCP1. The lysine residues indicated in these IκBs are the sites of ubiquitination that mark the proteins for proteasomal degradation. For p100 and p105, the full structure comprising the Rel homology domain (RHD) at the N-terminus is not shown, to emphasize the IκB-like part of these molecules. Phosphorylated serines in these proteins are located C-terminal to the ankyrin repeats as indicated. The blue box labeled GRR show the position of a glycine-rich repeat, located between the DNA binding RHD and the inhibitory ankyrin repeat domains, that is involved in constitutive processing.

cytoplasmic NF-κB was demonstrated by microinjection experiments into Xenopus oocytes. In these studies nuclear injection of recombinant IκBα resulted in partially restoring NF-κB to the cytosol.[11]

An important role for nuclear IκBα was firmly established by the identification of two nuclear export sequences (NES) in this protein.[12-14] Both sequences (Fig. 1) are leucine/isoleucine-rich motifs that interact with the exportin receptor CRM1 in the presence of GTP-bound Ran protein.[15] Proteins that contain such motifs bind to CRM1 in the GTP-rich nuclear environment and the complex is brought to the nuclear pore for translocation to the cytoplasm. Upon hydrolysis of the Ran-bound GTP to GDP the complex falls apart and the cargo protein is delivered to the cytoplasm. This vectorial transport process critically depends upon the Ran (GTP)/Ran(GDP) gradient between the nucleus and the cytoplasm. The CRM1 exportin is conserved from yeast to metazoa, though the metazoan species is selectively inhibited by the drug leptomycin B (LMB),[16] which is widely used to study CRM1-dependent nuclear export.

The IκBα NESs determine sub-cellular location of IκBα itself, as well as IκBα-associated Rel proteins, in yeast and mammalian cells. A green fluorescent protein (GFP) IκBα fusion protein is located mainly in the cytoplasm of transfected mammalian cells or transformed yeast strains.[14] However, inhibiting mammalian CRM1 with LMB, or attenuating yeast CRM1p genetically, leads to exclusively nuclear localization in both situations.[13,14] These observations suggest that that GFP- IκBα is in a state of dynamic flux between the nucleus and the cytoplasm. The appearance of most of the protein in the cytosol, either by direct visualization or in cellular extracts, thus reflects a snapshot of the state at which these measurements are made. Continuous movement in and out of the nucleus requires a nuclear localization sequence, to direct the protein to the nucleus, in addition to the NES that directs it out of the nucleus. A classical lysine-rich NLS has not been identified in IκBα. Rather, nuclear localization function is attributed to the second ankyrin domain of IκBα.[17] Consistent with the suggestion of a nonclassical NLS, nuclear import in vitro has been shown to be independent of importin α, the major classical NLS-recognizing protein.[18] Of the two NESs present in IκBα, mutation of the N-terminal NES, but not the C-terminal NES, is sufficient to enforce nuclear localization.[13,14] These observations suggest that the N-terminal NES is the stronger determinant of sub-cellular localization of IκBα.

Coexpression of p65/RelA and IκBα results in cytosolic localization of both proteins. However, as in the case of IκBα alone, this is also a momentary representation of a fluxional state. LMB-mediated inhibition of CRM1 export in mammalian cells, or genetic mutation of the endogenous *crm1* gene in yeast, leads to a complete shift of the complex to the nucleus indicative of ongoing shuttling.[13,14] Two possible NLSs may mediate nuclear entry of the complex. First, the classical NLS of p65, though attenuated by interaction with IκBα, may still be available to interact with importin α. Accessibility of the p65 NLS in complex with IκBα is indicated in biochemical[19] as well as X-ray crystallographic studies.[5,6] Alternatively, the second ankyrin domain of IκBα may still be available for import duty. There is no direct experimental support for either model at present. Presumably, the two pathways can be distinguished by determining whether nuclear import is dependent, or independent, of importin α. Nuclear export of the complex is mediated by the IκBα NESs as well as an NES in the C-terminal domain of p65/RelA (see below).[20] In coexpression studies, mutation of either element does not significantly alter cytoplasmic localization, however, the p65/ IκBα complex is predominantly nuclear when both IκBα and p65/RelA NESs are simultaneously mutated.[21] Thus, homodimers of p65 are not sequestered in the cytoplasm but shuttle continuously. While identification and characterization of sequences that regulate subcellular distribution of Rel complexes were carried out in transfection studies, the principles apply for endogenous Rel/IκBα complexes as well. For example, IκBα relocates to a primarily nuclear location in LMB-treated mammalian cells, and p65/RelA levels in the nucleus are also elevated under these conditions.

IκBα has also been shown to associate with hnRNP1,[22] a nucleocytoplasmic shuttling protein that is involved in mRNA export from the nucleus. The interaction requires an RNA binding domain in hnRNP1 and C-terminal sequences in IκBα. A functional role for this interaction was assessed in an erythroleukemia cell line that lacks hnRNP1. NF-κB activation

induced by the latent membrane protein (LMP1) of Epstein-Barr virus was reduced in these cells, and restored upon reconstitution with hnRNP1. At present it is unclear whether other NF-κB inducing stimuli also utilize this pathway, or the mechanism by which it works.

IκBε is also a nucleo-cytoplasmic shuttling protein.[23] Like IκBα, IκBε nuclear import is mediated by a nonclassical NLS in the ankyrin domains. Hannink and colleagues have identified a functional NES in the C-terminal domain of IκBε that interacts with CRM1 and mediates cytoplasmic retention and post-induction repression of Rel proteins. In vitro binding assays show that the association of IκBε to CRM1 via its NES is comparable to the strength of IκBα/CRM1 binding, suggesting that the export potential of both these proteins are similar. Thus, IκBα and ε share many common features of sub-cellular regulation of Rel proteins. One important difference between these two is the kinetics with which they are reexpressed after signal-induced degradation,[23] and thus their efficacy in terminating the NF-κB response. The rapid re-expression of IκBα suits it for rapid shut-down of NF-κB-dependent gene transcription, while the slower kinetics of IκBε re-expression make it more suitable for terminating long-lived NF-κB responses. How NF-κB-dependent promoters are distinguished for early, or late, shut-down by IκBα, or IκBε, respectively, remains an important question for future studies.

The properties of IκBβ differ considerably from those of IκBα and IκBε. Most importantly, from the perspective of this chapter, IκBβ-associated Rel proteins do not shuttle between the nucleus and cytoplasm; rather, these complexes are sequestered in the cytosol as envisaged in the classical pathway of NF-κB/IκB function.[19] One of the mechanisms postulated for the lack of nuclear entry of IκBβ-associated p65 is a closer association of IκBβ with the p65 NLS. More effective blockade of the NLS may be mediated by κB-Ras, a small Ras-like G protein, that interacts with IκBβ via the 40 amino-acid loop located in the third ankyrin repeat of IκBβ. Ghosh and colleagues have shown that deletion of this loop makes IκBβ a shuttling protein.[8] While nuclear entry in the absence of κB-Ras association may be explained by exposure of an NLS, it is less clear how an IκBβ-associated complex is exported from the nucleus since an export sequence has not yet been identified in this protein. One possibility is that IκBβ/p65 complexes may be exported using the NES located at the C-terminus of p65. This mechanism would not apply to c-Rel associated complexes because c-Rel does not contain an NES. Alternatively, IκBβ may associate with other proteins that direct it out of the nucleus.

Another difference between IκBα and IκBβ is that the latter is more refractory to signal-induced degradation compared to IκBα.[7,24,25] This has also been attributed to association with κB-Ras which, in addition to contacting the IκBβ loop, also requires an intact N-terminus of IκBβ for efficient binding.[26] Biochemical experiments indicate that κB-Ras-associated IκBβ is not efficiently phosphorylated by IκB kinase β(IKKβ) in vitro; conversely, IκBβ degradation in response to TNFα is enhanced in 293T cells in which endogenous κB-Ras is down-modulated by siRNA. Because only a proportion of cellular IκBβ is associated with κB-Ras, a testable prediction of this model is that the portion of IκBβ not degraded after TNFα treatment should be enriched in κB-Ras associated IκBβ. Another possibility discussed below is that the absence of nucleo-cytoplasmic shuttling of IκBβ plays a role in its reduced responsiveness to NF-κB inducing signals.

Signal Induced Degradation of IκB Proteins

Cellular stimulation leads to phosphorylation of IκB proteins and their degradation by the 26S proteasome.[1,27,28] The signal transduction pathway that activates IκB kinases is discussed in Chapters 2 and 3 of this book and will not be further considered here. Phospho-IκBs are targeted to the proteasome via poly-ubiquitination of a specific lysine residue at the N-terminus of each IκB (Fig. 1). The ubiquitination process is initiated by the β-TrCP protein that is the recognition component of the multi-subunit ubiquitin-conjugating enzyme SCF (Skpsl-culin-1-F-box).[29-31] By binding to phosphorylated IκB, $SCF^{\beta TrCP}$ brings to it a particular ubiquitin conjugating enzyme (UbcH5) that ligates a "string" of ubiquitin moieties to IκB, thus making it a substrate for proteasomal degradation.[27] Though some components of the

IκB kinase play a role in the nucleus (see below), most of the evidence suggests that IκB phosphorylation is a cytoplasmic event.[32-35] For IκBα-associated complexes this means that phosphorylation must occur as the shuttling complex passes through the cytoplasm. Indeed, the best evidence that phosphorylation must be a cytosolic event comes from the observation that nuclear IκBα/NF-κB complexes, produced in LMB-treated HeLa cells, are refractory to signal-induced degradation.[33] Since IκBβ-associated complexes reside in the cytoplasm, sub-cellular compartmentalization of the kinase and the IκB is less of an issue in this case.

Recognition of the phosphorylated IκB is the next essential step towards NF-κB activation. A quandary arises from the observation by Ben-Neriah and colleagues that β-TrCP1, the major phospho- IκBα recognizing protein, is located primarily in the nucleus.[36] The phospho-protein recognition domain of nuclear β-TrCP1 is complexed to a ubiquitous phospho-ribonucleoprotein hnRNP-U; this interaction, however, does not lead to ubiquitination and degradation of hnRNP-U (thus, it is referred to as a pseudo-substrate). Davis et al showed that a phospho-peptide comprising a part of the N-terminal domain of IκBa binds better to β-TRCP1 than the corresponding phosphopeptide from hnRNP-U. They suggested that the higher affinity of the IκBα phosphopeptide displaces the hnRNP-U peptide in activated cells. The shuttling property of IκBα complexes provides a ready mechanism by which phospho-IκBα/NF-κB complexes bind to nuclear β-TrCP1. Specifically, once NF-κB/IκB complexes are phosphorylated in the cytoplasm they re-enter the nucleus and bind to β-TrCP1. However, it has not been experimentally verified that phospho-IκBα/NF-κB complexes continue to shuttle. Studies with β-TrCP1-deficient lymphocytes show that IκBα degradation is reduced but not eliminated, indicating that the closely related β-TrCP2 protein may partially compensate for the lack of β-TrCP1.[37] Phosphorylated IκBβ is also recognized by β-TRCP1 en route to being ubiquitinated and degraded.[30] How cytosolic IκBβ-containing complexes meet up with nuclear β-TRCP1 remains unclear. One possibility is that β-TRCP2, which is mainly located in the cytoplasm, may serve as the major IκBβ recognizing protein. A prediction of this model is that IκBβ degradation would be less affected in β-TRCP1-deficient cells. Though β-TrCP2 has been shown to bind phospho- IκBβ, IκBβ degradation is affected more severely than IκBα in β-TrCP1-deficient thymocytes.[37] The relative contributions of the two β-TRCPs for the degradation of shuttling, and nonshuttling, IκBs thus remains murky.

Several possibilities may be considered for what happens subsequent to nuclear recognition of phospho-IκBα/NF-κB by β-TrCP1. First, the β-TrCP1-associated SCF may lead to poly-ubiquitination in the nucleus, and degradation via nuclear proteasomes giving rise to DNA binding NF-κB in the nucleus. Alternatively, the β-TrCP1 associated complex may be exported to the cytoplasm to undergo the more typical poly-ubiquitination and proteasomal degradation; the DNA binding NF-κB would then translocate to the nucleus to activate genes. We favor the hypothesis that the complex is exported out to the cytoplasm to be degraded, in part because of conflicting data regarding nuclear NF-κB activation. A possible complication is that the NES of IκBα, which is located close to the phosphorylation sites, may be obscured by the associated $SCF^{\beta TrCP}$ complex. We suggest that an alternate export mechanism, such as that mediated via hnRNP-1 associated with the C-terminus of IκBα, may predominate under these circumstances to bring the complex to the cytosol. Once in the cytosol, poly-ubiquitination and IκBα degradation would release DNA binding NF-κB.

An interesting aspect of this model is that the shuttling cycle predicts an increase of β-TrCP1 in the cell cytosol, which transiently puts it in the same sub-cellular compartment as IκBβ. This cytosolic β-TrCP1 can bind to phosphorylated IκBβ leading to its poly-ubiquitination and degradation. The need for cytosolic β-TrCP1, generated by IκBα degradation, may in part be the reason that IκBβ is degraded more slowly, and less efficiently, compared to IκBα in response to most signals. Ghosh and colleagues have previously proposed that the reduced responsiveness of IκBβ is due to association of κB-Ras protein with the loop of IκBβ, which inhibits IκBβ phosphorylation by the IKK complex.[26] Perhaps reduced phosphorylation, as well as reduced recognition of phospho-IκBβ, contribute to the relative stability of IκBβ in stimulated cells.

Shuttling of IκB Kinase Components

The first indication that signaling components in the NF-κB activation pathway are also in a state of dynamic flux between the nucleus and cytoplasm came from the observations of Birbach et al.[38] They used leptomycin B to block nuclear export and examined the subcellular distribution of several "upstream" activators of NF-κB. Of the various proteins examined, the NF-κB inducing kinase (NIK) rapidly shifted to the nucleus in response to LMB, as did the IκB kinase α subunit (IKKα), though with slower kinetics. More recently the adapter subunit IKKγ (NEMO) has been shown to accumulate in the nucleus of HeLa cells in response to LMB.[39] However, IKKβ, the dominant kinase involved in NF-κB activation in response to cytokines such as TNFα and IL-1, appears to be strictly located in the cytoplasm.

The relevance of NIK nucleo-cytoplasmic shuttling for NF-κB induction is unclear at present. This kinase has been implicated in the nonclassical NF-κB activation pathway which leads to the processing of p100 in a IKKα-dependent manner and release of p52/Rel B heterodimers for gene transcription.[40,41] The proposed mechanism involves NIK-induced phosphorylation of p100 at a C-terminal domain, which recruits IKKβ resulting in additional p100 phosphorylation. This form is recognized by β-TrCP and eventually results in degradation of the C-terminal ankyrin repeat domain by the proteasome. The need for nucleocytoplasmic shuttling is not apparent in this series of steps. However, it is intriguing to note that NIK, p100 and IKKα can all be located in the nucleus under appropriate conditions, raising the possibility that shuttling between compartments may play a role in coordinating this outcome. In analogy with the model proposed above for nuclear association of phosphorylated IκBα and β-TrCP, phospho-p100 may also interact with β-TrCP in the nucleus.

Sub-cellular dynamics of NEMO provide a satisfactory mechanism for the activation of NF-κB in response to DNA damage. Miyamoto and colleagues showed that a small fraction of cellular NEMO is modified by covalent attachment of the ubiquitin-like molecule, SUMO, in response to camptothecin, an inhibitor of DNA topoisomerase I.[42] Because sumoylation is believed to be a nuclear phenomenon,[43] the shuttling of NEMO in and out of the nucleus allows the substrate to be present in the right compartment, without the need to invoke the additional step of DNA-damage induced NEMO translocation. SUMO-modified NEMO is a target of the ATM kinase which, by an as yet undetermined mechanism, leads to replacement of SUMO with ubiquitin. Lysines 277 and 309 on NEMO have been identified as possible sites of these modifications. The nuclear accumulation of sumoylated NEMO may be due to retention in the nucleus or retardation of export from the nucleus. In contrast to sumoylated NEMO, the ubiquitin-modified form is exported to the cytoplasm where it presumably associates with and activates IκB kinases to induce IκB phosphorylation.

Nuclear NEMO has also been proposed to play a transcription regulatory role.[39] The work of Gaynor and colleagues shows that NEMO interacts with an N-terminal domain of the transcriptional coactivator CBP. This part of CBP also interacts with p65/RelA and IKKα, leading to the idea that NEMO may sequester CBP and prevent it from interacting with, and thereby activating, p65-dependent gene expression. Taken together these observations strongly support an important role for NEMO in the nucleus.

Immunofluorescence studies demonstrate that IKKα is located in the nucleus and cytoplasm of cells, and LMB treatment slowly shifts the balance towards nuclear localization. One of the functions of IKKα in the nucleus is the activation of NF-κB-dependent gene transcription. This occurs by recruitment of IKKα to the promoters of these genes via an association with the CBP coactivator and, presumably, other promoter-bound transcription factors. At the promoter IKKα likely phosphorylates histone H3 on Ser 10, causing transient increase of phosphorylated H3.[44,45] Promoter phosphorylation then triggers histone acetylation by histone acetyl transferases such as CBP. The model is well substantiated by the loss of promoter phosphorylation, and reduced histone acetylation, in IKKα-deficient cells. These observations are consistent with the idea that nucleocytoplasmic shuttling of IKKα ensures that some of this protein is in the right cellular compartment to be recruited to appropriate

promoters in response to cell stimulation. The mechanism of recruitment, however, remains unclear. Because IKKα directly associates with the N-terminus of CBP, one possibility is that a IKKα/CBP complex is recruited to the promoter. Alternatively, IKKα may interact with one or more promoter-bound transcription factors, though it does not interact with p65/RelA directly. It is also not clear whether IKKα is required at all or only a subset of NF-κB-dependent promoters; conversely, it is likely that IKKα may also activate NF-κB-independent promoters via its interaction with CBP, and perhaps other HATs. It is noteworthy that several other histone H3 kinases have been previously identified that phosphorylate Ser 10. The rules that govern the circumstances under which each kinase activates a set of genes remains to be determined.

Shuttling of Rel Proteins

Features of the Rel proteins themselves also contribute to their sub-cellular location and, importantly, distinguish between the two closely related family members p65/Rel A and c-Rel. The RHDs of these two proteins are sufficiently similar that it has been difficult to identify DNA sequences that specifically bind one, or the other, protein. There is less similarity in the C-terminal domains and it is well established that the transcriptional activation potential of p65/Rel A is substantially higher than that of c-Rel (at least when assayed with multimerized κB sites-containing promoters). In addition to the differences in transactivation potential, p65/Rel A contains a nuclear export sequence located in the C-terminal domain,[20] but c-Rel does not.[21] Location of an NES close to the transactivation domain has also been observed in NF-AT family members,[46] though the functional relevance of this juxtaposition in either protein is unclear.

The subcellular distribution of NF-κB/IκB complexes is determined by the cumulative action of NLSs and NESs present in a particular multi-protein complex. For example, ectopically expressed p65 protein, that is present as a homodimer, is located primarily in the nucleus, presumably because the two NLSs dominate over the two NESs. Coexpression of IκBα together with p65 drives the homodimer to the cytoplasm, presumably as the result of attenuating p65 NLSs via association with IκBα and the provision of a strong IκBα NES to the complex (this heterotrimer contains two attenuated NLSs and three NESs). Interestingly, coexpression of p65 with an NES-mutated IκBα also leads to cytoplasmic localization of the complex.[21] The observation indicates that the weaker (than IκBα) NESs of p65 are sufficient to skew the balance towards the cytoplasm when the p65 NLSs are attenuated by interaction with IκBα. In a heterotrimer that contains no NESs, where p65 and the IκBα NESs have been mutated, nuclear expression of the p65/IκBα complex increases. Studies with p65 deletion mutants have also confirmed that the NLS of p65 is functionally attenuated in complex with IKBα, as suggested by the X-ray crystallographic studies. Because c-Rel does not contain an NES, its subcellular distribution closely parallels that of an NES-mutated p65. Two things change when p65 is complexed to p50. First, the complex contains one less NES (from p65) and secondly, the p50 NLS, that is not in contact with IκBα, is stronger than the attenuated NLS of p65; both changes favor nuclear localization of the Rel heterodimer. In the p50/p65/IκBα heterotrimer, however, the additional strong NES of IκBα results in net cytosolic expression based on nuclear export prevailing over nuclear import.

Though the relative importance of various import and export sequences to sub-cellular distribution were dissected in ectopic expression assays, the behavior of endogenous cellular complexes corroborates the observed patterns. Thus, cytoplasmic p65/IκBα complexes in lymphocyte cell lines redistribute to the nucleus when nuclear export is blocked by leptomycin B.[21] Not all of the p65 goes to the nucleus however, presumably because a part of it is associated with IκBβ and therefore does not shuttle. Consistent with this observation, LMB-induced c-Rel redistribution is only observed in cells where c-Rel is associated with IκBα, but not in cells where it is predominantly associated with IκBβ.[21]

Greene and coworkers have recently identified an additional novel mechanism that regulates nuclear localization of p65/Rel A.[47-49] Following up on the observation that the histone de-acetylase inhibitor trichostatin A increases NF-κB-dependent transcription, they found that p65/Rel A is acetylated at several lysine residues (K122, K123, K218, K221 and K310) in the RHD, by the coactivator CBP. This presumably occurs in the nucleus during p65-dependent gene transcription. Acetyl modification of the RHD prevents interaction with IκBα and, as a consequence, p65/Rel A remains in a transcription competent state in the nucleus for a longer time. Thus, down-regulation of p65/Rel A from the nucleus requires removal of the acetyl group as well as re-expression of IκBα following cell stimulation.

The complex pattern of NLSs, NESs and post-translational modifications of Rel proteins that regulates cellular location is likely to be important for appropriate function of these proteins. Based simply on the numbers of NLSs and NESs it is likely that there is graded nuclear propensity amongst Rel family homo-and hetero-dimers. The homodimer of p65 is the least likely to be localized to the nucleus in the presence, or absence, of IκBα, with heterodimerization increasing the probability of nuclear location. This idea provides a plausible explanation for the subunit composition (p50/c-Rel) of constitutive NF-κB in the nuclei of mature B cells. We have previously proposed that nuclear p50/c-Rel in B cells may be the result of more efficient p65 export from the nucleus after generation of free pools of both p65 and c-Rel as a consequence of enhanced IκBα degradation.[21] It is also likely that this hierarchy dictates the order in which Rel proteins are removed from the nucleus by IκBα (or IκBε) after cellular activation. That is, p65 homodimers would be removed first (2 NESs and attenuated NLSs), followed by c-Rel homodimers (only attenuated NLSs). Heterodimers with p50 would tend to be more nuclear compared to the homodimers because of the unattenuated p50 NLS; but, the additional NES of p65 would increase its tendency to be cytoplasmic compared to p50/c-Rel. In this context it is interesting to note that p65, with the strongest transcription activation domain amongst Rel family members, may be the one most likely to lead to unwanted gene expression if present in the nucleus for longer than "required".

In summary, the central theme of this review is to highlight the dynamic nature of NF-κB and associated proteins in the cell. In the unstimulated cell continuous nucleo-cytoplasmic shuttling of several components maintains a nuclear environment that is free of functional transcription factor, yet provides the means for cytosolic or nuclear post-translational modifications that are essential for a variety of stimulus-induced NF-κB activation. In cells responding to stimulus, the same features of intracellular movement provides a means to terminate the response. While the duration of NF-κB-dependent gene expression may vary depending on the cell-type, or the activating stimulus, the underlying principles outlined here represent a framework in which to analyze cellular responses.

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CHAPTER 5

NF-κB in Lymphopoiesis

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NF-κB has long been recognized as a critical mediator of acute immune and stress responses, poised to coordinate the defensive response of the host to pathogenic threats. Beyond these roles, NF-κB is increasingly recognized for its roles in lymphoid organogenesis and development of hematopoietic cell lineages. These functions have been discovered primarily through analyses of mouse models deficient in NF-κB factors, inhibited for NF-κB activity or lacking key components required to signal to NF-κB.

Here we review NF-κB contributions in developing T and B lymphocytes (summarized in Tables 1 and 2). For the purpose of this review we will consider development to be completed once lymphocytes have matured into naïve, peripheral, long-lived recirculating cells. Discussed elsewhere in this book are NF-κB's roles in responses of mature lymphocytes, such as in their antigen-driven activation, proliferation and differentiation. As will become evident in this review, NF-κB is particularly important for the survival of developing lymphocytes, helping to rescue these cells from default death pathways at various stages of their development. However, NF-κB also contributes to differentiation and may even have a role in apoptosis in some situations. Thus NF-κB exhibits context-dependent functions that differ depending on the developmental stage. The structure, regulation and signaling pathways for activation of NF-κB are discussed in other chapters of this book.

NF-κB in Early Stages of Lymphocyte Development

Hematopoietic precursor cells lacking individual NF-κB or IκB proteins can generate mature B and T cells, indicating that no single subunit or inhibitor is essential for development of mature lymphocytes.[1-4] The resulting cells are, however, functionally deficient. Mice lacking RelA died about 7 days before birth from acute TNF-induced liver apoptosis, so lymphopoiesis could only be studied in radiation chimeras made by transfer of fetal liver cells (containing hematopoietic precursors) from RelA-deficient embryos into lethally irradiated wild-type mice.[5] The chimeric mice survived with hematopoietic cells derived from transferred precursors. They developed peripheral lymphocytes, albeit significantly diminished in numbers and impaired in function, when compared to chimeras with wild-type fetal liver cells (to be discussed later below).

In contrast to the RelA single knockout (KO), chimeras generated from mice deficient in both RelA and NF-κB1 (p50/p105)[5] essentially failed to develop lymphocytes. Similarly, lymphopoiesis was absent in radiation chimeras generated with hematopoietic precursors from mice lacking IKKβ.[6] IKKβ is the primary catalytic component of the IKK complex for most NF-κB activating signals and specifically for inflammatory cytokine signals.[3,4,7] Like RelA KO mice, IKKβ KO mice died in utero due to massive apoptosis of hepatocytes.[6] Finally, chimeras generated with fetal liver cells deficient in both RelA and c-Rel appeared to be largely, though

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Table 1. Cell-autonomous roles of NF-κB in developing T lymphocytes

Stage	Role
T Lymphocyte Precursors	*Probably needed to protect precursors from death induced by high levels of TNFα.*
Double Negative $CD4^- CD8^-$ *thymocytes.*	*pre-TCR induced activation of NF-κB important for survival during progression of DN thymocytes from stage IIIL to IV and to DP* Inhibition of NF-κB by IκBα superrepressor triggers apoptosis.
Double Positive $CD4^+ CD8^+ TCR^+$	*Self-antigen/TCR-induced NF-κB activity may help set the threshold for signal strength during positive and negative selection of DP thymocytes.* Inhibition of NF-κB by IκBα superrepressor interferes with negative selection (conversion of a strong to a weaker signal, leading to survival?), but also with positive selection (further reduction of a weak signal, leading to death by neglect?). May explain pro- and anti-apoptotic roles of NF-κB, depending on the in vivo assay. *Different hypothesis suggests negative selection mediated by TCR-induced expression of $I\kappa B_{NS}$.* $I\kappa B_{NS}$ may act like a supperrepressor to inhibit NF-κB in strongly self-reactive DP thymocytes, thus inducing death by eliminating the protection from apoptosis by NF-κB.
Single Positive $CD4^+$, $CD8^+$ Thymocytes and Peripheral T cells	*Required for survival of T cells as they progress from SP thymocytes to long-lived peripheral T cells.* Conditional knockout of NEMO or knockin of dominant negative IKKβ induces apoptosis of SP thymocytes (especially $CD8^+$) and leads to complete loss of peripheral T cells. Less severe loss of NF-κB activity in IKKβ knockouts and IκBα superrepressor transgenic mice results in partial reduction of peripheral T cells ($CD8^-$). *IKKβ-mediated activation of NF-κB required for development of T_r and NKT cell subsets.* IKKβ knockouts lack T_r and NKT cells in thymus. T_r also reduced in NF-κB1 and c-Rel dKO and thymic NKTs reduced in IκBα superrepressor transgenic.

not completely, impaired in generation of peripheral lymphocytes.[8] While these data imply an intrinsic role for NF-κB in developing hematopoietic cells, this role was not cell-autonomous. Cotransfer of wild-type hematopoietic precursors together with those from the above double knockouts (dKOs) or the IKKβ KO mice significantly rescued lymphopoiesis of mutant cells.[5,6,8,9]

Subsequent work has shown that NF-κB's critical function in early lymphopoiesis may be to prevent excessive formation of cytotoxic TNFα. If the IKKβ knockout was placed on a TNFR1-deficient background, this not only rescued the resulting embryos from early liver apoptosis and death, it also relieved the block in lymphopoiesis.[6] Lack of IKKβ was associated with increased granulopoiesis, the likely cause of excessive TNFα production. In addition though, TNFα may have been particularly effective in inducing apoptosis in the developing mutant lymphocytes, since these lymphocytes lacked the anti-apoptotic protection normally afforded to them by NF-κB. So, NF-κB probably also has a cell-autonomous, anti-apoptotic role during lymphopoiesis. Excessive granulopoiesis was seen in radiation chimeras generated with precursors from mice deficient in IKKβ, or RelA and NF-κB1 or RelA and c-Rel.[5,6,8,9] Most

Table 2. Cell-autonomous roles of NF-κB in developing B lymphocytes

Stage	Role
B Lymphocyte Precursors	*Probably needed to protect precursors from death induced by high levels of TNFα.*
Pre-B	*pre-BCR-induced NF-κB aids survival of pre-B cells during progression from large to small pre-B and immature B cells.* B cells deficient in NF-κB1 and NF-κB2 or containing an IκBα superrepressor generate fewer small pre-B and immature B cells (rescued with Bcl-x_L). *NF-κB may also contribute to light chain locus demethylation/ germline transcription in small pre-B cells.*
Immature	*(Self-antigen induced signaling via BCR in immature B cells may induce apoptosis during negative selection due to limited signaling to NF-κB by immature BCR complexes). Possible role in homing to spleen.*
Transitional (T1, T2)	*NF-κB essential for survival and for full phenotypic maturation. Activation of the alternative pathway by BAFF/BAFFR and the classical pathway by unknown signal important for survival of T1 and subsequent stages.* Complete development block at T1 in NF-κB1 and NF-κB2 dKO and at T1/T2 in RelA and c-Rel dKO due to apoptosis. Survival and partial maturation rescued with Bcl-2 anti-apoptotic transgene, but not full maturation. Reduction of transitional B cells in conditional NEMO KO or dominant negative IKKβ knockin mice (incomplete loss of wild-type gene/protein at this stage).
Marginal Zone (MZ)	*Needed for generation of MZ B cells, probably for survival and maturation. Signals likely to include BCR, BAFFR.* Single KOs of NF-κB1, NF-κB2 or RelB lack MZ B cells, and RelA or c-Rel KOs have reduced numbers.
Mature (follicular, recirculating)	*NF-κB needed for survival through BCR and BAFFR activation and other signals.* Reduction of mature B cells in mice deficient in BCR signaling components dedicated to activation of NF-κB. Loss of NEMO or IKKβ in B cells in conditional knockouts leads to complete elimination of mature B cells. Partial reduction of mature B cells harboring an IκBα superrepressor transgene (incomplete inhibition), or lacking IKKα (required for activation via the alternative pathway).

likely, NF-κB-dependent extracellular factors are involved in limiting granulopoiesis and thus excessiveTNFα production.

The NEMO (IKKγ) subunit is required for IKKβ activation in the classical pathway of NF-κB activation.[1,3,4,7] In contrast to the above factors, NEMO appeared to be required for the generation of peripheral lymphocytes even when wild-type hematopoietic cells were present.[10,11] In female mice in which one X chromosome carried a NEMO mutation, random lyonization produced hematopoietic precursors both with and without NEMO. Despite this mixture, which mimics a mutant/wild-type chimera, only NEMO-expressing circulating lymphocytes were observed. Similar skewing occurred in female human patients with Incontinentia

Pigmenti, where one NEMO allele is mutated.[12] It is not known at what stage of their development NEMO-deficient lymphocytes failed to thrive. Since loss of NEMO more completely blocks NF-κB activation than either loss of IKKβ or two NF-κB factors,[7] the absence of circulating NEMO-deficient lymphocytes may reflect a need for NF-κB in early lymphopoiesis. However, NEMO-deficient ES cells were able to generate some IgM$^+$ immature B cells in an in vitro differentiation system, suggesting that early development of B cells per se is not completely blocked.[13] On the other hand, the in vitro generated cells survived poorly. The true extent of this defect can only be assessed in vivo, however, under conditions where mutant cells are forced to compete with wild-type cells and where they encounter many other cells and signals.

Double Negative, PreTCR$^+$ Thymocytes

T lymphocyte development occurs largely in the thymus. The most immature thymocytes (distinguished by cell surface markers) are termed CD4$^-$CD8$^-$ double negative (DN), since they lack the coreceptors, CD4 and CD8. These cells mature to become CD4$^+$CD8$^+$ double positives (DP), then CD4$^+$ or CD8$^+$ single positive (SP) thymocytes, which finally migrate to the periphery.[14] DN thymocytes bearing the heat-stable HSA or CD24 antigen are subdivided into four stages by their expression of CD44 and IL-2Rα (CD25): (I) CD44$^+$CD25$^-$, (II) CD44$^+$CD25$^+$, (III) CD44$^-$CD25$^+$, and (IV) CD44$^-$CD25$^-$.[14]

T cell receptor (TCR) β chain rearrangement is necessary for stage III to IV transition.[15] In cells with rearranged TCR β chains, β associates with the pre-Tα chain to form the pre-TCR, which is thought to signal in the absence of any known ligands. TCRβ$^+$ stage III cells are referred to as "L" cells (about 15% of stage III cells) and are larger than the still unrearranged stage III, "E" cells. NF-κB was constitutively activated in thymocytes, with highest activity in stages IIIL and IV.[16,17] A further increase in NF-κB activity in IKKβ-transgenic mice generated more stage IV cells. Moreover, IKKβ expression in RAG1$^{-/-}$ thymocytes, which are blocked in pre-TCR assembly, still enabled some differentiation through stage IV to DP cells. Finally, mice with an IκBα superrepressor transgene had fewer stage IV thymocytes and NF-κB inhibition in isolated stage III L and IV cells triggered apoptosis.[17] Thus, pre-TCR signaling appears to activate NF-κB, providing a survival signal for stage III L and IV thymocytes, although involvement of other receptors has not been rigorously excluded. The pre-B cell receptor (BCR) may play an analogous role in B cell development (see below).

Expression of the IκBα superrepressor blocked development only partially at this stage,[17] possibly due to incomplete inhibition and/or alternative pre-TCR-generated survival signals. Thus, NF-κB certainly contributes to, but may not be essential for T cell development at this stage. Cells escaping the block eventually populate later stages, and continued impairment of NF-κB activity may actually obscure the earlier defect. This compensatory effect appears to be due to a pro-apoptotic role for NF-κB at the DP stage, such that repression of NF-κB may actually increase cell numbers at the later stage (see below).

Double Positive Thymocytes

DP thymocytes express both CD4 and CD8 chains, as well as TCRα and β chains (a mature TCR).[14] They are subject to both positive and negative selection. DP thymocytes with TCRs that strongly recognize self-peptides (in association with MHCs) are generally eliminated by apoptosis, as are those thymocytes that fail to see any peptides (death by neglect), while those with weak but measurable recognition of MHC-associated peptides are positively selected and progress to the SP stage. Multiple reports imply critical roles for NF-κB in DP thymocytes and even specifically in positive and negative selection, but to date no consensus has emerged as to exactly which biologic processes NF-κB regulates and by what mechanisms. This may reflect differences in the biologic assays employed in the various studies, as well as differences in the quality and quantity of NF-κB inhibition achieved. The situation may be complicated if NF-κB makes several distinct contributions that influence numbers and progression of thymocytes.

DP thymocytes underwent massive apoptosis in mice treated with dexamethasone, which also functions as an inhibitor of NF-κB, suggesting a need for the transcription factor to protect DP cells against what are likely endogenous apoptotic insults.[18] c-myc may have been the anti-apoptotic target of NF-κB in this instance, since ectopic c-myc partially rescued DP cells after dexamethasone treatment.[19]

Early studies showed that inhibition of NF-κB with IκB transgenes in T cells led to increased, rather than decreased numbers of DP thymocytes.[20,21] While this could have been due to a block in progression/maturation to SP cells, which were somewhat reduced in numbers,[21,22] it could also reflect a pro-apoptotic role of NF-κB in DP thymocytes. Indeed, IκBα superrepressor-expressing DP thymocytes resisted apoptosis normally induced by anti-CD3 administration in vivo,[23] as did DP thymocytes expressing dominant negative IKKβ.[24] However, this does not prove a cell-autonomous, anti-apoptotic activity of NF-κB. It is possible that the resistance to apoptosis was the result of defective expression of cell-extrinsic factors, normally dependent on NF-κB. T cells and thymocytes carrying the superrepressor[23,25] or dominant negative forms of both IKKα and IKKβ[24] are known to be significantly impaired in induced expression of extrinsically-acting cytokines, cytokines which might indirectly affect thymocyte numbers.

While in vivo administration of anti-CD3 is not likely to faithfully model negative selection, another report directly implicates NF-κB in this process.[26] Male mice expressing a TCR transgene directed against an MHC class I restricted H-Y (male) self-antigen represent a model for negative selection, since self-reactive DP thymocytes are largely eliminated, preventing the development of SP self-reactive T cells. However, if such negatively selected T cells also expressed an IκB superrepressor transgene (partially inhibiting NF-κB activity), then negative selection was blunted and many more DP thymocytes were observed. Thus NF-κB was concluded to have a pro-apoptotic effect. The aforementioned study also suggested a role for NF-κB during positive selection, however.[26] Expression of the TCR and IκB superrepressor transgenes in positively selecting mouse background models resulted in fewer than normal positively selected TCR^+ thymocytes.

It is conceivable that inhibition of NF-κB with the superrepressor might have interfered with both negative and positive selection by reducing TCR signaling strength. TCR-induced phosphorylation of the receptor-proximal ZAP-70 kinase was reduced when NF-κB was inhibited, suggesting that NF-κB normally enhances this critical early signaling step in some unknown way.[26] Inhibiting NF-κB therefore might have dampened the strong TCR signal, thereby also preventing apoptosis, while the lesser signal normally resulting in positive selection might have become so weakened that the result was death by neglect. Thus, NF-κB might normally contribute to both negative and positive selection of DP cells.

Given such potentially complex scenarios, this transcription factor might well appear to have different functions, depending on the experimental conditions employed (quality and strength of NF-κB inhibition; the cell types in which NF-κB is inhibited/reduced; the biologic assay). Not surprisingly then, some studies come to different conclusions than the ones cited above. One study failed to note any effects of an IκB superrepressor on negative selection, while positive selection, especially of $CD8^+$ SP cells was demonstrated to be significantly impaired.[21] That latter result is also consistent with the loss of peripheral T cells and of $CD8^+$ cells in particular, which has been noted in a number of studies involving IκB transgenes.[20-23,27] Two studies failed to note any significant perturbations of T cell development upon inhibition of NF-κB activity, one involving an IκB superrepressor[25] and another dominant negative-acting IKKα and/or IKKβ transgenes.[24]

Finally it has been reported that negative selection of DP thymocytes may be mediated by specific inhibition of NF-κB. In vivo administration of a negatively selecting peptide into mice bearing the matched TCR transgene (VSV8 peptide into N15 TCR transgenic $RAG2^{-/-}$, $H\text{-}2^b$ mice) induced the expression of $I\kappa B_{NS}$ in DP thymocytes, but, interestingly, not in mature T cells.[28] This novel IκB-like protein inhibited NF-κB activity and acted like a superrepressor,

since it lacks the phosphorylation and ubiquitination sites found in the signal-responsive IκBα, β and ε proteins. Ectopic expression of this protein led to a partial reduction of DP and SP thymocytes as well as a marked increase in apoptosis of thymocytes in response to anti-CD3 stimulation. It remains to be confirmed that this IκB-like protein is critical to negative selection, but if so, it suggests that negative selection induces death via inhibition of NF-κB. While this seems at odds with the reported pro-apoptotic roles of NF-κB in negative selection discussed above, it still remains possible that expression of $I\kappa B_{NS}$ is in some way controlled by NF-κB. If so, this transcription factor would again have pro- and anti-apoptotic activities, depending on the timing and context. NF-κB may have multiple, complex inputs into DP thymocyte selection, where small differences in the level and/or quality of NF-κB activity could make the difference between life and death.

As discussed above, transgenic expression of dominant negative IKKβ prevented the massive apoptosis of DP thymocytes upon in vivo administration of anti-CD3 antibodies.[24] However, the opposite effect was observed with a dominant negative IKKα transgene, as this apparently made DP thymocytes even more susceptible to induced apoptosis.[24] This suggests the possibility that IKKα is part of an anti-apoptotic pathway, while IKKβ, the primary signaling kinase downstream of the TCR, may communicate an apoptotic signal, even if only indirectly. RelB complexes may be involved in mediating the anti-apoptotic role of IKKα. Firstly, IKKα regulates RelB activation via the so-called alternative or nonclassical/noncanonical pathway of activation.[3,4,7,29] IKKα, but not IKkβ or NEMO, is necessary for processing of the IκB-like inhibitor of RelB, p100, to p52. This results in nuclear translocation of the RelB/p52 dimer. Secondly, RelB-deficiency in mice resulted in fewer mature SP thymocytes, and this defect was cell-autonomous.[30] When cultured, the mutant thymocytes were more prone to apoptose, and when stimulated via the TCR, it was the DP and early SP mutant thymocyte populations in particular that exhibited higher rates of apoptosis when compared to their wild-type counterparts.

NF-κB's participation in DN thymocyte survival and DP apoptosis could also be inferred indirectly from studies of the HLH proteins, E2A and HEB. Transgenic mice, whose thymocytes expressed E2A/HEB inhibitors Id1 and/or Tal1 underwent apoptosis at the DP stage.[31,32] The late DN thymocytes of these mice exhibited very high NF-κB activity, which may have been activated by a strong pre-TCR-derived signal, particularly strong due to the absence of negative modulation exerted by E2A/HEB. Even without TCR rearrangements these DN thymocytes can progress to the DP stage, presumably due to a higher basal signaling in these cells. E2A/HEB may normally set a higher threshold for pre-TCR derived signals, although the mechanisms for this are unknown. Once at the DP stage, however, the abnormally high TCR activation signal may have been interpreted as stimulation by self-antigens, triggering apoptosis (equivalent to negative selection). The involvement of NF-κB in apoptosis in the Id1/Tal1 transgenic DP thymocytes is shown more directly by rescue from apoptosis by transgenic IκB superrepressor. During normal DN differentiation, pre-TCR signaling may induce some expression of the HLH inhibitor, Id3, allowing NF-κB activation and developmental progression. TCR stimulation at the DP stage by self-antigens may cause higher levels of Id3, leading to even more NF-κB activation and thus triggering apoptosis,[32] either directly or indirectly (see above).

Single-Positive Thymocytes and Peripheral T Cells

Several early reports noted that expression of IκB superrepressor transgenes in developing thymocytes resulted in decreases in peripheral SP cells, in particular of $TCR\alpha\beta^{high}$ $CD8\alpha\beta^{+}$ peripheral T cells.[20-23,27,33,34] Loss of IKKβ (on a TNFRI deficient background, so mice survived, see above) decreased numbers of thymocytes and peripheral T cells, and this was speculated to be due to a defect in TCR-induced proliferation based on in vitro observations.[6] Loss of IKKα, on the other hand, did not effect numbers of developing or mature T cells.[35,36] Finally, radiation chimeras generated with c-Rel and RelA double-deficient hematopoietic pre-

cursors contained fewer peripheral T cells, a defect not rescued by a Bcl-2 transgene.[8,9] These early observations supported a role(s) for NF-κB in SP thymocytes/peripheral T cells.

More recently the roles of NF-κB in development and maintenance of T cells have also been explored in CD4-promoter driven, cre-mediated conditional T cell knockouts of IKKβ and NEMO/IKKγ, and in knockins of an activation-deficient IKKβ (which functions as an IKK dominant-negative).[37,38] While this approach would seem well suited to test the roles of IKKs during DP thymocyte selection, it was useful for assessing the need for NF-κB only after DP selection has largely been completed. This is so because developing thymocytes in this conditional knockout model retain at least some IKK activity until they have progressed to the early SP stage, as only then the vast majority of cells have completed cre-mediated recombination and the wild-type protein has been lost.[37]

Mere deletion of IKKβ did not grossly affect the generation and maintenance of naïve peripheral T cells, although peripheral T, especially $CD8^+$ T cells were somewhat reduced,[37] in line with results obtained with transgenic IκB superrepressor mice (see above).

However, the absence of a more pronounced effect is most certainly due to compensation by IKKα, since conditional deletion of NEMO or knockin of the activation-deficient IKKβ completely prevented the appearance of any peripheral mutant T cells.[37] Furthermore, even SP thymocytes, especially $CD8^+$ ones, were markedly reduced in the conditional NEMO knockouts, and this was accompanied by increased apoptosis of these thymocytes.[37] Therefore, a cell-autonomous IKK activity, mediated by either IKKα or IKKβ is critical for maturation/survival of SP T cells. Interestingly, elimination of TCR-induced activation of NF-κB in Bcl-10 knockouts did not lead to loss of mature T cells, suggesting that it was not the TCR, but an as yet unknown signal that was responsible for NF-κB activation and thus generation/survival of mature T cells.[37] This signal may be analogous to the BAFF/BAFFR survival signal that is required in maturing and mature B cells (see below).

While loss of IKKβ activity was largely compensated by IKKα during development of the bulk population of T cells, regulatory T cells (T_r)[37] and NKT[38] cells were exquisitely sensitive to loss of IKKβ, as were memory T cells in the periphery.[37] Therefore, IKKβ appears to have a unique function in these subpopulations that cannot be substituted for by IKKα. Support for the notion that these T cell subtypes may require greater NF-κB activity also comes from the observed reduction of regulatory (and memory) T cells in NF-κB1 and c-Rel double-deficient mice,[39] and of NKT cells in mice transgenic for the IκB superrepressor.[39] In the latter case thymic as opposed to peripheral NKT cells were preferentially reduced, probably the result of incomplete inhibition by the superrepressor in the thymus, allowing some cells to escape into the periphery and eventually fill this niche. The generation of NKT cell thymic precursors is also dependent on expression of RelB in stromal cells.[40,41]

NKT, T_r and memory cells exhibit a more activated phenotype.[38] Quite possibly these cells have an increased need for NF-κB activity, such that loss of IKKβ cannot be sufficiently compensated by IKKα. DP thymocytes serve as precursors for T_r cells. Unlike regular DP thymocytes, which are eliminated by recognition of self-antigens, T_r cells instead are positively selected by self-antigens, probably due to the identity and context of the antigen-presenting cells. $CD4^+CD25^+$ T_r cells act as supressors of autoreactive effector T cells in the periphery and thus are critical to maintain tolerance and prevent autoimmunity. NKT cells too are positively selected by recognition of self-antigens in the thymus, in this case self-lipids presented by the nonclassical MHC class I-like molecule CD1d.

Although development of T_r, NKT and memory cells depends on antigen, only T_r and $CD4^+$ memory cells could be shown to depend on TCR-driven NF-κB activation.[38] This conclusion is based on the absence of these latter cells in mice deficient in components needed for TCR signal-induced NF-κB activation, such as Bcl-10.[38] T_r cells are missing in both thymus and periphery in Bcl-10 knockouts. In contrast, NKT cells showed no such dependence on these signaling components. This suggests that TCR-independent signals are responsible for NF-κB activation in developing and peripheral NKT cells, although it remains theoretically

possible that NKT TCRs activate NF-κB via an unknown signaling pathway. While NKT cells were normal in CARMA1/Card11, PKCθ or MALT1 knockouts (all are required for TCR-induced NF-κB activation), peripheral though not thymic NKT cells were reduced in Bcl-10 knockouts.[38] Therefore, surprisingly, an unknown peripheral, nonTCR signaling pathway may involve Bcl-10. (The main contributions of NF-κB to T cell development are summarized in Table 1.)

Pre-B Cells

After birth, early B cell development takes place in the bone marrow. The earliest recognized precursors are termed pre-pro-B[42] (for nomenclature used in this review see Hardy et al[43]). In late pro-B cells, immunoglobulin μ heavy chains undergo rearrangement and association with surrogate light chains (Vpre-B and λ5) to form the pre-B cell receptor (pre-BCR). Cells advance to the large pre-B stage and expand. Large pre-B cells eventually progress to become noncycling small pre-B cells that no longer express the pre-BCR, but which begin to rearrange κ or λ light chains. Once successfully rearranged, a κ or λ chain combines with the μ heavy chain to form a B cell receptor (BCR). Expression of a BCR (IgM) on the cell surface marks the beginning of so-called immature B cells, which eventually leave the bone marrow to complete their full maturation in the spleen.[43]

Several lines of evidence suggest that NF-κB participates in both κ and λ rearrangement, although proof for an essential role remains elusive. The Igκ locus is regulated by a distal (3' Eκ) and by a proximal, intronic enhancer (iEκ).[44-46] The latter enhancer contains the first κB element identified.[47] These regulatory elements function as transcriptional enhancers in transfection experiments, although their in vivo role may be more complex[46] (see below). Homozygous disruption of either the iEκ[47] or the 3' Eκ partially impaired rearrangement of this locus, but did not completely abolish it.[46] However, disruption of both enhancers resulted in a near complete block of V to Jκ rearrangements.[46] This suggests that the two enhancers encode somewhat redundant activities in controlling the rearrangement process.

Work by Bergman and colleagues[48-50] specifically implicated the κB element within the iEκ enhancer in demethylation of the Igκ locus in B cells, a necessary step in the rearrangement process that occurs in small pre-B cells once chromatin has become 'accessible'. These investigators also proposed that 'opening' of chromatin and demethylation only occurred on one of the alleles, which would explain the allelic exclusion phenomenon. The rearranging allele may be marked early on by epigenetic mechanisms associated with the order of replication of the two chromosomes. The conclusion that the κB site controlled rearrangement via demethylation was based largely on the demethylation of a transfected, in vitro methylated part of the Igκ locus that included the iEκ enhancer. Demethylation occurred only on open chromatin,[50] and only with an intact κB element.[48-50] Furthermore, demethylation was dependent on activated NF-κB. S107 plasmacytoma cells are blocked in NF-κB activation in response to many signals and they lack constitutive activity. Accordingly, demethylation of the transfected piece of the Igκ locus was blocked in these cells, but could be induced by cotransfection of RelB. RelA failed to do so, probably because it was inhibited and remained in the cytoplasm in S107 cells. Thus RelB appeared to control the demethylation process, at least in these cells.[50]

Data obtained with Abelson murine leukemia virus transformed pre-B cells in culture has implicated NF-κB in control of Igκ locus germline transcription and rearrangement.[46] Germline transcripts precede and appear to be necessary for rearrangement, perhaps signifying the accessibility of chromatin. The Abelson virus encoded v-abl oncogene may impair NF-κB and other transcription factors. The transformed cells are frozen in a large pre-B-like state, since they cycle and fail to rearrange their Igκ locus. Stimulation with LPS activated NF-κB, increased binding to the iEκ κB site and induced κ germline transcription and rearrangement. Similarly, inactivation of the v-abl protein (using drugs or a temperature-sensitive (ts)-v-abl) resulted in germline transcription and rearrangement.[46,51] If the v-abl transformed cells were engineered to also express the IκBα mutant superrepressor, then LPS-induced or temperature

(ts-v-abl)-induced germline transcription and rearrangement of κ were blocked.[52] Thus rearrangement of Igκ induced by relief from the v-abl block did require NF-κB activity.

Not all observations appear to be entirely consistent with the hypothesis that activation of NF-κB in pre-B cells might regulate germline transcription and demethylation and thus rearrangement of the Igκ locus. Early in vivo footprinting studies demonstrated that the κB site in the iEκ enhancer was already occupied at the pro-B cell stage.[46] While this does not rule out a role for this site (or NF-κB) in germline transcription and/or demethylation, and thus in rearrangement, it appears to rule out that binding of newly activated NF-κB to the iEκ is an initiating event as cells transit from large to small preB cells. Instead, progression was correlated with changes in occupancy of various transcription factor sites within the 3'Eκ[46] (and not the iEκ). Therefore NF-κB and the κB element may contribute to making the Igκ locus more accessible or assist in the assembly of a demethylation complex, but they appear not to initiate these processes. Because proteins were bound to the iEκ κB (and surrounding sites) even prior to pre-BCR formation, occupancy of this site must be independent of the pre-BCR. However, it is also possible that a pre-BCR-derived signal modified or even exchanged the proteins that interacted with the iEκ κB site, given that in vivo footprinting could not identify the proteins bound.

NF-κB has been suggested to regulate λ light chain germline expression/rearrangement.[53] Three synergistic NF-κB sites were identified in the human λ enhancer[54] while the mouse λ enhancer was reported to contain only a mutated NF-κB site, which may explain its low activity.[55] Nonetheless, inhibition of NF-κB activity via the IκB superrepressor prevented not only κ but also λ rearrangement in response to temperatureinduced inactivation of ts-v-abl in transformed mouse pre-B cells.[53]

Another study failed to implicate NF-κB in κ or λ rearrangement or expression. Mice with IκB superrepressor under the control of the μ heavy chain enhancer and thus expressed in B lineage cells produced near normal levels of κ$^+$ and λ$^+$ cells.[56] However, the superrepressor may not have been very effective in this case since both precursor and mature B cells from these mice still exhibited significant constitutive NF-κB activity. In addition, the strong LPS signal still induced NF-κB in the superrepressor-bearing splenic B cells ex vivo, although the presumably weaker BCR-induced NF-κB activation was impaired.[56] Rearrangement may require only minimal NF-κB activity and given the plasticity of the developmental process, any decrease in the rate of production of κ$^+$ or λ$^+$ cells could have easily been covered up as precursors continue to be generated and as κ$^+$ or λ$^+$ cells may expand to fill later developmental stages. Mice deficient in both NF-κB1 and NF-κB2 generated κ+ or λ$^+$ cells as well, although at somewhat reduced rates[57] (see below). Once generated, these B cells had normal levels of light chain expression on their surface,[57] suggesting that these NF-κB factors did not regulate final light chain expression.

Our results and others, suggest that NF-κB may act as a survival factor during generation of small pre-B cells from pro-B cells[58] (Claudio and Siebenlist, unpublished). In adoptive transfer experiments, precursors lacking NF-κB1 and NF-κB2 produced reduced numbers of small pre-B and immature B cells in bone marrow as compared to the numbers of earlier-staged large pre-B/pro-B cells and this effect was greatly exacerbated in the presence of competing wild-type cells (Claudio and Siebenlist unpublished). Therefore, while the defect in mutant developing B cells was relatively mild, it became dramatic when these mutant cells were forced to compete with wild-type cells, presumably because the wild-type cells fill developmental niches more effectively. The transition of mutant pro-B to small pre-B was also partially blocked in bone marrow cultures ex vivo (Claudio and Siebenlist unpublished). In this assay bone marrow cells are stimulated with IL-7 for several days, during which time pro-B cells selectively expand to make up nearly the entire culture. Withdrawal of IL-7 then stops expansion and reveals B cells advancing towards later developmental stages. In cultures generated with NF-κB1 and NF-κB2 double-deficient bone marrow we observed relatively fewer small pre-B and immature B cells. Those that were generated appeared to be more prone to apoptosis. Feng et al[58] have also recently noted a reduction in small pre-B and immature B cells in radiation chimeras generated

with bone marrow cells transduced ex vivo with retroviruses encoding the IκB superrepressor (dominant-negative). A Bcl-X transgene expressed in these same B cells rescued the loss of cells during the pro-B to small pre-B/immature B transition, confirming the role of NF-κB as a survival factor during development of B cells in bone marrow and specifically in generation of small pre-B cells. These data suggest but do not prove that the pre-BCR signal may be responsible for activation of NF-κB at this stage.

NF-κB's role in generating small pre-B and immature B cells may be analogous to that in T cells, where it is needed for optimal survival at the preTCR stage (see above).[17] Supporting this notion, btk$^{-/-}$ mice (lacking Bruton's tyrosine kinase (BTK), a member of the BCR signal pathway involved in activation of NF-κB) have fewer small pre-B cells.[59] In humans the block in small pre-B cell generation is complete in the absence of a functional BTK.[60] NF-κB may enhance survival of small pre-B cells or their immediate precursors, with the pre-BCR providing the initial activating signal.

Further evidence for a role for NF-κB in pre-BCR signaling comes from Blk-, Lyn- and Fyn- triple-deficient mice.[61] pre-B cells from these mutant mice were impaired in anti-Igβ-stimulated pre-BCR-mediated NF-κB signaling (apparently via PKCλ), while signaling via tyrosine-phosphorylation of Syk remained intact. The triple-deficient mice were blocked in pro- to pre-B transition, generating few small pre-B and immature cells and scarcely any mature B cells. The remaining triple knockout pre-B cells exhibited increased apoptotic rates.

Additional evidence points to activation of NF-κB in pre-B cells or their immediate precursors. Analysis of B cells during their progression from pro-B to small pre-B and immature B cells in bone marrow cultures (see description of culture above) revealed activation of NF-κB in response to withdrawal of IL-7, which stopped proliferation of pro-B cells. The NF-κB activity consisted mostly of p50/RelA and RelA/cRel dimers.[62] Pre-BCR formation is the only known positive signaling in this system and may therefore be the trigger for NF-κB activation.

Immature B Cells

IgM first appears on immature B cells. IgM$^+$ B cells with strong self-antigen reactivity may apoptose or undergo further light chain rearrangement to change their IgM specificity. Immature/Transitional B cells then migrate to the spleen where they mature.[43] BCR stimulation in immature B cells induces apoptosis, while it induces proliferation in mature B cells,[63] suggesting that these cells differ in their linkage from the BCR to downstream targets.

The WEHI 231 tissue culture cell line resembles immature B cells based on cell surface markers and thus provides a possible model for negative selection at the immature/transitional B cell stage. BCR stimulation of WEHI 231 transiently induced NF-κB activity beyond already high basal levels, but this was then followed by a significant decrease in activity to below basal levels and the cells apoptosed.[64] Lowered NF-κB activity correlated with down-regulation of c-myc and cyclin D2 and with increased cyclin-dependent kinase inhibitor (CKI) p27 (Kip1) synthesis.[65] This caused growth arrest and apoptosis.[65] Apoptosis could be blocked by costimulation with CD40 ligand, which induced NF-κB and one of its targets, c-myc.[66] This suggests that BCR signaling in WEHI 231 immature B cells ultimately down-regulates NF-κB to effect negative selection, which contrasts with up-regulation of NF-κB in mature B cells.

Whether the transformed cell line WEHI 231 with constitutively high NF-κB activity can be a model for normal physiologic negative selection of immature B cells in vivo is questionable. Nevertheless, it has been suggested that ex vivo immature B cells may not induce NF-κB to the same extent as mature cells (unpublished observations reported in review by Monroe[63]). Although calcium influx and tyrosine-phosphorylation events in immature B cells were largely similar to those seen in mature B cells, only mature B cells recruited the BCR into lipid rafts after stimulation, while immature cells failed to do so. This is likely the reason why BCR stimulation in immature B cells occurred in the relative absence of phosphatidyl inositol biphosphate hydrolysis and thus generation of diacylglycerol (DAG). DAG is essential for activation of many Protein Kinase C (PKC) family members. Importantly, PMA-induced activation of PKCs rescued immature B cells from BCR-induced apoptosis.[63]

These data do not prove that lack of PKC activation and consequently impaired NF-κB activation are the reasons why BCR-stimulated immature B cell apoptosed. However, in BCR-stimulated mature B cells activation of the DAG-dependent PKCβ isoform appeared to assure survival via activation of NF-κB. BCR-stimulated, mature PKCβ$^{-/-}$ B cells exhibited normal tyrosine-phosphorylation events (including upregulation of early cell cycle entry proteins), but did not proliferate well due to poor survival.[67-69] Activation of NF-κB in response to BCR stimulation was specifically impaired in these mutant cells, but not in response to CD40.[67-69] In consequence, BCR-induced expression of NF-κB controlled survival genes, such as Bcl-x, was abrogated. Further analysis showed that PKCβ was required to recruit the adaptors CARMA1 and Bcl-10 into lipid rafts, which ultimately recruit and activate the IKKs and NF-κB.[70] PKCβ may play a role analogous to that of PKCθ in T cells (see above). PKCβ-deficient B cells resemble B cells from XID mice (mutated in BTK) in their response to BCR stimulation, reportedly because BTK, among other functions is responsible for sustained BCR-induced activation of PKCβ and thus NF-κB.[70] In any case, it remains to be shown whether BCR-induced activation of NF-κB and its targets is significantly impaired in immature B cells when compared to mature B cells.

Generation and Maintenance of Mature B Cells

Immature B cells leave the bone marrow and migrate to the spleen. These cells are so-called transitional B cells. Final development occurs in the spleen where transitional B cells evolve through as many as three distinguishable stages (transitional 1 through 3) before they become mature, naïve B cells.[71,72] Transitional B cells also give rise to marginal zone B cells, which do not recirculate. Transitional 2 B cells move into and accumulate in B cell follicles, a stage when B cells first respond positively to stimulation via their BCR, so the transition from stage 1 to 2 is functionally highly significant.[43,71,72] After reaching full maturity in the B cell follicles, these cells recirculate through the entire periphery, including lymph nodes and bone marrow.[43] Development of transitional B cells in the spleen requires NF-κB, since mice deficient in NF-κB1 + NF-κB2[57] or in RelA + c-Rel[8,9] are blocked in B cell development just before/at the transitional 2 stage, when transitional B cells become more like mature cells; these mutant mice are totally devoid of any mature follicular/recirculating B cells. NF-κB1 + NF-κB2 dKO mice also lack peritoneal B1 B cells (Claudio and Siebenlist, unpublished results). The mechanisms behind these developmental blocks will be discussed below.

Immature/transitional B cells migrating from the bone marrow to the spleen in NF-κB1, NF-κB2 double knockout (dKO) mice may already be somewhat at a disadvantage before they reach their final block in the spleen (see above and below). Relatively fewer transitional 1 B cells were observed in the spleen than expected, based on the number of immature B cells generated in dKO bone marrow[73] (Park and Siebenlist, unpublished results). This relatively mild loss became more apparent in radiation chimeras, when mutant immature/transitional B cells were forced to compete with adoptively cotransferred (and differentially marked) wild-type B cells. Based on these results we speculated that B cells might have a defect in homing to spleen, a defect intrinsic to B cells, since the adoptively transferred host mice produced normal levels of stroma-derived chemokines in the wild-type spleen. Immature/transitional B cells from NF-κB1, NF-κB2 dKO mice were previously noted to lack expression of the chemokine receptor, CXCR5 (BLR1).[74] In addition, the chemokine receptor CCR7 was impaired in its expression in mutant immature/transitional B cells (Park and Siebenlist, unpublished observations), consistent with prior data linking high CCR7 expression with high NF-κB activity in Hodgkins Lymphomas.[75] Both receptors have been suggested to contribute to entry of B cells into the spleen, even though this is not their primary function.[76] Therefore, although NF-κB1, NF-κB2 dKO B cells were still able to migrate into the spleen, lack of both CXCR5 and CCR7 may have been responsible for the lower efficiency, which was much more noticeable in the presence of wild-type cells.

The developing NF-κB1, NF-κB2 dKO B cells that did migrate into the spleen were severely impaired in survival; they were especially prone to undergo apoptosis when placed in

culture, even without any stimulation.[73] RelA, c-Rel dKO developing B cells were also impaired in survival, based on their very rapid turnover in spleens in vivo.[9] Furthermore, both NF-κB1, NF-κB2 dKO and RelA, c-Rel dKO B cells were largely lacking in expression of the anti-apoptotic proteins A1 and Bcl2.[9,73] Together these findings suggested that impaired survival in both compound knockout B cells was responsible for the complete developmental block encountered during the transitional phases. The block in both mutants is intrinsic and autonomous to their B cells, based on adoptive cotransfer experiments of mutant together with wild-type bone marrow.

The notion that impaired survival is responsible for the block in the compound mice is supported also by the rescue of developmental progression in the presence of a Bcl-2 transgene. Exogenous expression of the anti-apoptotic Bcl-2 protein in NF-κB1, NF-κB2 dKO(Claudio and Siebenlist, unpublished) and RelA, c-Rel dKO[9] mice allowed mutant B cells to accumulate in spleen and express mature B cell markers. It did not, however, promote full maturation nor did it restore the ability of these B cells to proliferate after BCR stimulation[9] (Claudio and Siebenlist, unpublished). Expression of the mature markers CD21 and CD62L, for example, was not fully restored in NF-κB1, NF-κB2 dKO B cells by a Bcl-2 transgene and these cells remained functionally impaired in basal (and antigen-specific) immunoglobulin production (Claudio and Siebenlist, unpublished). NF-κB is therefore critical not only for survival of transitional B cells, but also for their full functional maturation.

What are the relevant targets of NF-κB that assure survival of developing B cells? Bcl-2 is likely to be one of these targets, based on the data discussed above and additional data presented below. Furthermore, several reports have concluded that that the Bcl-2 gene can be directly regulated by κB elements that may bind several different NF-κB complexes.[77-79] Bcl-2 expression normally rises as B cells progress through the transitional B cell phase. Bcl-2 is not essential for development, however, since Bcl-2 knockout mice were able to generate mature B cells, albeit at significantly reduced levels, coupled with loss of the remaining cells' long-term survival.[80,81] A1 is a known direct target of in particular c-Rel/NF-κB, but c-Rel is not essential for development and A1 is unlikely to be essential. Mice deficient in either A1 or c-Rel had normal B cell development.[80,81] However, only one of three potentially expressed isotypes of the A1 gene was eliminated, so questions regarding A1 remain. Nevertheless, the most likely hypothesis is that various NF-κB factors and their anti-apoptotic targets have at least partially redundant activities to insure optimal survival, such that elimination of only one factor or one anti-apoptotic target does not obviously block development of B cells, especially when there is a continuous influx of newly formed transitional B cells into the spleen.

Although single knockouts of NF-κB factors showed no significant defects in development of regular follicular B cells, all single knockouts were nearly completely (NF-κB1, NF-κB2, RelB) or partially (RelA, c-Rel) devoid of marginal zone (MZ) B cells, indicating that the generation/maintenance of this mature B cell population is highly dependent on NF-κB activity[82,83] (Claudio and Siebenlist unpublished). In addition, even though mature follicular B cells were generated in the single KOs, these cells were partially impaired in survival in at least some situations, revealing the importance of all NF-κB factors in overall B cell survival. For example, while B cells matured normally in NF-κB1 single KOs, the mature cells turned over more rapidly in vivo and showed decreased survival in unstimulated cultures ex vivo.[84] B cells also matured normally in c-Rel-deficient mice, but the mature cells were impaired in proliferation and survival ex vivo when stimulated via the BCR.[84] Loss of RelA resulted in a significant decrease of Bcl-2 and cFLIP, making these mutant B cells highly sensitive to TNF-induced apoptosis; in the presence of high levels of TNF few RelA-deficient B cells were generated.[85] B cell development was also largely normal in NF-κB2-deficient mice, save for a small reduction in numbers of mature cells, but the survival of mature and, even more surprising, of transitional B cells was impaired ex vivo.[73] The life of wild-type immature/transitional B cells in ex vivo cultures could be significantly extended by the addition of BAFF, a member of the TNF family, but this factor had no effect on NF-κB2-deficient immature/transitional B cells (see below).

The latter results suggested that NF-κB2 was already important quite early in the life of transitional B cells, at least ex vivo, which may explain why NF-κB1, NF-κB2 dKO B cells were blocked at the transitional 1 stage and thus slightly earlier than B cells deficient in RelA and c-Rel.[81] The latter mutant cells were reported to progress to the transitional 2 stage, albeit with much reduced numbers. Transitional B cells may be exposed to several different survival signals as they progress through the different stages, signals that appear to be partially redundant, but may mediate their effects in part via distinct NF-κB complexes.

A striking advance in our understanding of the factors underlying B cell development was the elucidation of the role of the B cell Activating Factor (BAFF). BAFF was found to be critical for B cell survival at the transitional stage, as well as for long-term survival of mature cells.[86] This ligand engages three receptors, TACI, BCMA and BAFF receptor (BAFFR). The first two receptors are also engaged by a BAFF-related ligand, APRIL and their deletion in knockout mice did not block B cell generation. However, B cell development in BAFF$^{-/-}$, BAFFR$^{-/-}$ and TACI-Ig transgenic mice (which produce a decoy receptor for BAFF and APRIL) was blocked during progression from transitional 1 to transitional 2 B cells (T1 to T2). There were few T2 B cells and a complete absence of mature B cells.[86-90] Consistent with the survival function of BAFF signaling in B cell development, transgenic over-expression of anti-apoptotic Bcl-2 restored the mature follicular B cell population in mice containing a TAC I transgene (in which BAFF function is inhibited) and in BAFFR$^{-/-}$ mice.[88,90] However, expression of some of the mature B cell markers was still somewhat impaired and marginal zone B cells were not restored at all, suggesting roles in addition to survival for BAFF.[88,90]

As discussed, BAFF-promoted survival of transitional B cells ex vivo was dependent on NF-κB2. BAFF not only failed to extend the life of transitional B cells derived from NF-κB2-deficient and BAFFR mutant (A/WySnJ) mice, but also from *aly/aly* mice (mutated in the NF-κB-inducing kinase, NIK).[73] This indicated that BAFF/BAFFR signaling was mediated, at least in part, by the alternative (non-classical, non-canonical) pathway of activation, in which the NIK and IKKα kinases cooperate to induce the processing of p100 to the p52 form of NF-κB2. BAFF indeed induced p100 processing in mature and in developing B cells, starting with immature/transitional 1 B cells, which led to nuclear accumulation of p52/RelB and possibly other complexes. BAFF-induced processing and NF-κB activation was completely independent of NEMO (IKKγ), which together with IKKβ is a necessary component of the classical, IKK-complex-mediated pathway.[73]

Loss of BAFF or BAFFR led to a nearly complete block in B cell development from the T1 to T2 transition onward, although the functionally inactivating mutation of the BAFFR in A/WySnJ mice was slightly less restrictive.[89] As discussed, a BAFF/BAFFR-induced survival signal for transitional (and mature) cells is transmitted via the alternative activation pathway (NIK - IKKα - NF-κB2). However, functional loss of NIK (*aly/aly* mutation) or IKKα or NF-κB2 did not cause as severe a block in B cell development as loss of BAFF or BAFFR.[35,36,73,89,91] Radiation chimeras with NIK-mutant or IKKα-deficient (or IKKα-mutant) bone marrow reduced, but did not eliminate the mature B cell population, and chimeras with NF-κB2-deficient bone marrow contained near-normal numbers of mature B cells.[35,36,73,91] (One report also noted a mild reduction of transitional B cells in IKKα-deficient chimera[36]). Therefore, signaling via BAFF/BAFFR may contribute to survival (and/or differentiation) via a second pathway, independent of the alternative activation pathway. The reason why specifically those B cells lacking NF-κB2 appeared less impaired in vivo than those lacking NIK or IKKα may be due in part to the loss of the inhibitory p100 precursor protein in the former, but not the latter knockouts (p100 is the main inhibitor of RelB).

It is not known what other pathways may be activated by the BAFF receptor. One report suggests a very weak, but functionally significant stimulation of the classical NF-κB activation pathway by BAFF in mature B cells, a pathway that reportedly involved NF-κB1.[92] If such occurred also in transitional B cells, it might explain why only loss of both NF-κB2 and NF-κB1 led to a block in B cell development during the transitional stage. However, the block in these

doubly deficient mutant B cells occurred slightly earlier than that seen in BAFF- or BAFFR-deficient mice since the latter contained some T2 cells. In addition, BAFF failed to promote survival of transitional B cells lacking only NF-κB2, but containing NF-κB1, while those lacking NF-κB1 responded well to BAFF.[73] Regardless of whether BAFF can transmit some signal via the classical pathway, this NEMO/IKKβ-dependent pathway is likely to contribute to survival of transitional B cells in vivo (presumably in response to some signal). CD19 promoter-Cre recombination enzyme-driven conditional knockouts of NEMO or conditional knockins of an IKKβ mutant in B cells resulted in noticeably fewer transitional B cells in the spleen (in addition to loss of mature B cells, see below).[93] As discussed previously here, such conditional knockouts/knockins are more difficult to interpret: although loss of the targeted DNA segment increases as B cells progress, it is never complete, and some cells escape. Aside from that, even cells in which the DNA was recently deleted will continue to express the protein for some period of time thereafter while cells progress. It is therefore difficult to pinpoint exactly when loss of classical pathway (NEMO/IKKβ) first interferes with developmental progression. However in the case of the conditional NEMO knockout and IKKβ mutant knockin the first problem must have occurred during the transitional stage (or even earlier), since B cells present at this developmental stage were apparently already somewhat counter-selected for those that had escaped recombination.

In transitional B cells ex vivo, BAFF led to increased expression of Bcl-2 via an NF-κB2-dependent (alternative) pathway of activation.[73] In mature B cells BAFF has been reported to increase expression of in particular A1 and Bcl-x_L.[94] It is thus possible that transitional and mature B cells differ in their response to BAFF, possibly due to differences in expression of other receptors for BAFF. Recently an additional new mechanism of action for BAFF in mature B cell survival has been suggested by a report that BAFF in an unknown way blocks the (spontaneous) nuclear localization of PKCδ, thereby preventing the pro-apoptotic function of this protein in the nucleus.[95]

Apart from BAFF, to what other signals do transitional B cells respond? The MHC class II chaperone, invariant chain li, has been reported as essential for B cell maturation via a mechanism in which a proteolytically released cytoplasmic fragment of li promotes RelA-mediated transactivation.[96] These findings were based on initial studies with li-deficient mice in which B cell maturation appeared to be largely blocked during in the final transitional stages. However, new studies suggest that while li-deficient mice do have reduced numbers of mature follicular B cells, they actually have normal or even increased numbers of marginal zone B cells; furthermore the reduction of mature follicular B cells is due to a shortened B cell life span rather than a developmental block per se. More importantly, li-deficient compound knockouts in which expression of MHC class II molecules is also severely reduced or completely eliminated exhibited normal B cell development, suggesting that li-free MHC class II molecules specifically interfered with B cell survival through an unknown mechanism and that neither li nor MCH class II are required for normal B cell development.[97,98]

The B cell receptor (BCR) is important for B cell development and it can signal activation of NF- κB, but does it play a role in transitional B cells? The BCR is certainly essential for maintenance of mature B cells,[99] as the CD23 promoter-Cre recombinase-driven conditional loss of the BCR leads to disappearance of mature B cells within days. The importance of BCR signaling during at least the final transitional maturation can be inferred from the phenotype of mice deficient in Bruton's tyrosine kinase, a component of the BCR pathway in mature B cells which mediates its functions in part via NF-κB.[100] These mice were impaired in the last stage of B cell maturation (transitional stage 2 to mature B cells) and T2 cells accumulated; they also were partially impaired in generation of B1 B cells.[100] Further evidence for the importance of the BCR in late (transitional) B cell development comes from mice deficient in CARMA-1/CARD 11, Bcl-10 or MALT-1. These adaptor proteins act downstream of the BCR (and TCR) in the signal pathway to NF-κB. Mice deficient in CARMA-1, Bcl-10 or MALT-1 generated few peritoneal B1 and MZ B cells and reduced numbers of mature B cells.[101,102] There is

however presently no evidence for a role of BCR or this pathway during the earlier transitional phase of B cells (T1).

The maintenance or long-term survival of mature B cells depends on signaling from the BCR and BAFF receptors (see above) and possibly other receptors, mediated at least in part by the classical and alternative pathways of NF-κB activation. Conditional knockouts of the main components of the classical pathway, namely NEMO[93] and IKKβ[103] revealed that as cells progressed and ongoing deletion of targeted alleles should have neared completion, the small population of mature and in particular of marginal zone B cells still had a high percentage of wild-type NEMO/IKKβ alleles, as these 'escapees' were they only ones that had survived. Furthermore, mature B cells were impaired in survival ex vivo and exhibited increased turnover in vivo as even escaping cells eventually lost the wild-type alleles. Finally, if generation of newly maturing cells was blocked by administration of IL-7 antibodies, then all previously generated mature B cells disappeared, long before such cells would normally expire, as the Cre-mediated deletion of NEMO or IKKβ alleles approached completion in the remaining cells. Thus loss of the classical NF-κB activation pathway is incompatible with long-term survival and thus maintenance of all mature B cells, including B2 and B1 cells.[93,103] This conclusion is consistent with other data. A superrepressor IκBα transgene expressed in B cells resulted in dose-dependent reduction of the mature, recirculating B cell pool.[56] c-Rel, NF-κB1 double knockout mice had slightly fewer B2 B cells and a more sizeable reduction of B1 B cells, although the authors suggest that this may have been due to impaired proliferation on top of a defect in survival of mature B cells already noted in single NF-κB1 knockout mice.[104] A dominant-negative IKKβ mutant transgene expressed in B cells appeared to have no dramatic effect on development, most likely due to insufficient blocking of the wild-type activity.[105] But proliferation and antibody responses were impaired, which was also noted across the board not only for KOs of members of the classical NF-κB activation pathway, but also for single KOs of the various NF-κB factors, suggesting that functions of mature cells are even more sensitive to loss of the classical pathway or of NF-κB factors. Radiation chimeras generated with fetal liver cells from IKKα knockouts[36] or mutant IKKα knockins[35] (IKKα KOs die in utero due to non-hematopoietic defects) were reported to have a reduction in the mature, recirculating pool of B cells, with those cells present exhibiting increased turnover, reduced expression of anti-apoptotic A1, reduced survival ex vivo and impaired processing of p100 NF-κB2 to p52. Together with results showing a requirement for BAFF in survival of mature B cells in the periphery, the BAFF-induced processing of p100 mediated by IKKα appears to be essential for the proper maintenance of the peripheral B cell pool. (The main contributions of NF-κB to B cell development are summarized in Table 2.)

Perspectives

NF-κB is a critical contributor to the development and maintenance of lymphocytes. It does so primarily via intrinsic and cell-autonomous functions in lymphocytes, but also via non-cell autonomous mechanisms and activities in other cells. Activation of NF-κB transcription factors in response to signals encountered by developing lymphocytes leads to their survival; without such signals these lymphocytes undergo apoptosis by default. As lymphocytes mature, NF-κB's intrinsic and cell-autonomous roles appear to increase and to include contributions to differentiation and eventually proliferation. Upon maturation the maintenance of these cells also requires NF-κB. While both T and B cells critically rely on this transcription factor for their maturation and differentiation into distinct mature subsets, B cells appear to depend on a broader range and quantity of NF-κB activity.

Many important questions remain unanswered. Particularly intriguing are the likely complex functions of NF-κB in positive and negative selection of T lymphocytes, the yet-unknown activation signals that guide B cell development in the spleen, the possible direct involvement of NF-κB in demethylation and the specific gene targets of NF-κB at developmental junctions.

Throughout most of development individual NF-κB factors appear able to substitute for each other's cell-autonomous survival functions. Only certain compound knockouts or loss of entire NF-κB activation pathways result in noticeable deficits of mutant lymphocytes, and even then in some cases mature lymphocytes may be formed. However, the effects of the loss of a given factor or factors have usually been observed in the context of the original mutant mouse or in radiation chimeras. This tends to minimize the degree to which mutant lymphocytes are impaired, since they do not have to compete with their wild-type counterparts. When mutant cells are forced to compete, important contributions of NF-κB are readily revealed; wild-type cells increasingly fill available niches during developmental progression and ultimately mutant cells are almost entirely lost from the periphery. This mechanism may have evolved to ensure that only cells with properly functioning NF-κB systems reach or persist in the mature pool, given the importance of this transcription factor system in host defense. While only minimal NF-κB activity may be sufficient to enable progress to full maturity per se, mutant lymphocytes in which NF-κB activity was only partially impaired may be eliminated in competition with wild-type cells, since their developmental progression is comparatively inefficient.

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Chapter 6

NF-κB and Immune Cell Effector Functions

Hsiou-Chi Liou,* Biao Feng, Wenzhi Tian, Shuhua Cheng and Constance Y. Hsia

Abstract

Initially identified as a constitutive nuclear factor of kappa light chain immunoglobulin in B lymphocytes (NF-κB), the NF-κB transcription factor family now consists of 5 mammalian members (p50/NF-κB1, p52/NF-κB2, p65/RelA, c-Rel, and RelB). Individual knockouts of each NF-κB subunit in mice have shown that NF-κB is functionally expressed in nearly every tissue and cell type. The best documented roles for NF-κB lie in their ability to modulate the development, activation, and effector functions of immune cells. NF-κB participates in both innate and adaptive immunity through regulation of target genes including anti-apoptotic molecules, cell cycle regulators, cytokines, surface receptors, and various other immune modulators. This chapter will focus on the contribution of each NF-κB member to immune cell differentiation and effector function, particularly with regard to macrophages, dendritic cells, T lymphocytes, and B lymphocytes.

Brief Overview of the Immune System

The primary purpose of the vertebrate immune system is to protect the host from infection by foreign pathogens such as bacteria, viruses, and parasites. Distinct immune cell types carry out two main branches of host immunity, namely the innate and adaptive arms of immune regulation. However components of the innate system also play an important part in shaping the adaptive system. Here we review key elements of the immune system that will be relevant to this chapter.

Immune responses are initiated by host cells which recognize foreign antigens as potentially harmful substances in the body. Innate responses are typically generated against microbial antigens through recognition of invariable patterns expressed on molecules produced by pathogens. To identify these antigens, phagocytes such as macrophages and neutrophils bear pattern-recognition receptors (PRR) on their cell surface. The breadth of antigenic responses induced upon recognition by PRRs is restricted however, as only a limited number of fixed structures are effectively detected. As a consequence, the adaptive immune system has evolved to allow more specific recognition of pathogenic antigens, and entails use of antigen-specific receptors to identify these unique epitopes. Antigen-specific receptors are found only on the surface of lymphocytes, and are generated through genetic recombination to produce receptors bearing variable regions that can recognize a potentially infinite number of epitopes. This complex strategy invites a perplexing dilemma as to how the immune system distinguishes "foreign" versus "self" antigens. Mechanisms must therefore be in place to spare the host from

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attacking its own tissues. To avoid harmful autoimmune responses, nature has evolved a fail-safe method to remove self-reactive lymphocytes via a process termed negative selection. Negative selection ensures that only nonself reactive lymphocytes are allowed to populate peripheral lymphocyte pools in the body.

As it stands, the immune system is prepared to guard against foreign invasion and has developed similar methods for the surveillance of abnormal cell growth (i.e., tumors). Modern medicine, not surprisingly, is developing approaches to manipulate these mechanisms for combating cancer, or conversely to enable organ graft tolerance by suppressing undesirable immune reactions. Failure or dysregulation of the immune system therefore has multiple health implications including the development of immunodeficiency, tumorigenesis, hypersensitivity, graft rejection, or autoimmune disease. An understanding of how the immune system operates holds tremendous potential for the treatment of these and many other serious conditions.

NF-κB and Innate Immunity

Innate and adaptive immunity comprise two important aspects of host defense against microbial infection. Innate immunity occurs through phagocytosis of pathogenic material and secretion of anti-microbial or inflammatory mediators by cells such as macrophages and neutrophils. This response typically occurs within minutes or hours of infection. Phagocytes recognize pathogens through several forms of pattern-recognition receptors, including mannose receptors, scavenger receptors, and the more recently identified family of Toll-like receptors (TLRs). Recognition by these receptors leads to (1) enhanced phagocytic activity, (2) production of anti-microbial products such as nitric oxide (NO), defensins, and proteolytic enzymes, (3) production of anti-inflammatory cytokines such as TNF-α, IL-1, and IL-6, (4) production of chemokines or trafficking and adhesion molecules, and (5) expression of costimulatory molecules.[1]

Notably, signaling through all members of the TLR family converge upon the activation of NF-κB through shared signaling pathways (see chapter on Receptor and Adaptor Signaling). Multiple studies support the role of NF-κB in the production of inflammatory mediators that control innate immune responses against bacterial infection. NF-κB induced expression of NO synthase (iNOS) for instance, has been well-documented and highlights one of the key pathways involved in respiratory NO production.[2] Subsequent studies in NF-κB knockout mice have shown that deletion of p65 and c-Rel decreases production of iNOS, as well as the expression of TNF-α, IL-1, and IL-6 in macrophages.[3,4] These reports implicate NF-κB in the regulation of inflammatory cytokines and the respiratory burst in phagocytes. Other studies in mice lacking p50 illustrate the physiological significance of NF-κB-mediated host defense where an inability to clear L. monocytogenes and greater susceptibility to infection with S. pneumoniae is seen.[5] The observed immunodeficiencies are attributed to a hyporesponsive reaction to bacterial lipopolysaccharide (LPS) through a TLR4-dependent recognition pathway. These data suggest that intact NF-κB protein is essential for TLR signaling during activation of macrophages and neutrophils in the clearance of bacteria.

Corroborating these findings is a recent report on a human subject experiencing a recurrent bacterial infection. The study showed that leukocytes derived from the patient displayed profound hyporesponsiveness to LPS and IL-1 concurrent with diminished downstream NF-κB activation.[6] Evidence suggests that the patient carried specific mutations in the IRAK-4 molecule, a kinase downstream of the TLR4 and IL-1R pathways that is required for NF-κB activation (see chapter on Receptors and Adaptors for NF-κB Signaling). Thus, studies such as this support the notion that regulation of inflammatory cytokines, chemokines, and costimulatory molecules by NF-κB is critical to phagocyte activation, maturation, trafficking to inflamed tissues, as well as the ability to present pathogenic antigens to infiltrating T lymphocytes. NF-κB therefore constitutes an important aspect of frontline defense during infection.

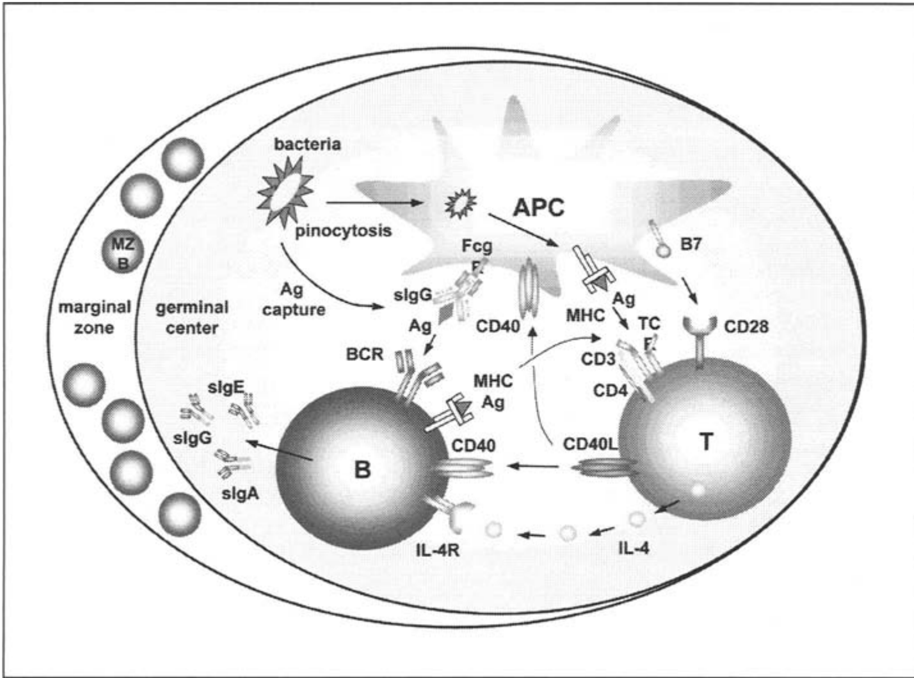

Figure 1. Germinal center response in secondary lymphoid tissues.

NF-κB in Dendritic Cells

Whereas innate immunity can provide initial protection from pathogenic infection, many bacteria and viruses have evolved intricate mechanisms to conceal their identity. Gram-negative bacteria, for instance, are encapsulated by a thick sugar coat that prevents the contents of the cell from being recognized by immune cells. Viruses, on the other hand, may hide inside cells by integrating into host DNA. Mechanisms to accommodate the recognition of disguised pathogens in what is known as the adaptive immune response have subsequently evolved in most higher organisms.[7] The adaptive response involves processing of pathogenic antigens into small peptides that are then presented by MHC molecules to reactive T cells (Fig. 1). MHC class I is expressed on the surface of all cells, but enhanced expression of MHC I and II is found on antigen presenting cells (APC). Antigen receptors on T cells, known as the T cell receptor (TCR), recognize these peptides presented on MHC molecules through APC-T cell interactions. Importantly, this differs from the recognition of native or whole antigens by the B cell receptor (BCR; see below). Soluble BCR is also known as antibody or immunoglobulin, and is produced by B lymphocyte lineages to allow direct recognition of antigens in their unprocessed forms.

The most common type of APCs are dendritic cells (DC) which serve to bridge innate and adaptive immune responses by capturing and processing intracellular antigens for presentation to T lymphocytes in local lymph nodes.[8,9] DCs originate from a common myeloid and lymphoid hematopoietic precursor in the bone marrow and migrate to the periphery as "immature" dendritic cells. Recent studies have devoted much effort to describe the signals that lead to DC maturation, as it appears that the quality or status of DC maturation can determine whether a T cell response is primed or suppressed. During pathogenic infection, numerous signals effectively induce DC maturation including microbial products that bind TLRs on DCs, pro-inflammatory cytokines (such as TNFα and IL-1) produced by infected tissues and

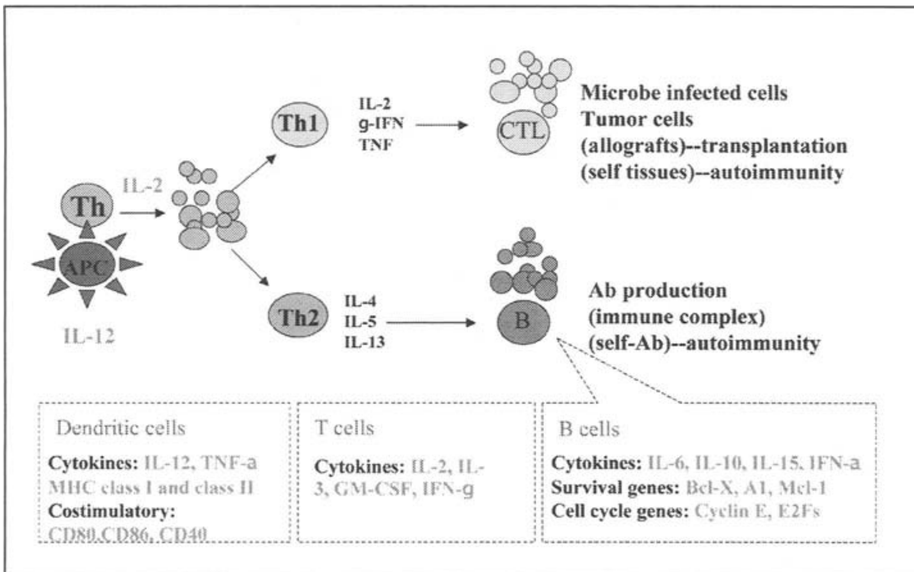

Figure 2. NF-κB/Rel and immune cell effector function.

activated macrophages, and other necrotic cellular components.[8,9] Mature DCs are characterized by enhanced expression of MHC class I and II molecules, costimulatory receptors such as B7-1 (CD80), B7-2 (CD86), and CD40, as well as adhesion molecules such as ICAM-1. Each of these proteins is critical to priming and maintaining strong interactions with T cells via complementary receptors on the lymphocyte (TCR, CD28, CD40L, and LFA-1 respectively).[8-10] DCs may also produce IL-12 and IL-23 to polarize differentiation of T helper cells into T_H1 cells that are crucial for activating cytotoxic T lymphocytes during attack against intracellular pathogens[11] (Fig. 2).

At the intracellular level, NF-κB plays a pivotal role in mediating both DC development and maturation. It was initially observed that RelB knockout mice exhibit defective development of thymic DC and myeloid-derived DEC205$^-$ CD8$^-$ DCs.[12-14] Subsequent studies on other NF-κB knockout mice show that loss of both p50 and p65 abolishes the formation of lymphoid and myeloid DCs.[15] Interestingly however, deletion of c-Rel or both p50/c-Rel does not affect DC development, but does impair the activation of mature DCs. These studies therefore suggest that different NF-κB members serve distinct roles in DC function and development i.e., p50, p65, and RelB are required for DC development, whereas c-Rel and p50 are important for DC survival and costimulatory function.

Since NF-κB is one of the key mediators of TLR and CD40 signaling (critical DC maturation factors), deletion of NF-κB would appear to compromise DC function (Fig. 2). As mentioned above, DC maturation is a crucial aspect of antigen presenting ability via upregulation of MHC and costimulatory molecules. The promoter regions of B7.1, B7.2, and MHC class II genes all contain NF-κB binding motifs. It is believed that NF-κB controls transcriptional regulation of each of these molecules, and blocking NF-κB activity by IkBα overexpression or using pharmacological inhibitors significantly reduces surface expression of each of these receptors on DCs.[16,17] Yet interestingly, individual knockouts of p50, p65, or c-Rel only minimally reduces expression of B7.1, B7.2, and MHC class II receptors on DCs,[15,18] suggesting that NF-κB members may compensate for each other during transcription of these genes, or alternatively that other transcription factors can participate in this capacity.

Studies also implicate NF-κB in the regulation of DC survival and IL-12 cytokine expression. It appears that during DC-T cell interaction, DCs receive survival signals from T cells via CD40L and TRANCE (Fig. 1). DCs lacking both p50 and c-Rel are particularly vulnerable to apoptosis, an effect attributed to diminished Bcl-X anti-apoptotic gene expression.[15] Other studies indicate that c-Rel is an important regulator of IL-12 expression by controlling the transcription of IL-12p40 and IL-12p35 subunits in macrophages and dendritic cells upon TLR signaling.[19-23] Deficient IL-12 production in c-Rel knockout DCs may explain why c-Rel deficient DCs produce insufficient amounts of T_H1 and T_H2 cytokines in vitro. Furthermore, these DCs fail to stimulate both allogeneic and antigen-specific T cell responses.[18] The defect in IL-12 production is consistent with the finding that c-Rel knockout mice are protected from autoimmune encephalomyelitis, a condition primarily mediated by T_H1 responses. The protection afforded through c-Rel deletion is manifested at two levels: reduced production of IL-12 by DCs and decreased production of IFN-γ by T cells (see chapter on Roles of NF-κB in Autoimmunity).[20-26]

NF-κB in T Cell Development and Selection

Multiple studies support the role of NF-κB in shaping the T cell compartment by influencing both thymocyte development and peripheral T cell effector functions. During T cell development in the thymus, $CD4^-CD8^-$ double negative (DN) thymocytes transit through a $CD4^+CD8^+$ double positive (DP) stage before maturing into $CD4^+$ or $CD8^+$ single positive (SP) cells. Selection begins in the DN stage (the first checkpoint) where signaling through a precursor TCR (preTCR) consisting of a genetically-rearranged TCRβ chain and an invariant surrogate α chain (pTα), is necessary for the subsequent rearrangement and expression of the TCRα chain. Once expressed, the TCRα chain pairs with the TCRβ chain to form a functional TCR receptor on the cell surface. Only cells expressing functional TCRs at the surface are allowed to progress further for development.

In vitro and in vivo experiments demonstrate that NF-κB is critical to the survival and proliferation of immature thymocytes.[27,28] Studies reveal that crosslinking the preTCR can activate molecules such as p56-lck, ZAP70, ras, and PLC-γ, resulting in Ca^{+2} flux and downstream activation of NF-AT and NF-κB. It is hypothesized that proliferation-inducing cell cycle regulators (e.g., cyclins) and anti-apoptotic survival genes (e.g., Bcl-2 family) are activated by NF-κB in DN thymocytes, and may occur through mechanisms similar to those previously described for mature lymphocytes.[29-32,33,34] For instance, in T cell specific IkBα-overexpressing mice (IκB broadly inhibits most NF-κB isoforms), a reduction in the total number of DP and SP thymocytes as well peripheral $CD4^+$ and $CD8^+$ T cells is observed.[35-38] Individual and combinatorial NF-κB knockout studies would help confirm these findings, however only c-Rel and p65 knockout studies have been performed thus far. Deletion of either or both of these genes appears to have little effect on early thymocyte development, and may reflect redundant contributions by other NF-κB family members that potentially compensate for c-Rel or p65 deletion in early thymocytes.[39,40]

The second checkpoint takes place during the DP stage where thymocytes failing to express TCR die by neglect while those expressing a functional TCR undergo positive and negative selections.[41,42] T cells expressing different TCRs are initially circulated through thymic epithelium where they encounter peptides presented on MHC molecules of thymic epithelia. Together this MHC-peptide complex binds to TCRs with varying degrees of avidity. Evidence suggests that positive selection involves weak interaction (low avidity) of the TCR with polymorphic residues on the MHC coreceptor.[41] In contrast, TCRs exhibiting strong avidity for MHC-peptides are signaled to die via apoptosis (negative selection), in what is thought to be a mechanism for deleting potentially self-reactive T cells. Newer studies, however, suggest that some T cells may instead become regulatory T cells which exhibit suppressive activity.[41]

The strength and threshold of TCR signaling has been researched to some extent and data from these studies indicate that these factors may be important in determining the fate of DP

and SP thymocytes.[41,43,44] The manner in which cells interpret TCR signal strength is not known however. It is unclear, for example, whether all TCR signals activate a defined set of signaling pathways such as Ca^{+2} mobilization or activation of NF-κB and NF-AT transcription factors. Furthermore, the role of NF-κB in positive and negative selection can only be speculated at present, but emerging evidence suggests that NF-κB proteins do participate to some extent.

A clue to NF-κB's involvement in positive selection emerged from studies using IκBα transgenic mice where broad inhibition of NF-κB in T cell lineages led to a severe reduction of DP and SP thymocytes as well peripheral $CD4^+$ and $CD8^+$ T cells.[35-38] Moreover, deletion of c-Rel and/or p65 has also been associated with decreased peripheral T cell numbers.[39,40] These studies indicate that NF-κB is required for survival of thymocytes, and thereby constitutes an essential requirement for positive selection. With regard to negative selection, it is well documented that thymic epithelial cells present abundant self-peptides, presumably to aid the negative selection of self-reactive thymocytes. Interestingly, both cortical and medullary thymic epithelial cells not only express common self-antigens but also tissue-specific self-antigens that are often expressed only in restricted tissues (i.e., insulin in pancreatic β-cell). This rather promiscuous form of gene expression in the thymic environment is thought to provide a means for eliminating harmful self-reactive thymocytes through either cell death or the generation of regulatory T cells.

A connection between NF-κB and negative selection was first reported in the study of aly/aly mice that harbor a mutation in the NF-κB Inducing Kinase (NIK) gene.[45] The data showed that $NIK^{aly/aly}$ mutant mice develop autoimmune diseases and multi-organ inflammation with disorganized thymic structure, much like RelB knockout mice.[46] More recent reports describe a reduction in $CD4^+CD25^+$ regulatory T cells in these mice, and that this deficiency is associated with impaired processing of the p52 precursor protein p100, as well as low levels of RelB production.[45] Autoimmune conditions in $NIK^{aly/aly}$ mice are rescuable however, by transferring $CD4^+CD25^+$ regulatory T cells into $NIK^{aly/aly}$ hosts, thereby implying that the suppressive function of these cells is relevant to the prevention of self-reactive T cell activation. Further data from the same study suggest that NIK activation (e.g., via RelB and p52) may contribute to the generation of $CD4^+CD25^+$ regulatory T cells through promiscuous expression of temporally controlled tissue-specific genes in thymic stromal cells. Validation of the hypothesis that NF-κB is involved in negative selection or immune tolerance could ideally be tested in p52/RelB knockout mice.

Finally, a recent study suggests that p50 and c-Rel may also contribute to the generation of $CD4^+CD25^+$ T-regulatory cells.[47] c-Rel is a known transcriptional activator of IL-2 in activated T cells and both c-Rel and p50 have been detected at the CD28 responsive element in the IL-2 promoter.[48,49] Considering that numerous reports now suggest a role for IL-2 and STAT5 in the generation of T-regulatory cells,[43,50-55] NF-κB dependent activation of IL-2 my serve as the potential mechanism by which this occurs. Further work to determine whether individual NF-κB knockout mice develop autoimmune disease, as evidenced for IL-2 knockout, IL-2 receptor knockout, and STAT5 knockout mice, will be critical to establishing this relationship.[52-55]

NF-κB in T Cell Effector Function

Key features of adaptive immunity include (1) the ability to recognize an almost infinite number of antigens, (2) clonal selection and expansion of antigen-specific lymphocytes, and (3) development of lymphocyte memory to ensure a more rapid and efficient response upon reencounter with the same antigen. Antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes exist at low frequency in naïve hosts. Upon infection or immunization, clonal expansion of antigen-specific T cells is necessary to generate large numbers of effector cells. A small portion of effector cells will also differentiate into memory cells to ensure rapid recall response. Evidence suggests that NF-κB controls lymphocyte clonal expansion and effector function through the regulation of cell survival proteins, cell cycle regulators, cytokines, chemokines, surface

receptors, and adhesion molecules.[56] Studies also suggest that development of memory and T-regulatory cells may involve NF-κB.[47]

Cytokines serve unequivocal functions in shaping T effector cell development. $CD4^+$ T cells exist primarily to aid the expulsion of pathogens through cytokine-dependent activation of phagocytes, cytotoxic T cells (CTL), natural killer cells (NK) and B cells (e.g., antibody-secreting B cells) (Fig. 2). Upon TCR ligation, naïve $CD4^+$ T cells differentiate into two distinct subsets: T helper 1 (T_H1) and T helper 2 (T_H2). Each of these cells is characterized by the production of a specific set of cytokines. T_H1 cells express IFN-γ and TNF-α which promote phagocyte, NK, and CTL responses, while T_H2 cells express IL-4, IL-5, IL-9, and IL-13 that largely aid the activation of B cells.[57,58] Mice deficient in T_H1 cells demonstrate that T_H1 dependent responses are essential to protect the host against intracellular pathogenic infection,[59,60] whereas T_H2 deficiency results in loss of protection against extracellular pathogens.[61,62] Hence the action of $CD4^+$ T helper cells is primarily supportive in nature. In contrast, $CD8^+$ T cells mediate active cytotoxic killing through the production of cytolytic enzymes such as perforin and granzyme B and/or the production of IFN-γ and TNF-α.[63]

During the presentation of antigen to T cells, only antigen-specific cells undergo clonal expansion into effector cell populations. This initial wave of proliferation is mediated by the expression of IL-2 upon TCR and CD28 signaling. IL-2 is perhaps the most influential T cell cytokine during this phase that not only drives the replication of T cell clones, but also promotes downstream differentiation of $CD4^+$ T cells into helper cells. When combined with IL-12 produced by DCs, IL-2 promotes differentiation of $CD8^+$ T cells into cytotoxic T lymphocytes and production of IFN-γ.[64-67] The importance of IL-2 and IFN-γ is demonstrated by the absence of T_H1 and CTL mediated anti-viral immune responses in IL-2 knockout mice.[63,68] Studies show that upon LCMV infection, viral specific $CD8^+$ T cells fail to proliferate in IL-2 knockouts, correlating with an observed decrease in IFN-γ production and a reduction in CTL mediated killing of infected cells.[69,70]

IL-2 expression in T cells is coordinately regulated by NF-κB, NF-AT and AP-1 transcription factors.[49,71-73] In particular, studies show that the CD28 responsive element in the IL-2 promoter possesses high affinity for c-Rel homodimer binding,[49] whereby c-Rel deficient T cells exhibit impaired expression of IL-2, proliferate poorly in response to TCR signals, and are unable to generate $CD8^+$ effector T cells.[74-76] c-Rel is not only essential to expression of IL-2, but regulates the expression of other cytokines such as IL-3, GM-CSF, and IFN-γ as well.[74-76] Given that c-Rel mediates IL-2 and IFN-γ expression during T_H1 responses, there is precedence that loss of anti-viral responses and decreased memory cell production occur in the absence of c-Rel or other NF-κB members (also see chapter on Regulation of Immunity to Infection). Certainly such hypothesis could be tested in relevant NF-κB knockout systems.

Recent studies have identified several transcription factors that are regulated through antigen receptor signaling, as well as cytokines, which direct T_H1/T_H2 differentiation. For example, T-bet appears to be required for T_H1 polarization, whereas GATA-3 is required for generation of T_H2 cells.[57,58,77] Reports indicate that different NF-κB members may contribute to either T_H1 or T_H2 development as well. Initially, researchers thought that NF-κB participated only in the development of T_H1 responses as studies in c-Rel deficient mice showed defective production of T_H1 cytokines (IL-12 and IFN-γ),[19-21,23] and IκBα transgenic mice displayed impaired T_H1 responses correlating with defective survival, clonal expansion, and loss of T_H1–mediated IFN-γ production.[78,79] Further examination however, reveals that p50 deficient T cells exhibit impaired production of T_H2 cytokines associated with decreased GATA3 expression as well.[80] These studies suggest that p50 acts upstream of GATA3 gene expression.[80] More recent studies with Bcl-3 knockout mice (an IκB member) further demonstrate that Bcl-3, possibly in complex with p50, drives GATA-3 expression and participates in T_H2 differentiation. By comparison, RelB appears to control T_H1 differentiation by regulating T-bet gene expression (Mark Boothby, manuscript in preparation). Hence the contribution of different NF-κB or IκB members to either T_H1 or T_H2 responses is more complex than originally believed.

NF-κB in B Lymphocyte Clonal Expansion and Cell Fate Determination

Individual NF-κB factors are implicated in various stages of B cell differentiation ranging from immature cell to effector cell development (for discussion of early B cell development, see chapter on NF-κB and Lymphopoiesis). This section will therefore focus on the signaling consequences of antigen-activated B cells and how interactions among various B cells, T cells, and DCs contribute to germinal center formation and terminal differentiation of B cells into antibody-producing plasma cells and memory B cells (Fig. 1).

There are several means by which mature B cells can encounter foreign antigen. Antigens captured by DCs in tissue can be passed to B cells after DC migration to the lymph node whereas antigens circulating in peripheral blood can be trapped in lymphoid organs through high endothelial venules (HEV). Upon antigen binding in lymphoid follicles, mature B cells undergo clonal expansion in a manner similar to T cells. Notably, the BCR and TCR share analogous signaling pathways including homologous tyrosine kinases and adaptor molecules that induce activation of NF-κB, NF-AT, and AP-1 transcription factors (see chapter on Receptors and Adaptors for NF-κB Signaling). Among these factors, NF-κB dimers (particularly c-Rel/p50 heterodimers) appear to be absolutely required for lymphocyte survival and cell cycle progression.[56] Numerous reports conclude that c-Rel expression in B cells is critical to expression of pro-survival molecules (e.g., Bcl-X, Bfl-1, Mcl-1),[29-32] cell cycle regulators (e.g., cyclin E, E2F3a),[33,34] and cytokines (e.g., IL-6, IL-10, IL-15)[32,56] (Fig. 2).

More and more evidence suggests that the level of NF-κB activation may dictate the fate of lymphocytes upon antigen encounter, i.e., clonal expansion vs. anergy or tolerance. In T lymphocytes, chronic exposure to antigenic signals in the absence of costimulation can lead to anergy (state of unresponsiveness). Anergy of T cells is associated with activation of NF-AT without coactivation of NF-κB and AP-1.[81] Subsequent studies show that NF-AT dimers, in the absence of NF-κB and AP-1, can induce expression of molecules that negatively regulate TCR signaling (and potentially BCR signaling), including Cbl-b and Itch (both E3 ubiquitin ligases) and a ubiquitin-binding component which cooperatively target PLC-γ and PKC-θ for degradation, thus leading to termination of TCR signaling.[82,83] These studies suggest that the primary function of costimulatory signals is to perhaps facilitate activation of NF-κB and AP-1, which together with NF-AT enable the induction of a different set of target genes such as cytokines and chemokines that collectively lead to immune reactivity rather than immune tolerance.[83,84] A similar phenomenon is described in B lymphocytes whereby B cells anergic to self-antigen exhibit specific blockade of c-Rel, p65, and JNK activation.[85]

It has been proposed that differential recruitment of CARMA1 or Cbl adaptor proteins to the intracellular BCR signaling tail (and potentially the TCR) promotes formation of an immunosome or tolerosome, respectively, and ultimately leads to opposite outcomes, i.e., immunogenic response vs tolerogenic response.[86] A notable feature of the immunosome is the ability of CARMA1 to recruit various NF-κB signaling complexes, Bcl-10, MALT1, PKC, and IKK to the intracellular proximal receptor chains where signaling is amplified and sustained within lipid rafts (see chapter on Receptors and Adaptors for NF-κB Signaling). By comparison, the tolerosome is composed of Cbl ubiquitin ligase, E2-conjugating enzyme, and endophilin complexes that target proteins into endosomal compartments for degradation. Hence, an antigenic signal may result in an immunogenic response if NF-κB signaling components are recruited to the BCR-lipid raft, while a tolerogenic response may result in the absence of recruitment.

Cell fate determination has also been investigated in immature B cell development. While mature B cells are signaled to proliferate upon BCR ligation, it was observed that immature B cells undergo growth arrest and apoptosis instead.[87,88] The propensity for BCR-induced death in this manner potentially ensures that immature B cells exhibiting self-reactivity are deleted prior to their appearance in the periphery. Molecules expressed in high concentration in the bone marrow, such as self-antigens, will thus bind the BCR with high affinity and result in

apoptosis before the cells have a chance to emerge from the bone marrow. Conversely, those that are not deleted presumably do not recognize self-antigens and are therefore less prone to induce autoimmunity.

Only until recently it became clear that immature B cells are defective in their ability to mobilize several key signaling components necessary for NF-κB activation, including PKC, PI3K, and Akt,[89] (Cheng S, Feng B, Hsia C, and Liou H-C, manuscript in preparation). It is proposed that immature B cells destined to die fail to express critical survival genes (e.g., Bcl-X) and cell cycle progression factors (e.g., E2F, cyclin E) lying downstream of these signaling pathways. Indeed new evidence now suggests that both Bcl-X and E2F3a are regulated by NF-κB in mature B lymphocytes.[33,34] In attempt to better understand the decision to respond to antigenic signals or else undergo tolerance, comparison of NF-κB induced target genes under certain conditions or states of development will be necessary. Recent insights have been offered through microarray profiling of c-Rel dependent targets which provide a glimpse of relevant genes including transcription factors, adhesion molecules, intracellular signaling proteins, C-type lectin-like receptors, and metabolic enzymes (Hsia, C, Owyang, A, Tian, W, Hsu, J, Xiang, J, and Liou manuscript in preparation). These findings indicate that the role NF-κB plays in controlling lymphocyte fate extends beyond the regulation of proliferation or survival and may include differentiation, trafficking, metabolism, and cell-cell communication.

NF-κB in the Germinal Center Immune Response

During microbial infection, dendritic cells capture antigens and migrate to the T-cell zone in lymphoid tissue where they activate antigen-specific T cells (Fig. 1). Antigen-specific B cells are similarly trapped in the T-cell zone upon encounter with BCR-specific antigens. Numerous studies document the importance of $CD4^+$ T cell help in promoting B cell proliferation, differentiation, and the formation of germinal centers.[90] Germinal centers are specialized microenvironments within lymphoid tissue where interaction among follicular DCs, T cells, and B cells is highly concentrated. The result of these interactions may lead to vigorous B cell proliferation, Ig somatic hypermutation, and Ig class switching. Importantly, the T cell-B cell interface involves multiple pairs of receptor-ligand interactions including CD40L-CD40, CD28-B7, and CD30L-CD30. These pairings effectively determine the outcome of an immune response, controlled in large part by cytokines produced within the interaction environment. T_H2 cytokines such as IL-4, IL-5, IL-6, IL-13, and TGFβ for example, facilitate Ig class switching to IgG, IgE, and IgA subclasses.[91]

Not surprisingly, certain NF-κB knockout mice display defective germinal center and antibody phenotypes. Poor germinal center formation is observed in c-Rel deficient mice for instance, correlating with defective Ig class switching and failure to develop memory B cells upon immunization.[30-34,74,92] While this defect appears to be cell-intrinsic, other NF-κB knockout studies show that p52 and RelB deficiency results in both intrinsic and extrinsic defects of B cell follicles including disorganized formation of follicular dendritic cell networks. Interestingly, the extrinsic effect is attributed to loss of NF-κB dependent BLC chemokine expression,[14,93,94] a gene expressed on lymphoid stromal tissue which normally provides chemotractant signals for B cell retention in the lymphoid follicles.

The ultimate purpose of B cell activation is to generate antibodies that will bind and neutralize microbial antigens through various means. This can be achieved through immunoglobulin class switching from IgM/IgD to soluble IgG, IgA, or IgE isotypes. Depending on the isotype, each immunoglobulin carries out different effector functions such as neutralization, opsonization, or activation of the complement system. Class switching to a particular isotype depends upon the accessibility of transcription factors to enhancer regions within the heavy chain (CH) constant locus. In particular, NF-κB binding sites have been identified within the Cγ3, Cγ1 and Cε chain enhancer, and thus implicated in the germline transcription of IgG3, IgG1, and IgE isotypes.[95,96] Cells lacking c-Rel for example, exhibit loss of IgG1 and IgE production resulting from impaired Cγ1 and Cε germline transcription.[97,98] Similarly, impaired germline transcription of Cγ3 and Cε in p50 or p65 deleted B cells results in defective

Table 1. NF-κB function in immune cells

Immune Cell Type	NF-κB Function
Phagocytes (macrophages,neutrophils)	Phagocytosis
	iNOS, TNFα, IL-1, IL-6
	Chemokines
	Costimulatory molecules
Dendritic cells	Development (p50, p65, RelB)
	Maturation (p50, c-Rel)
	Survival (p50, c-Rel)
	Cytokines (IL-12, IL-23)
	Costimulatory molecules (B7.1, B7.2)
	MHC class II
NK cells	Unknown
T lymphocytes	DN thymocyte expansion
	DP, SP thymocyte survival
	Positive selection
	Negative selection (unknown)
	Survival and proliferation
	Cytokines (IL-2, IL-3, IFN-γ)
	CD4+ T_H1 differentiation (c-Rel, RelB)
	CD4+ T_H2 differentiation (p50, Bcl-3)
	CD8+ CTL effector function
	Memory T cell (unknown)
	T-regulatory cell (unknown)
B lymphocytes	Development and maturation (p50, p65, p52)
	Survival (Bcl-x, Bfl-1, Mcl-1) (c-Rel)
	Proliferation (cyclin E, E2F3a) (c-Rel)
	Cytokines (IL-6, IL-10, IL-15)
	Germinal center formation
	Ig class switching
	Plasma cells (unknown)
	Memory B cells (unknown)

Note: Listed in the parentheses are NF-κB members required for carrying out described functions, not excluding the potential participation of or cooperation with other NF-κB

switching to IgG3 and IgE.[99,100] These selective defects demonstrate specific NF-κB requirements for individual isotypes, some of which overlap.

Concluding Remarks

The NF-κB transcription factor family plays an important role in innate and adaptive immunity by regulating various aspects of immunological development and effector cell function (Table 1). Although NF-κB members display significant overlapping activities, each member is also unique with regard to differential responses to receptor signals and distinct target gene profiles. Future challenges include systematic analysis of NF-κB activity on downstream targets in various immunomodulatory capacities including inflammatory diseases, autoimmune diseases, transplant rejection, as well as host defense against pathogenic infection. As such, these research efforts hold significant promise toward the discovery and development of therapeutic targets for the prevention of immunopathological conditions via manipulation of NF-κB signaling.

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CHAPTER 7

Roles of NF-κB in Autoimmunity

Stacey Garrett and Youhai H. Chen*

Abstract

Autoimmune diseases are the result of improper and uncontrolled immune responses against self-antigens. The NF-κB/Rel family of transcription factors is known to play important roles in the initiation and regulation of immunity against pathogens and foreign antigens. Over time, evidence has accumulated implicating NF-κB as a mediator of autoimmunity and potential therapeutic target for treating autoimmune diseases. In this chapter, we discuss the role of NF-κB in apoptosis and cell proliferation as well as tolerance and autoimmunity.

Introduction

Once described as "horror autotoxicus", autoimmunity has become a well-recognized phenomenon.[1] At its surface, autoimmunity may be described simply as the incomplete tolerance of the host's immune system to self antigens. Studies showing that autoantibodies and autoreactive T cells can be detected in healthy mice and humans render the previous definition of autoimmunity overly simplistic.[2-5] Recent studies indicate that autoimmunity does not always lead to autoimmune disease leading researchers to conclude that genetic and environmental factors are also required for the development of autoimmune diseases.

Autoimmune diseases occur on two scales: systemic and organ-specific. Additionally, different diseases are driven by different cell types, primarily T or B cells. Autoimmunity is essentially an immune response, albeit an uncontrolled and improper one. The NF-κB family of transcription factors has been implicated as one of the master controllers of the immune response. As detailed in other chapters of this book, these molecules have complex effects on the processes of lymphocyte proliferation, cytokine production, and apoptosis. Each of these cellular processes is intricately involved in immunity and autoimmunity.

Roles of NF-κB in T Cell Development

Proper thymic development is critical for normal immune function. The thymus is responsible for positively and negatively selecting T cells as they progress from the CD4+CD8+ (double positive) stage to the single positive stage. Through the years, the role of NF-κB in this process has been hotly debated.

Early studies involving deletion of single NF-κB family members or blockage of c-Rel and Rel A activation via transgenic expression of mutated IκBα indicated that NF-κB played no role in thymocyte development or selection.[6-9] By contrast, the absence of a functional IκB kinase causes a dramatic reduction in the total numbers of thymocytes, presumably due to its

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role in protecting cells from TNF-α induced apoptosis.[10] Recently these findings have been questioned in models employing TCR transgenic mice.

By crossing the IκBα(ΔN) transgenic mouse (which blocks c-Rel and RelA activation in the T cell lineage by out competing endogenous IκBα) to the DO11.10 TCR transgenic mouse, Mark Boothby and colleagues found that positive and negative selection were inhibited. This inhibition was characterized by diminished ZAP-70 phosphorylation in response to stimulation. This study differs from others in that it examined thymocyte development on two different genetic backgrounds – H-2d x H-2d allowed for examination of positive selection while H-2d x H-2b allowed for study of negative selection. The use of IκBα(ΔN) may be superior to that of other dominant negative transgenic IκBα molecules in that it is expressed at a higher level and therefore achieves a more complete blockade of c-Rel and RelA activation. While IκBα(ΔN) protects thymocytes in vivo from negative selection, this study supports signal strength-based theories of negative selection by showing that high levels of negatively-selecting peptide stimulation in vitro can bypass the protective effects of IκBα(ΔN).[11]

Additional evidence for a role of NF-κB in mediating central tolerance has been shown through studies of the aly/aly strain of mice. The aly mutation renders the NF-κB inducing kinase (NIK) incapable of binding to IKK-α. As a result, the development of lymphoid organs is abnormal and these mice experience autoimmunity characterized by chronic inflammation of several organs.[12-14]

Fumiko Kajiura and colleagues performed crucial studies to illustrate the mechanism by which this mutation leads to autoimmunity. These researchers showed that disease identical to that of aly/aly mice could be transferred to nude mice via thymic engraftment. The phenotype can also be rescued by transfer of normal CD4+ T cells and the inflammation is not completely resolved if only CD4+CD25- T cells are transferred. They went on to demonstrate by FACS that the total number of CD25+ regulatory T cells are decreased in the periphery and thymi of aly/aly mice, although these cells are functional in vitro at suppressing T cell proliferation. To support the argument that regulatory T cell production was affected by this NIK defect, they showed that the total levels of FoxP3, a protein currently believed to be specific to regulatory T cells, was decreased in both CD4+ T cells and the thymi of aly/aly mice.[15] Additionally, PCR analysis of AIRE, a family of transcription factors responsible for the expression of peripheral antigens in the thymus, demonstrated that defects in central tolerance were directly related to defects of negative selection in the aly/aly thymi. AIRE message was decreased and the expression of specific peripheral antigens, like salivary protein 1, was completely absent in the thymi of aly/aly mice.[16] As a result, negative selection to peripheral antigens was incomplete.

This study provides critical information about the role of NF-κB in the processes by which the thymic microenvironment establishes central tolerance. A critical question remains as to the role of NIK in the production and maintenance of regulatory T cells though. This study does not answer the question as to whether the decrease in regulatory T cell numbers is due to dysregulation of AIRE and peripheral antigen expression in the thymus or to the role of NIK in the generation of regulatory T cells themselves.

These studies demonstrate that NF-κB does have a role in the development of T cells in the thymus. However, only in the case of the aly/aly strain of mice do these defects actually lead to autoimmune disease. Yet the connection between NF-κB and autoimmunity is supported by the fact that single gene knockouts of NF-κB members affect lymphocyte function to the point that these mutations render mice resistant to the development of autoimmunity. One exception to this statement is the RelB-/- mice which develop diseases similar to the aly/aly mice due to the effects of TNF-α.

Roles of NF-κB in Mature Lymphocytes

Several studies have shown increased NF-κB activity in autoimmune diseases like myasthenia gravis, rheumatoid arthritis, and multiple sclerosis while systemic lupus erythematosus patients have been described to have decreased NF-κB activity in lymphocytes.[17-19] Schmid and Pahan demonstrated that activated NF-κB dimers could be found in the spinal cords of

rats with experimental allergic encephalomyelitis (EAE).[20] These observations as well as the critical role of NF-κB in the initiation of effector immune responses and lymphocyte survival have made NF-κB an intriguing target of autoimmune research in recent years. Additionally, our laboratory has shown that deficiencies in NF-κB1 and c-Rel render mice resistant to the development of autoimmunity.[21-23]

One of the key studies providing a link between NF-κB dysregulation and autoimmunity demonstrated aberrant cytokine regulation in autoimmune mice. It had long been observed that MRL and NZB mouse strains, both of which are prone to the development of autoimmunity, produced reduced levels of IL-1 in vitro. This observation was similar to that found for monocytes of lupus patients (rev in 24). Hartwell and colleagues showed that the expression of inflammatory cytokine transcripts like IL-1 and TNF-α occurred with a fatser kinetics and disappeared earlier than in control Balb/c cells. This correlated with a more rapid repression of IL-1 transcriptional activity in MRL/+ macrophages. This rapid repression was accompanied by accelerated reduction of nuclear NF-κB as shown by EMSA while the faster loss of cytokine mRNAs was not associated with decreased mRNA stability in the MRL/+ mice. Thus, researchers concluded that differences in transcription were directly due to the amount of NF-κB in the nucleus resulting in a decrease of IL-1 protein expressed by MRL/+ macrophages in vitro.

Beller's laboratory followed up these findings by showing that the dysregulation of NF-κB was the cause of the abnormal IL-12 production observed in NOD and NZB/W mice. Interestingly, these two strains show different patterns of IL-12 production for different reasons. NOD mice display elevated levels of IL-12 as is generally associated with Th1 mediated organ specific diseases while NZB/W show a reduction in IL-12 before the development of pathology similar to systemic lupus.

Using thioglycolate elicited peritoneal macrophages, Liu and Beller showed that these defects were due to unique binding patterns of Rel dimers to the IL-12p40 promoter. In NOD mice, there was a preferential association of c-Rel with the p40 κB site due to hyperphosphorylation of c-Rel dimers. In contrast, p50 was found bound to the p40 κB site in NZB/W mice. This difference appeared to be due to elevated levels of IκB specifically sequestering c-Rel dimers in the cytoplasm. These findings demonstrated that different patterns of cytokine production can be directly explained by the effects of dysregulation of NF-κB mediated transcription.[25]

Roles of NF-κB in Apoptosis, Proliferation and Autoimmunity

Perhaps one of the strongest links between NF-κB and autoimmunity is the role of this family of transcription factors in regulating both proliferation and apoptosis. One of the most relied upon treatments for autoimmunity involves the use of prednisone to decrease inflammation as well as to prime lymphocytes for apoptosis. As a result, there has been much study of the link of Rel family members to both of these processes over the years.

Several studies have shown a link between NF-κB and apoptosis.[8,26,27] Cells deficient in RelA/p65 show an increased sensitivity to TNF-α and other mediators of apoptosis like radiation (rev in 28). Additionally, c-Rel deficiency results in enhanced B cell apoptosis following mitogen stimulation unless both the BCR and CD40 are signaled.[29] NF-κB also controls many pro-survival genes like c-IAP, A20, and c-Myc.[28]

This led Vallabhapuvapu and colleagues to determine if blocking NF-κB activity with a mutant IκBα molecule targeted to the T cell lineage would alleviate disease in gld/gld mice. Gld mice lack functional FasL, develop lymphadenopathy and autoimmunity characterized by increased serum immunoglobulin and autoantibodies (reviewed in ref. 30). Use of this transgenic mutant IκBα resulted in a dramatic reduction of lymphadenopathy and a complete elimination of the abnormal thy1+B220+CD4-CD8- T cells. Additionally, reduced proliferative responses and an increase in apoptosis of peripheral T cells were shown resulting in partial correction of B cell abnormalities. Nuclear extracts revealed increased amounts of p50 homodimers in unstimulated and stimulated nuclear extracts of the mutant IκBα gld/gld mice.[31] This study

provides intriguing possibilities of targeting NF-κB activity in specific lymphocyte compartments for the treatment of autoimmune diseases.

Another interesting relationship between NF-κB, apoptosis and autoimmunity has been demonstrated recently by Mei Wu's laboratory. They generated a transgenic (Tg) mouse in which immediate early response gene X-1 (IEX-1) was constitutively overexpressed in the lymphoid lineage which resulted in susceptibility to a lupus-like autoimmune disease due to accumulation of activated T cells. Additionally, they showed that IEX-1 Tg T cells are resistant to apoptosis mediated by ligation of Fas and TCR.[32] Furthermore, the IEX-1 promoter is specifically regulated by NF-κB in coordination with p53 and c-Myc. The IEX-1 promoter can be activated by multiple Rel dimers including p65/c-Rel, p65/p50, and p50 homodimers.[33]

This data combined with previous studies, which showed that IEX-1 is upregulated in response to mitogens and NF-κB activating cytokines, argues that IEX-1 is an important NF-κB target which functions to promote T cell survival during an immune response. Further studies need to be performed to determine if it possesses similar functions in other cell types since there is evidence indicating that IEX-1 can promote apoptosis in cultured cells under certain conditions (reviewed in ref. 32). Given the lupus-like phenotype of IEX-1 Tg mice, this gene may be a promising therapeutic target for autoimmune diseases.

Roles of NF-κB in Autoimmune Encephalomyelitis and Diabetes

The link between c-Rel transcriptional control of IL-12p35 and p40 led our laboratory to examine the role of c-Rel and NF-κB1 in both experimental autoimmune encephalomyelitis (EAE) and type I diabetes.[34-36] We found that mice deficient in NF-κB1 experienced significantly less EAE due to reduced proliferation and effector function of myelin oligodendrocyte glycoprotein (MOG)-specific T cells.[21] Similarly, c-Rel deficient animals experienced significantly less disease than controls as well as a fourteen day delay in disease onset following MOG immunization. The c-Rel knockout group also showed only a 16 percent incidence of disease. Splenocytes from MOG-immunized c-Rel deficient mice showed deficiencies in proliferation, IL-2 and IFN-γ production as have been previously described in response to other stimulations.[22,37-39] Interestingly, we also observed a significant increase in IL-4 production as well.

This led us to compare the levels of interferon-γ versus IL-4 production of NF-κB1 deficient B6:129 mice, c-Rel deficient C57BL/6 mice, and their respective controls to determine if the skewing of Th cytokines was a result of the strain background of the different knockouts. We found that control B6:129 mice produced slightly more IL-4 than control C57BL/6 mice, but both produced similar amounts of IFN-γ. In contrast, the c-Rel knockout B6 mice generated as much IL-4 as the B6:129 strain control while producing almost no IFN-γ. The NF-κB1 knockout mice produced a similar level of IFN-γ as its control while making almost ten fold less IL-4. This data indicates that NF-κB1 and c-Rel may influence the Th differentiation of T cells. Mechanistically, it is unclear if this Th effect is T cell intrinsic or the result of NF-κB transcriptional regulation of the microenvironment in which the T cell is primed.

An argument for the effect of NF-κB on the microenvironment of the T cell was made by showing that deficiency in c-Rel reduces the amount of IL-12p40 produced by bone marrow derived dendritic cells, microglia and astrocytes. Also, lack of c-Rel decreases the amount of IL-23 p19 message produced by antigen presenting cells stimulated with LPS while the level of T-bet expression by T cells is unchanged compared to wild type cells. IL-23p19 has been implicated as necessary and sufficient for induction of EAE suggesting that the defects in antigen-presenting cell function in c-Rel deficient mice may be more influential than the T cell defects.[40-42] This is supported by data demonstrating that wild type T cells stimulated with anti-CD3 in the presence of c-Rel deficient DCs produce 5 fold less IFN-γ than controls. Despite this finding, supplementation of IL-12 to c-Rel-/- CD4+ T cells stimulated with anti-CD3 and anti-CD28 does not increase IFN-γ production indicating that the role of c-Rel in Th differentiation is not limited to its affects on the priming DCs.

Similarly, using low dose streptozotocin to induce type I diabetes, we showed that both c-Rel and NF-κB1 deficient mice experienced significantly reduced incidence of diabetes as well as a delay in disease onset. Splenocytes from c-Rel deficient mice displayed increased IL-4 production as well as increased IL-10 production in response to anti-CD3 stimulation. Additionally, both c-Rel-/- and NF-κB1-/- bone marrow derived dendritic cells stimulated with LPS produced significantly reduced TNF-α. To further investigate the mechanism of diabetes resistance in these mice, apoptosis of monocytes was studied. In the absence of GM-CSF and IL-4, NF-κB1 deficiency resulted in a two fold increase in apoptosis of bone marrow derived DCs while apoptosis was increased in c-Rel deficient granulocytes and macrophages. Stat-1 expression in NF-κB1-/- macrophages was also decreased. This study provides further evidence for a significant role for NF-κB family members in shaping the innate immune response and its impact on the development of autoimmune diseases.[22]

In the streptozotocin-induced diabetes model, the loss of c-Rel and NF-κB1 have multiple effects on factors affecting disease development. Beyond the well-known effects on T cells, it is significant that NF-κB1 deficiency also affected the survival of antigen presenting cells. This effect could very well decrease the amount of T cell priming in vivo due to decreased antigen presentation in lymphoid organs. The decreased TNF-α production could also slow disease progression by decreasing the number of lymphocytes trafficking to both lymphoid organs and the pancreas. This effect could also explain the decreased disease in the c-Rel deficient animals. More implications of this study may become clear as the exact mechanism of streptozotocin-induced diabetes is determined.

Conclusion

The role of NF-κB in cell proliferation and survival has long been known. NF-κB dimers influence the transcription not only of growth factors and cytokines, but also regulators of cell cycle (reviewed in ref. 43). Multiple studies of NF-κB knockout mice have demonstrated decreased lymphocyte proliferation in response to mitogens as well as defects in Th differentiation. These results combined with evidence implicating NF-κB's role in immune tolerance establish NF-κB as a potential mediator of autoimmunity and therapeutic target. However, evidence is also accumulating suggesting that some of the NF-κB members or regulators can prevent autoimmune diseases under certain circumstances. Thus, both RelB and NIK may protect against autoimmunity through their control of TNF-α signaling. Depending on the cell types involved in the initial stages of autoimmune diseases and the types of the NF-κB activated, the roles of the NF-κB family may vary. Environmental and genetic factors which result in hyper-responsiveness or a shift in NF-κB species activation can tip the fine balance from normal immune function to autoimmunity. Further studies are needed to better understand the mechanisms of action of different members of NF-κB in promoting and inhibiting autoimmunity.

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CHAPTER 8

The Central Role of NF-κB in the Regulation of Immunity to Infection

Cristina M. Tato and Christopher A. Hunter*

Introduction

The NF-κB family of transcription factors is a group of evolutionarily conserved proteins involved in lymphoid organogenesis, the development of immune cells as well as the coordination of many aspect of innate and adaptive immunity to infection.[1-10] Although the events involved in the activation of these transcription factors is covered in greater detail in other chapters of this book, a brief overview here will highlight some key points. In particular it is important to note that numerous stimuli which act through a variety of receptors initiate the activation of distinct pathways that recruit unique combinations of scaffolding and signaling proteins that ultimately converge on the IκB kinase (IKK) complex. IκB is a protein that is bound to dimers of NF-κB and which retains these transcription factors in the cytoplasm. Activation of IKK leads to the phosphorylation of IκB and its degradation that allow nuclear localization of NF-κB.[3,11-13] Depending on the stimulus and the cell type, different combinations of homo- and hetero-dimers of these transcription factors are activated. Once in the nucleus, NF-κB is involved in the regulation of numerous genes involved in immune function including the production of IκB proteins that provide a feedback mechanism to limit NF-κB activity.[14,15] In part, because of the central role of these transcription factors in the regulation of the immune system this pathway has become one of the best studied signaling pathways and become a focus of drug discovery for the treatment of inflammatory diseases and cancer. Nevertheless, the aim of this chapter is to focus on the principal role of NF-κB in the development of protective immunity to infection.

Though numerous stimuli lead to the activation of NF-κB, some of the best characterized are associated with pathogens. The binding of diverse microbial products (LPS, bacterial DNA, peptidoglycans, and parasite mucins) that contain pathogen associated molecular patterns (PAMPs) to pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) or NOD proteins, results in the activation of NF-κB which initiates distinct profiles of gene expression associated with innate responses to pathogens.[16-21] Some of the best examples of genes that are regulated by these events are cytokines, such as IL-12 and TNF-α, that have a prominent role in innate immunity. Other events that fall into this category include the expression of chemokines and adhesion molecules which are necessary for the migration of immune cells into sites of inflammation.[22-27] NF-κB signaling also regulates the production of effector molecules such as nitric oxide and perforin, which are directly involved in the control of pathogens.[28-30]

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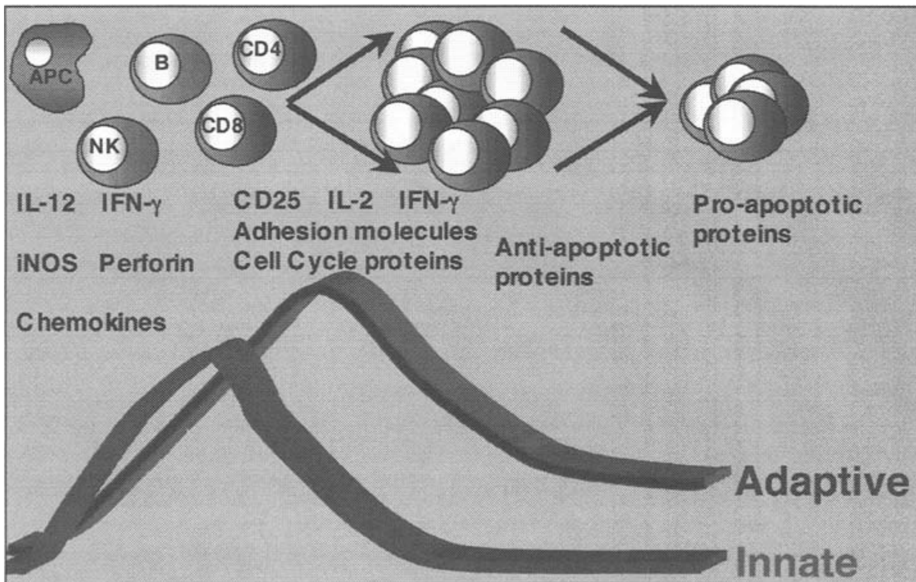

Figure 1. NF-κB target genes. As the immune response transitions from innate to adaptive immunity, NF-κB activation leading to the transcription of target genes is necessary for almost every aspect of resistance to a variety of pathogens. NF-κB promotes activation of innate immune cells such as macrophages and NK cells, and the production of key cytokines, such as IL-12 and IFN-γ, important for differentiation into a Th1 response. Development and expansion of a T cell effector population, as well as the proliferation and isotype switching of plasma cells for specific antibody production are all dependent on signaling pathways leading to NF-κB activation. Finally, the ability of effector cells to contract and down-regulate the immune response, along with the ability to maintain an antigen-specific memory population also involve NF-κB mediated gene transcription.

The activation of NF-κB is not restricted to microbial stimuli or innate responses and many receptors that activate NF-κB initiate events that directly influence adaptive functions of the immune system. Historically, NF-κB activity was first described in B cells[31,32] and it is clear that these transcription factors regulate many aspects of humoral immunity. Specifically BLyS signaling through NF-κB_2 regulates B cell homeostasis while CD40-mediated activation of NF-κB plays an essential role in plasma cell class switching required for production of high affinity antibody.[33-43] It is also clear that the other major arm of adaptive immunity, the T cell response, is dependent on NF-κB. For example, the ability of microbial stimuli to induce dendritic cell maturation and upregulate Class II expression required for efficient antigen presentation to T cells is dependent on NF-κB.[44-46] T cell responses can also be divided broadly into type 1 and 2 responses. Type 1 immunity is associated with cell mediated responses dominated by the production of IFN-γ and resistance to intracellular pathogens. In contrast, type 2 immunity is more commonly associated with T cell production of cytokines such as IL-4, 5 and 13 which influence humoral responses and resistance to helminth parasites. The development of these events depends on many cytokines and their receptors, as well as proteins involved in the regulation of T cell proliferation. The events which influence the polarity of a T cell response are closely linked to the innate NF-κB-dependent production of pro-inflammatory cytokines, in particular IL-12. Many of the genes for these factors have NF-κB binding sites in their promoters, and molecular studies have correlated the binding of specific NF-κB members to these sites and with gene expression,[22,23,36,47-50] (summarized in Fig. 1). In addition, stimulation through the T cell receptor, costimulatory molecules as well as many cytokine receptors,

such as those for TNF-α and IL-1, result in NF-κB activity which affects the response of T cells.[50-60] Many of these effects of NF-κB on lymphocyte function can be attributed to its role in the regulation of life and death of these cells. Thus, NF-κB regulates expression of anti-apoptotic (Bcl-x_L) and cell cycle proteins (cyclin D1) that have a prominent role in the expansion and contraction of the immune response.[61-69] Since these events are known to influence the development of memory responses, it seems likely that these transcription factors have a role in directing the development and maintenance of immunological memory, but there are few studies which directly address this issue.[70-72]

The previous paragraph associates NF-κB with the innate recognition of invading microorganisms as well as the regulation of many facets of the adaptive immune response. However, in order to appreciate the complexity of this system in resistance to infection, it is necessary to draw from a variety of experimental systems and studies. In this chapter we will briefly review several aspects of the evolutionary history of NF-κB signaling and innate immunity, and highlight how studies with NF-κB deficient mice have helped to define the role of individual family members in resistance to different pathogens. The last two sections deal with case studies of natural infection in humans and the ability of pathogens to subvert NF-κB signaling in order to promote their own survival.

NF-κB: An Evolutionarily Conserved System Associated with Innate Immunity

The Toll receptor in *Drosophila melanogaster* was first identified as having an important role in the development of dorsoventral patterning in embryos,[73,74] but the recognition that Toll was homologous to the human IL-1 receptor implicated it as having an additional role in immunity. This was illustrated by studies in which signaling through Toll was shown to promote production of anti-microbial peptides such as defensins, essential for resistance to bacterial and fungal infections in flies.[73,75,76] One of the strengths of Drosophila as an experimental system was the availability of forward and reverse genetic approaches which helped to identify many of the events downstream of Toll. The use of these techniques revealed that stimulation of Toll leads to activation of the kinase Pelle, the subsequent degradation of the inhibitor Cactus and the activation of the transcription factor Dif. Each of these proteins is evolutionarily conserved with mammalian homologues in the NF-κB pathway: Pelle is homologous to the IL-1 receptor associated kinase (IRAK); Cactus is homologous to IκB; and the transcription factors NF-κB1 and RelA are equivalent to Dif.[77-80] The strong similarities between the signaling induced by Toll and IL-1 was important in developing the concept that NF-κB represented an evolutionarily conserved system associated with innate immunity.

Although Toll had a role in resistance to fungal and gram positive bacterial infections it was not required for resistance to gram negative bacteria. Subsequent studies led to the recognition that the immune deficiency gene, *imd*, was required for resistance to gram negative bacteria but not the protective responses to fungi and gram positive bacteria.[81,82] Furthermore, the identification of a death domain within the adaptor molecule encoded by *imd* revealed a striking similarity of this pathway to mammalian TNF signaling.[83] Moreover, similar to the Toll pathway, other genes identified in the imd pathway also had mammalian homologues involved in the NF-κB pathway. The drosophila kinases dIKK-β and dIKK-γ were homologous to IKK-β (IκB kinase-β) and IKK-γ whereas Relish was equivalent to the precursors of NF-$κB_1$ and NF-$κB_2$.[3,84-88] Thus, for Drosophila, two receptors that lead to the activation of pathways homologous to NF-κB were found to have a central role in distinguishing between different classes of pathogens and directing the development of appropriate anti-microbial responses.

Toll-Like Receptors and Innate Immunity

In the early 90's Charles Janeway proposed that receptors encoded in evolutionarily conserved germ-line were involved in the recognition of microbial products and that these affected subsequent adaptive responses.[89] Given the role of Toll receptors in the innate recognition of

pathogens, and their ability to activate conserved signaling pathways associated with immunity, these molecules represented candidates that could form the basis for the recognition of pathogens in mammals. This hypothesis led to the identification of a human Toll-like receptor (TLR) based on homology with Toll and studies which established that signaling through this receptor led to the activation of NF-κB and was associated with the innate activation of genes (IL-6 and B7) that influenced adaptive responses.[90,91] To date 11 human TLRs have been cloned which recognize a wide variety of microbial products from bacteria, parasites and yeast (TLR1,2,4,5,6,9,11),[92-101] to heat shock proteins and viral and fungal products (TLR4,7,8).[102-108] In contrast, only one or two ligands have been identified for others (TLR3,5,7,8,9) including double- and single-stranded RNA or unmethylated CpG DNA.[105-110] Moreover, for some TLRs (TLR2,4) there are coreceptors required for recognition of PAMPs.[93,96,98] There are additional complexities to this system that include the formation of heterodimers by several TLRs (TLR1,2,6) that are required for recognition of ligands[95] and major differences in cell and tissue distribution of certain receptors. For example, these receptors can be expressed not only on lymphoid cells (TLR4,7,10) such as B cells, macrophages and dendritic cells[90,105,107,108,111,112] as well as T and NK cells,[113] but also epithelial cells of the intestine, liver, kidney and bladder (TLR5,11).[99,101] Nevertheless, while the distribution of these pattern recognition receptors contributes to the development of pathogen–specific responses, downstream of these receptors there are also complex signaling pathways that converge on the generic activation of NF-κB. However, downstream of NF-κB activity there is evidence that different TLRs promote distinct patterns of gene expression associated with different pathogens.[114] The molecular events that tailor this response to different classes of pathogens is poorly understood. Indeed, it is unclear whether different PAMPs lead to the activation of different NF-κB homo- or hetero-dimers and affect the strength and duration of this signaling cascade, or whether these latter events influence the response to different PAMPs. In addition, another level of regulation that is likely to affect the events downstream of TLR signaling are epigenetic changes that alter gene accessibility in different cell types. Clearly, additional studies are needed to address these questions, but the major point of these studies is that the immune system has evolved a variety of germ line encoded receptors linked to NF-κB, that distinguish invading microorganisms and direct the development of appropriate immune responses.

The Role of NF-κB in Resistance to Infection

During infection, the coordination of innate and adaptive immunity is a complex process and, as discussed earlier, NF-κB has been implicated in many of these processes. However, the most striking evidence for the importance of NF-κB in immune function is provided by the generation of mice deficient in various components of this pathway. In the last decade there has been a remarkable growth in the number of studies that have been performed to assess how mice that lack TLRs, cytokine receptors, adaptor molecules used in these pathways or individual NF-κB family members respond to infection, and some of these are summarized in Table 1. While these reports establish the critical role for NF-κB in the development of immunity to infection, several studies have provided unexpected insights into the biology of NF-κB.

Initial studies that linked TLR4 with the recognition of LPS provided an important conceptual breakthrough that linked Toll with the best characterized microbial product that induced inflammation.[115] Although there are multiple TLRs and several TLR deficient mice, it has with notable exceptions[101] been difficult to link a particular TLR with susceptibility to a particular micro-organism or class of pathogens. A partial explanation for this may be that pathogens tend to express more than one PAMP and there seems to be some overlap in what different TLRs can recognize. Nevertheless, there has been much more success in linking signaling machinery downstream of TLR ligation with specific pathogens. One example is provided by the TLR/IL-1R (TIR) signaling pathway which recruits the kinases IRAK-1/4 which are necessary for IKK and TRAF-6 activation which in turn lead to NF-κB dependent transcription of pro-inflammatory cytokines.[116] Consistent with this central role in the TIR pathway, studies using IRAK$^{-/-}$ mice revealed that they were more susceptible to infection with

Table 1. Murine gene KO infection phenotypes

Genotype	Increased Susceptibility	Immune Phenotype	
NF-κB1	*L. monocytogenes* *S. pneumoniae* *L. major* *T. muris* *C. jejuni** *H. hepaticus**	Decreased: Antibody production; Proliferation IFN-γ; Th1/Th2 response	Increased: Apoptosis of infected cells
NF-κB2	*L. monocytogenes* *T. gondii* *L. major* *T. muris*	Decreased: Antibody; Proliferation Macrophage function IL-12; IFN-γ Th2 response	Increased: IL-2 GM-CSF Apoptosis DC activation
c-Rel	*L. monocytogenes* *S. pneumoniae* *T. gondii* *L. major* *T. muris* Influenza	Decreased: Antibody response Proliferation IL-2, IL-12, IFN-γ APC response	
RelB	Lymphocytic choriomeningitis virus *L. monocytogenes* T. gondii	Decreased: Antibody production IFN-γ	Increased: Inflammation Chemokine production
Bcl-3	*L. monocytogenes* *S. pneumoniae* *T. gondii* Influenza	Decreased: Antibody response; Macrophage function; Th1 response	Increased:
IκBα(DN)	*T. gondii* Reovirus	Decreased: NK activation; Proliferation; IFN-γ; Cytotoxicity	
IRAK	CMV *P. acnes*	Decreased: NK function; Th1 response; IFN-γ	
MyD88	*C. albicans* Gram(+) bacteria Gram(-) bacteria *M. tuberculosis*; *M. avium* *B. burgdorfei* *T. gondii*	Decreased: Macrophage function; Cytokine production: IL-12, IFN-γ and TNF-α	
NIK	*T. spiralis* *M. leprae* vesicular stomatitis virus	Decreased: Antibody production; Th2 response Mast cell function; IL-2; IL-6; IL-18; IFN-γ	

murine cytomegalovirus (CMV) or *Propionibacterium acnes*.[117] Similarly, MyD88, an adaptor protein associated with IRAK signaling, is required for optimal resistance to a wide variety of pathogens including bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*, as well as the intracellular parasite *Toxoplasma gondii* and the spirochete *Borrelia burgdorferi*.[118-127] However, interpretation of these studies can be difficult as IL-1, IL-18 and multiple TLRs use

MyD88, which can make it hard to distinguish which specific pathway is required for protective responses. Nevertheless, a common finding of these studies was that the susceptibility of MyD88$^{-/-}$ mice was associated with a profound defect in macrophage and dendritic cell function, consistent with an important role for MyD88 in TLR-induced signaling.

Although NF-κB plays an important role in innate recognition of infection, immune cells involved in the development of adaptive immunity employ a variety of receptors that recruit specific kinases that lead to NF-κB activity. For example, stimulation through the T cell receptor recruits NIK (NF-κB inducing kinase) which in turn initiates NF-κB signaling.[128] Mutations in this kinase result in a significant immune deficiency that leads to increased susceptibility to diverse pathogens such as the helminth *Trichinella spiralis* and the bacterium *Mycobacteria leprae*.[129,130] During these infections these mice are unable to mount an appropriate cell mediated response, consistent with an impaired antigen specific response by T cells and typically exhibit a lack of antibody production. Other studies have shown that the NIK mutation causes a specific defect in B cell class switching during infection with vesicular stomatitis virus (VSV), but that the susceptibility of these mice to lymphocytic choriomeningitis virus (LCMV) is actually due to structural defects in secondary lymph node formation and is not cell intrinsic.[131] This latter study illustrates the difficulty in using genetically manipulated mice that may exhibit defects that are not intrinsic to the NF-κB signaling pathways, but are a secondary cause of abnormal tissue development and structure.

While the section above illustrates how defects in the NF-κB signaling pathway can lead to increased susceptibility to infection, many of these kinases and adaptor molecules are also involved in the activation of other signaling pathways. As a consequence, some of the most definitive data on the role of NF-κB in resistance to infection have been dependent on mice that lack individual family members. However, many of these studies are associated with their own set of problems. For example, the prototypical NF-κB dimer is composed of RelA (p65) and NF-κB_1 (p50) but the ability to study the role of RelA in resistance to infection is compromised because the loss of RelA results in embryonic lethality. In contrast, deletion of NF-κB_1 does not affect viability, and this has allowed examination of the role of NF-κB_1 in resistance to infection. A combination of studies revealed that although NF-$\kappa B_1^{-/-}$ mice have normal responses to *Escherichia coli* and *Haemophilus influenzae* they are more susceptible to infection with *L. monocytogenes*, *Streptococcus pneumoniae* or *Leishmania major*.[132,133] For some of these studies, how the absence of NF-κB_1 leads to increased susceptibility has not been defined, but this transcription factor has been implicated in the development of type 1 (dominated by the production of IFN-γ) and type 2 (characterized by the production of IL-4) T cell responses. In one case, susceptibility to *L. major* was found to be the result of a defect in the production of IFN-γ, associated with reduced proliferation of antigen specific CD4$^+$ T cells.[132] Similarly, NF-$\kappa B_1^{-/-}$ mice infected with the gut dwelling helminth *Trichuris muris* failed to develop the type 2 responses required to expel these worms.[134] Interestingly, chronic infection with *T. muris* does not normally lead to the development of inflammation, but in the absence of NF-κB_1, infection results in a severe colitis. This particular phenotype does not appear to be unique to challenge with *T. muris* as NF-$\kappa B_1^{-/-}$ mice heterozygous for RelA expression (p65$^{+/-}$) develop gastroenteritis when infected with *Camplyobacter jejuni* or *Helicobacter hepaticus*.[135-137] The basis for these inflammatory phenotypes remains an open question, but they do indicate a role for NF-κB_1 as a negative regulator of inflammation in the gut, and these observations are pertinent to studies in human patients with colitis that are discussed in a later section.

A comparison of the canonical and non canonical pathways of NF-κB activation is outlined in detail in other chapters. However, one of the family members most closely associated with the noncanonical pathway is NF-κB_2 (p100/p52). Several immune stimuli, such as CD40L, BLyS and lymphotoxin-α which play a prominent role in B cell function activate this pathway and this is consistent with homeostatic defects in the B cell populations of mice that lack NF-κB_2.[33,35,42] In terms of infection, these mice display increased susceptibility to several intracellular organisms including *L. monocytogenes*, *T. gondii* and *L. major*.[35,138,139] Interestingly, mice deficient in Bcl-3, an IκBα family member that is involved in the processing of NF-κB_2,

are also susceptible to some of these same infections, but the basis for these phenotypes remains unclear.[140,141] In contrast, more is known about how the absence of NF-κB_2 affects the response to these pathogens. For example, NF-$\kappa B_2^{-/-}$ mice infected with *T. gondii* develop appropriate innate and adaptive responses during the early phase of infection, but as the infection progresses there is a loss of T cells that are required for long term resistance to this persistent parasite. As a consequence, there is reactivation of the infection and the development of severe disease in the brain.[138] Similarly, NF-$\kappa B_2^{-/-}$ mice infected with *L. major* develop a chronic disease characterized by nonhealing lesions associated with reduced production of IFN-γ. Resistance to *L. major* is dependent on a complex feedback loop in which the CD40L/CD40 interaction is required for the production of IL-12 which in turn stimulates T cell production of IFN-γ.[142] In the absence of NF-κB_2, macrophages stimulated through CD40 have reduced IL-12 responses and this provides a likely basis for the susceptibility of these mice to *L. major.*[139] However, NF-κB_2 is not just associated with the development of cell mediated immunity as NF-κB_2 deficient mice infected with *T. muris* fail to develop the type 2 responses required for resistance to this helminth.[134] Interestingly, in contrast to NF-$\kappa B_1^{-/-}$ mice, persistence of *T. muris* in the absence of NF-κB_2 is not associated with the development of colitis. The opportunity to directly compare the response of the NF-$\kappa B_1^{-/-}$ and NF-$\kappa B_2^{-/-}$ mice to this pathogen indicates distinct roles for these transcription factors in the development of type 2 responses and emphasize the unique role of NF-κB_1 in the regulation of inflammation in the gut.[134]

Another NF-κB member that is activated via the noncanonical pathway, is RelB. The observation that $RelB^{-/-}$ mice spontaneously develop a lethal inflammatory disease mediated by T cells[143] indicates a critical role for RelB in immune homeostasis, but has restricted research on their response to infection. Nevertheless, these mice have been used in a small number of studies and have been shown to be more susceptible to LCMV as well as *L. monocytogenes.*[144] Furthermore, susceptibility of $RelB^{-/-}$ mice to *T. gondii* is associated with an inability of T and NK cells to produce IFN-γ.[145] These studies suggest a prominent role for this family member in the development of Th1 responses to intracellular pathogens, but underlying defects in the immune system of $RelB^{-/-}$ mice make some of these results difficult to interpret.

The NF-κB family member, c-Rel, is widely expressed in lymphoid cells and has been implicated in the regulation of macrophage as well as T and B cell functions. However, c-$Rel^{-/-}$ mice do not have any major developmental defects in their immune system, although they lack marginal zone B cells[146] and are surprisingly immune competent. Nevertheless, there are several reports that establish a role for c-Rel in the regulation of multiple macrophage functions, in particular production of IL-12 in response to a large number of microbial and inflammatory stimuli.[53,147] Consistent with these reports are studies which revealed that in the absence of c-Rel, mice infected with *L. major* developed more severe lesions than wild type mice.[28] While c-Rel is important in the production of IL-12 there are c-Rel independent pathways to produce IL-12 and this is illustrated by studies with *T. gondii.*[53,148] However, mice deficient for c-Rel also failed to recover from infection with *T. gondii*, due to functional defects in both accessory cell and T cell populations.[148] Several studies have also indicated a role for c-Rel in B cell function[50,149] and while many aspects of acquired immunity are still intact in c-$Rel^{-/-}$ mice, during infection with the influenza virus, these mice have a defect in their ability to produce neutralizing antibodies.[150]

Together, these in vivo studies continue to highlight a critical role for NF-κB in the development and maintenance of the immune response to many pathogens. Yet, in all of these studies, the molecular basis for many of the observed defects remains unclear and few studies have managed to truly understand how defects in specific innate or adaptive compartments contribute to the phenotypes observed. One problem with the use of conventional gene knockout mice is that since the gene of interest is absent from all lymphoid cells, the ability to distinguish direct versus indirect effects of the missing gene can be extremely difficult. One way to address this issue is to generate transgenic mice in which a degradation deficient form of IκBα, (IκBα(ΔN)) which functions as a global inhibitor of NF-κB, is selectively expressed in

Table 2. Human genetic mutations

Gene	Mutation Type	Susceptibility	Prognosis	Immune Characteristics
NEMO	Hypomorphic Recessive	Pyogenic bacteria Gram(+) and gram(-) Fungal; Viral	Acute and recurrent 50% of cases develop disseminated disease.	XL-EDA-ID Hyper IgM; hypo IgG; Decreased NK activity; Impaired cell mediated responses.
IkBa	Hypermorphic Dominant	Pyogenic bacteria; Gram(+) and gram(-)	Chronic and recurrent infections	AD-EDA-ID hyper IgM; Lymphocytosis; Severe T cell immunodeficiency
IRAK-4	Amorphic Recessive	Pyogenic bacteria; Fungal; Opportunistic	Acute in early childhood. Severity decreases as child matures.	Complete TLR signaling deficiency. Decreased inflammatory cytokines.
p50	Single nucleotide polymorphism	Gram(+) and gram(-) bacteria	Chronic ulcerative colitis; IBD?	Increased local inflammatory response; mechanism unknown
Nod2	?Single nucleotide polymorphism	Gram(+) and gram(-) bacteria	Chron's Disease	Tissue specific, decreased innate immune function

different cell types. This approach has emphasized the global role of this transcription family in a tissue specific way for the development of innate and adaptive immune responses to a variety of pathogens.[151-153] Thus, mice in which the IκBα(ΔN) transgene is expressed in hepatocytes are more susceptible to *L. monocytogenes*. Similarly, mice in which the NK and T cells express this transgene are highly susceptible to infection with *T. gondii* as a consequence of decreased NK cell activation and antigen specific $CD4^+$ T cell expansion and subsequent failure to produce IFN-γ.[153]

Human Gene Deficiencies

The studies described in the previous section illustrate the key the role of NF-κB signaling in the coordination of the immune response required for resistance to different pathogens in diverse model systems. Nevertheless, the importance of these transcription factors during natural infection in humans is underscored by the identification of individuals with genetic defects in this pathway. With regard to infectious disease, there are currently four known categories of genetic mutations associated with NF-κB which result in mild to severe forms of immunodeficiency.[154] Table 2, contains a summary of these mutations, their phenotype and disease morbidity.

IRAK-4 Deficiency

As mentioned earlier, the kinase IRAK-4 is part of the TIR signaling pathway and plays a prominent role in the activation of NF-κB in response to many stimuli. To date, four individuals have been described with amorphic mutations in IRAK-4 that are associated with immunodeficiency.[155-158] Importantly, although NF-κB has been associated with development[33,159-162] and haematopoietic cell ontogeny,[8,9,151,163] patients with this mutation have no overt physical developmental defects or deficiencies in lymphocyte populations. However, affected individuals have severe infections with pyogenic bacteria, such as *S. pneumoniae* and *S. aureus* in early childhood associated with sepsis and cellulitis.[155,158] These individuals are also susceptible to

recurrent infections with fungi (*Candida albicans*) and other opportunistic infections.[164] In these patients susceptibility to gram(+) infections is characterized by a poor inflammatory response including reduced IL-6 and IFN-γ production.[158] Two of the four patients also exhibited impaired responses to LPS, or stimulation through TLRs 1-6 and TLR-9.[155-157,164] Moreover, consistent with a role for IRAK-4 in B cell function, antibody responses after vaccination to polysaccharide and protein antigens were below normal range and were shown to diminish after the last booster.[164] The reduced production of antibody by B cells could be the result of either an intrinsic defect in B cell TLR signaling, or due to a lack of T cell help. Interestingly, as these patients reach adolescence the severity and frequency of bacterial infection decreases significantly which may indicate the development of sufficient adaptive responses to cope with these challenges.[158]

Mutations in NEMO

As discussed at the start of this chapter, the IKK complex has an important role in the activation of NF-κB. NEMO is a central component of this complex and is required for NF-κB signaling in response to many developmental and inflammatory stimuli. The *NEMO* gene is located on the X chromosome, and similar to NEMO deficient mice, amorphic mutations primarily affect males, causing a lethal form of incontinentia pigmenti (IP) in utero.[165,166] Subsequently identified hypomorphic mutations were found to lead to a collection of developmental abnormalities associated with anhidrotic ectodermal dysplasia (EDA) which includes partial or total absence of teeth, conical teeth, sparcity of hair and an absence of sweat glands leading to dry skin.[167] Furthermore, immunodeficiency (ID) characterized by increased susceptibility to a variety of infectious diseases is a hallmark of this X-linked mutation.[168] The immunological phenotype of these patients is complex, in that most individuals have normal lymphocyte distribution and in vitro proliferation of PBMCs in response to mitogens, but impaired expansion in response to tetanus or diphtheria antigens.[169] Additionally, patients typically present with decreased IgG, but hyper-IgM in the peripheral blood, suggestive of a defect in CD40-mediated class switching in B cells.[168-174] Consistent with this observation, activation of B cells via CD40 ligation was found to be defective in most of the individuals studied.[173-175]

Given the central role of NEMO in the activation of NF-κB, it is not surprising that these patients are highly susceptible to a range of pathogens. Typically, boys with this mutation present with multiple, recurrent pyogenic bacterial infections early in life, including *S. pneumoniae*, *S. aureus* and *H. influenza*, with about 50% of identified cases succumbing to disseminated infection.[168,169,174] Mycobacterial, fungal and viral infections are less frequent in these patients, but are often severe when they occur.[169,173,174,176-178] NK cell cytotoxicity is also significantly impaired in the majority of these individuals, along with a defect in the production of the NK and T cell growth factor IL-2.[169,176] Since NK cells play a central role in resistance to many herpes viruses,[179] it is not surprising that these patients are also susceptible to this group of pathogens, but treatment with IL-2 was able to induce NF-κB activation and partially restore NK cell activity.[176] Overall inflammatory responses during infection were significantly impaired in all cases described[154,172] and characterized by a lack of responsiveness to LPS, IL-1β, IL-18, TNFα and CD40L.[174] Together, the identification of multiple immune defects in these patients and their susceptibility to multiple pathogens indicates the central role of NEMO for the activation of NF-κB.

Mutations in IκBα

The ubiquitination and proteasomal degradation of IκBα represents a major checkpoint in the activation of NF-κB. Hypermorphic mutations of the *IKBA* gene are associated with a dominant form of EDA-ID that results in a specific T cell immunodeficiency and increased susceptibility to infectious disease.[180] This mutation, which was not inherited, prevented IκBα degradation while IκBβ and IκBε degradation remained normal.[180] The patient described had a failure to thrive and presented with chronic diarrhea and recurrent bronchopneumonitis

from 2 months of age.[180] This child also suffered from numerous gram(+) and (-) pyogenic bacterial infections, the details of which have not yet been published. The profound T cell immunodeficiency associated with this mutation was characterized by a complete absence of γ/δ T cells and memory α/β T cells, and a lack of responsiveness of the naïve T cell population, indicating a role in development and maintenance of human lymphocyte populations as well as activation and effector function in vivo. In this patient, reduced NF-κB DNA binding activity in response to TIR and TNF-α stimulation was observed, which correlated with decreased production of IL-6, IL-2 and IFN-γ. Thus, the increased severity of disease in this patient is likely related to the ablation of multiple NF-κB signaling pathways in T cells.[180] Despite an overall lymphocytosis, there was a complete absence of serum antibody for specific antigens, indicating a defect in CD40 signaling in B cells, a common theme observed in the various groups of patients described so far.

Colitis and NF-κB1

The defects described above are the result of either deletions or missense mutations which lead to the production of mutated proteins with reduced or altered function. In contrast, recent studies have associated a single nucleotide insertion/deletion polymorphism (SNP) in the 5'-promoter region of the *NF-κB1* gene with chronic ulcerative colitis.[181] This unique polymorphism is linked to decreased binding of nuclear proteins to the *NF-κB1* promoter.[181] This result coincides with studies in NF-κB$_1^{-/-}$ mice that were shown to have an increased susceptibility to infection-induced colitis.[134,136,137] Because of the dual role of NF-κB$_1$ in transcriptional activation as well as suppression, the basis for this susceptibility remains elusive. One receptor which may be directly related to NF-κB$_1$ activation and surveillance of mucosal pathogens, is Nod2. This protein, along with the related molecule Nod1, works as a cytosolic receptor that recognizes peptidoglycans from invading bacteria and induces the activation of NF-κB.[55,182] Recently, a mutation in the gene encoding Nod2 has been linked to the pathogenesis of colitis in human and mouse studies[54,183,184] and supports a role for this bacterial "sensor" in regulation of NF-κB$_1$ activation. In contrast to the NF-κB$_1$ SNP that is associated with an increase in inflammatory cell activation, the Nod2 defect leads to a decrease in activation in response to bacterial LPS and peptidoglycan.[183,184] Clearly more studies are required to address the role of NF-κB in the development of this enteric disease.

Pathogens That Interfere with NF-κB

The sections above have focused on the role of NF-κB in the coordination of the immune responses required for resistance to infection. However, as a consequence of the critical role these transcription factors play in innate and adaptive immunity, this pathway acts as a strong selective pressure for pathogens. It is now recognized that microorganisms have developed specific strategies to block or enhance this intracellular signaling pathway in order to promote their own replication, survival and dissemination within the host. Indeed, almost every aspect of the NF-κB pathway has been targeted by pathogens and the following section along with (Table 3) highlight what is currently known about different pathogens that interact with NF-κB and the specific point in the pathway that each target.

One general strategy that has been developed by pathogens to reduce the ability of cells to become activated and/or respond appropriately to stimuli is to alter the surface expression of activating receptors on target cells. Certain viruses, such as CMV reduce NF-κB activity by suppressing surface expression of cytokine receptors, thus preventing infected cells from becoming activated, migrating and interacting with T cells.[185,186] Another example is provided by the bacterium *Ehrlichia chaffeensis* that is able to directly down regulate specific TLRs and their coreceptors on the surface of infected human monocytes.[187] These events would presumably lead to a reduced capacity of cells to sense the presence of these organisms and direct the development of protective immunity.

Table 3. Pathogen inhibition of NF-κB

Pathway	Pathogen	Proposed Mechanism	Target
Receptor Expression	MCMV[185]	NK	TNFR1/2
	huCMV[186]	NK	CCR7
	Ehrlichia chaffeenis[187]	NK	TLR2/4'CD14
	HIV[101]	NK	Mannose receptor
Adaptor Protein Complex Formation	Vaccinia virus	Dominant negative, A52R competes w/ host protein	MyD88
Kinase Activation	*Yersinia pestis* *Y. pseudotuberculosis* *Y. enterocolitica*[189-194]	YopJ, YopH, YopP and others interrupt kiniase and ubiquitan function	MAPK; SUMO-1; IKKs
	Uropathogenic *E. coli*[195]	Soluble factors	MAPK
IκBα Degradation	Measles virus[196]	NK	IκBα phosphorylation
	Pox virus[197]	NK	IκBα degradation
	HIV[198-199]	Viral protein, Vpu competes w/ host protein	β-TrCP
	Francisella tularensis[200]	23kDa protein	IκBα degradation
	Salmonella[201]	NK	IκBα ubiquitination
Nuclear Translocation	African swine fever virus[202]	A238L acts as non-degradable IκB homolog	NF-κB dimers
	M. ulcerans	Soluble toxin	RelA phosphorylation
DNA Binding	Epstein-Barr virus[204]	ZEBRA	RelA
	T. gondii[205-207]	NK	NF-κB dimers
	Schistosoma masoni[208]	Parasite factor	DNA binding complex
Unknown	*L. donovani*[209]	Ceramide production	NK

Another group of gram negative bacteria that are particularly impressive in their ability to interfere with NF-κB signaling are the extracellular bacteria *Yersinia*. These pathogens use a type III secretion system to inject virulence factors, known as *Yersinia* outer proteins (Yop), into target host cells such as macrophages, epithelial cells, fibroblasts and lymphocytes, and directly inhibit kinase activation. Thus, YopJ of *Y. pseudotuberculosis* targets MAPK kinases, which are upstream of IκB phosphorylation.[189] In addition, these proteins also have proteolytic activity and can target ubiquitin-like molecules and thereby disrupt the degradation of regulatory proteins, including IκB.[190] Similarly, YopP of *Y. enterocolitica* can bind to IKKβ and thereby prevent activation of NF-κB and cause apoptosis in macrophages.[192,193] *Y. pseudotuberculosis* uses YopH, a tyrosine phosphatase, that has been shown to block all antigen specific receptor signaling in T and B cells by inhibiting phosphorylation of proteins early in the pathway[194] and this virulence factor is likely able to inhibit TCR-mediated activation of NF-κB. It is thought that the inhibition of NF-κB activation not only limits the ability of these lymphocytes to produce cytokines, but may also promote their apoptosis and so inhibit activation of the immune system.[210] Together, these studies highlight a common theme among these pathogens: events upstream of IκB degradation represent good targets to inhibit NF-κB activation which emphasizes the importance of IκB as a major checkpoint in this pathway.

Epstein-Barr virus can differentially affect the activation of NF-κB, depending on the cell type in question. In infected T cells, a viral protein is able to bind to RelA and inhibit NF-κB activity most likely blocking transcription of anti-apoptotic proteins and rendering infected T cells susceptible to apoptosis.[204] In contrast, the viral transformation of B cells and the subsequent development of lymphoproliferative disease is associated with sustained NF-κB activation.[211,212] This strategy of transforming cells through sustained NF-κB activation which promotes proliferation and resistance to apoptosis has also been used by other viruses such as human T-cell leukemia virus (HTLV) and the parasite *Theileria parva*.[213-215] This process is analogous to the elevated NF-κB activity that is thought to contribute to the transformation of certain cancer cells. Nevertheless, regardless of the mechanism, it is striking to note the number of viruses, bacteria and parasites which have evolved ways to activate or inhibit the NF-κB system for their own benefit. This is likely a reflection of the long term evolutionary relationship between these micro-organisms and this conserved signaling pathway involved in the recognition and elimination of these pathogens.

Conclusions and Future Directions

In the last 20 years there have been many important advances in our understanding of the receptors and signaling molecules involved in the development of protective immunity to infection. The experimental and clinical studies discussed in this chapter provide several illustrations of the importance of NF-κB in resistance to infection. In particular, the identification of numerous germ line encoded receptors that activate NF-κB provides an insight into how the innate system distinguishes different classes of pathogens. Interestingly, pathogens have targeted almost every aspect of the NF-κB signaling pathway to promote their own replication. It seems possible that as we explore the specifics of how individual pathogens alter intracellular signaling, these studies may provide new insights into aspects of NF-κB signaling that are not well understood. Similarly, with the identification of a growing cohort of patients with defects in NF-κB signaling, it seems likely that the study of these natural mutants will provide a better understanding of the role of some of the individual molecules involved in this intricate pathway.

From an experimental perspective, attempts to delineate the role of individual NF-κB members in resistance to infection has been difficult for several reasons. With few exceptions the response of each knockout strain has yet to be evaluated for the same pathogens[134] and more complete comparative studies are still needed. Moreover, because all of the NF-κB family members are involved in so many aspects of the immune system in many different cell types that cross-regulate each other, it has been difficult to study mice deficient in single NF-κB family members and fully understand their phenotypes. The availability of inducible approaches to ablate specific elements of NF-κB signaling in a cell and tissue specific fashion[216-219] will lead to the development of more sophisticated tools to address specific questions and overcome some of the intrinsic difficulties with the approaches that have been widely used to date Lastly, it is important to recognize that because of its role in inflammatory processes and the development of cancer, the NF-κB system has represented an attractive target for the development of anti-inflammatory and anti-tumor drugs. However, it seems likely that the same knowledge that drives the development of new pharmaceuticals could be used to develop ways to promote and direct the development of protective immunity, either at the level of vaccine development or for the treatment of chronic infections.

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CHAPTER 9

Molecular Basis of Oncogenesis by NF-κB:

From a Bird's Eye View to a RELevant Role in Cancer

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Abstract

The Rel/NF-κB transcription factors are renowned for their fundamental contribution to normal immune, inflammatory and acute phase responses. A growing body of evidence also underscores their important role in the control of cellular gene expression, cell proliferation and apoptosis. Thus, it comes as no surprise that sustained Rel/NF-κB activity has emerged as a hallmark of many human cancers. Experimental evidence indicates a strong correlation between the transcriptional activity of Rel/NF-κB and its role in malignant cell transformation. The important role of NF-κB in the control of the apoptotic response also supports its participation in the resistance of tumor cells to therapeutic treatment. This review focuses on the mechanisms that underlie the contribution of Rel/NF-κB to cancer and highlights how appreciation of its role in this context has evolved from a bird's eye view to a true recognition of its RELevant function in oncogenesis.

Introduction

The Rel/NF-κB transcription factors have been the focus of numerous studies aimed at elucidating their role in the development and function of the immune system and at unveiling the signaling pathways that control their activity (see accompanying chapters by M. Karin, S.C. Sun, R. Sen, U. Siebenlist, H.C. Liou, Y. Chen, and C. Hunter). In recent years, there has been considerable progress in appreciating their contribution to oncogenesis and in understanding the mechanisms involved. Inappropriate Rel/NF-κB activity is observed in many different types of human cancers. Hyperactivation of the NF-κB signaling cascade, mutations that inactivate the inhibitory IκB subunits or chromosomal aberrations involving various *rel/nf-κb* genes have been noted in many human tumors.[1,2] Consistent with the transforming activity of the viral Rel/NF-κB oncoprotein v-Rel and its cellular homologue c-Rel in primary cells and in animal models, NF-κB is also critically involved in malignant cell transformation by viruses such as the human T-cell leukemia virus type I (HTLV-1) and Epstein-Barr virus (EBV).[3] Collectively, these findings justify the vast body of literature exploring the molecular basis for the role of Rel/NF-κB in cancer. Important findings center on its ability to regulate cellular gene expression, to affect cell proliferation and survival, and on important regulatory mechanisms that control its activity – all of which have important consequences for effective anti-cancer therapy.[4-7]

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Constitutive Rel/NF-κB Activity Is a Hallmark of Many Human Cancers

Sustained activation of NF-κB is a feature of many human leukemia, lymphoma and solid tumors.[1] Immunohistochemistry, gel mobility shift assays and gene expression profiling of primary tumor specimens and tumor-derived cell lines have highlighted the persistent nuclear localization of NF-κB subunits compared to normal controls (for example see refs. 8-11). The dimer comprised of the p50/p65 subunits is the most frequently reported NF-κB complex to be activated in human cancer, although there is evidence that clearly implicates c-Rel-containing complexes in certain tumor types, like breast cancer.[8,9] The important implication of sustained NF-κB activity for the survival and proliferation of tumor cells is underscored by the growth arrest and rapid onset of apoptosis observed in many tumor-derived cell lines upon introduction of a degradation-resistant form of IκB (IκB super-repressor) to inhibit endogenous NF-κB activity (for example see refs. 12,13).

Activation of the NF-κB Signaling Cascade

Persistent activation of the NF-κB pathway is observed in many different human cancers. By virtue of its ability to trigger the N-terminal phosphorylation of the NF-κB inhibitory subunit IκBα on serines 32 and 36, the IKK kinase complex promotes degradation of IκB via the ubiquitin/proteasome pathway. This enables NF-κB dimers to accumulate in the nucleus where they promote transcription of specific gene programs.[14] Although the detailed mechanisms responsible for sustained IKK activation in many human tumors remain unknown, there are several potential mechanisms (Table 1).[15,16]

IKK Complex Activation

Since no mutation has yet been identified to affect IKK subunits in human tumors, unrelenting activation of NF-κB is likely to result from alterations in upstream signaling components. In many types of cancer, sustained IKK activation is achieved via autocrine loops involving cytokines and growth factors that activate the NF-κB pathway and are themselves transcriptional targets of NF-κB (Tables 1, 2).[17] For instance, IL-1 activates NF-κB in pancreatic carcinoma cell lines and is in turn induced by NF-κB.[18] Likewise CD40, the receptor for CD40 ligand, constitutively activates NF-κB in malignant Reed-Sternberg (H/RS) cells of Hodgkin's disease (HD) and is upregulated in these cells.[19] Another mechanism for constitutive activation of the IKK complex involves deregulation of TRAF adaptor proteins in human tumors. TRAF2 is a critical component of receptor-triggered signaling pathways involving NF-κB, JNK and p38. Recent work showed that loss of the TRAF2- and IKKγ/NEMO-interacting tumor suppressor protein CYLD, a de-ubiquitinating enzyme for TRAF2, leads to constitutive activation of IKK coincident with increased cell resistance to apoptosis.[20-23] Loss of CYLD causes cylindromatosis, an autosomal dominant syndrome that predisposes patients to benign tumors of hair follicles and sweat and scent glands.

Interestingly, recent work unveiled a new NF-κB-independent role for IKK in cancer. IKKβ expression in primary breast cancer specimens is correlated with poor survival and studies in primary breast cancer cell lines showed that IKK negatively regulates the forkhead transcription factor FOXO3a, independent of NF-κB activation.[24] Indeed, IKK-mediated phosphorylation of FOXO3a promoted its nuclear export and proteolysis via the ubiquitin proteasome pathway to promote cell growth and tumorigenesis. It will be interesting to see if the newly reported abilities of IKKα and IKKγ/NEMO to localize to the nucleus and respectively modify histones and interact with CBP to regulate NF-κB gene expression imply that these subunits can also act on other nuclear targets to affect oncogenesis.[25-27]

Activation by Other Kinases, Oncogenes and Viruses

Other means to constitutively activate NF-κB signaling in human tumors entail various kinases other than IKK, as well as oncogenes and viruses (Table 1). One example involves the

Table 1. Mechanisms for constitutive NF-κB activation in human cancer

Mechanism		Type of Cancer
IKK complex activation		
Unknown mechanism		Hodgkin's lymphoma
		Childhood acute lymphoblastic leukemia
		Breast carcinoma
		Colon carcinoma
		Ovarian carcinoma
		Pancreatic carcinoma
		Thyroid carcinoma
		Bladder carcinoma
		Prostate carcinoma
		Melanoma
		Squamous cell carcinoma
Interleukin-1 autocrine loop		Pancreatic carcinoma cell line
Interleukin-13 autocrine loop		Hodgkin's lymphoma
Tumor necrosis factor-α autocrine loop		T-cell lymphoma
Loss of CYLD		Turban tumor syndrome
Activation by other kinases		
Bcr-Abl		Acute lymphoblastic leukemia
		Chronic myelogenous leukemia
Activation by oncogenes		
Ras		Acute lymphoblastic leukemia
		Chronic myelogenous leukemia
API2/MALT1		Mucosa-associated lymphoid tissue lymphoma
Her2/Neu		Breast carcinoma
Activation by viruses		
Human T-cell leukemia virus-1 (HTLV-1)		Adult T-cell leukemia
Epstein Barr virus (EBV)		Burkitt's lymphoma
		Nasopharyngeal carcinoma
		Hodgkin's lymphoma
		Immunoblastic lymphoma
		Gastric carcinoma
Hepatitis B virus (HBV)		Hepatocellular carcinoma
Human herpes virus-8 (HHV-8)		Kaposi's sarcoma
***iκb* gene mutations**		
iκbα	Mutation	Hodgkin's lymphoma
iκbε	Mutation	Hodgkin's lymphoma
bcl-3	Rearrangement / Overexpression	B-cell non-Hodgkin's lymphoma
		B-cell chronic lymphocytic leukemia
***nf-κb* gene alterations**		
c-rel	Amplification	Hodgkin's lymphoma
		Follicular B-cell lymphoma
		Diffuse large cell lymphoma
		Primary mediastinal B-cell lymphoma
	Rearrangement / Overexpression	Follicular lymphoma
		Diffuse large cell lymphoma
		Non-small cell lung carcinoma

Table continued on next page

Table 1. Continued

Mechanism		Type of Cancer
***nf-κb* gene alterations**		
relA	Rearrangement / Overexpression	B-cell non-Hodgkin's lymphoma
		Multiple myeloma
		Non-small cell lung carcinoma
		Thyroid carcinoma cell lines
	Amplification	Diffuse large cell lymphoma
		Squamous head and neck carcinoma
		Breast adenocarcinoma
		Stomach adenocarcinoma
nf-κb1	Rearrangement/Overexpression	Acute lymphoblastic leukemia
		Non-small cell lung carcinoma
		Colon cancer cell lines
		Prostate cancer cell lines
		Breast cancer cell lines
		Bone cancer cell lines
		Brain cancer cell lines
nf-κb2	Rearrangement/Overexpression	Cutaneous T-cell lymphoma
		B-cell non-Hodgkin's lymphoma
		B-cell chronic lymphocytic leukemia
		Multiple myeloma
		Breast carcinoma
		Colon carcinoma

PI3-kinase to Akt kinase signaling pathway in response to overexpression of the epidermal growth factor (EGF) receptor family member c-erbB2/Her-2/Neu in breast cancer.[28] IκBα degradation in this context is mediated by the protease calpain. Another example is casein kinase II (CKII) that phosphorylates serines in IκBα distinct from those targeted by IKK and triggers calpain-mediated cleavage of IκBα.[29] Upregulation of CKII activity was suggested as a possible contributing factor to hepatocarcinoma induced by TGF-β1.[30]

A number of oncogenes mediate their transforming function by virtue of NF-κB activation. These include the chimeric oncoprotein tyrosine kinase Bcr-Abl implicated in acute lymphocytic leukemia (ALL) and chronic myelogenous leukemia (CML), and the Ras oncogene. Bcr-Abl enhances nuclear translocation of NF-κB and the transactivation function of NF-κB subunit p65/RelA via MEKK1 and p38 MAPK and also partially requires Ras function.[31-33] Ras is another well-known oncogene mutated in human tumors that utilizes NF-κB to achieve oncogenesis.[34,35] The anti-oncogenic effect of lysyl oxidase on Ras-transformed cells was recently demonstrated to involve suppression of NF-κB activation.[36] An interesting new report showed that the API2/MALT1, a chimeric protein between inhibitor of apoptosis c-IAP2 and the MALT1 paracaspase, participates in the transformation process of mucosa-associated lymphoid tissue (MALT) lymphoma by activating NF-κB dimers comprised of RelB/p50.[37] It will be interesting to see whether other oncogenes act in a similar manner.

Lastly, many viruses achieve their oncogenic effects via the NF-κB signaling cascade (Table 1). A notable example relevant to human cancer is the human T-cell leukemia virus-1 (HTLV-1) implicated in acute T-cell leukemia (ATL). Persistent activation of NF-κB by HTLV-1 Tax causes nuclear accumulation of NF-κB dimers, helps to overcome their inhibition by the p105/NF-κB1 subunit, and is an essential step in the transformation of T cells.[38-41] Additionally, Tax stimulates phosphorylation-dependent processing of NF-κB2/p100, and hence activates both

Table 2. A sample of NF-κB-regulated gene products implicated in human cancer

Function	Protein	Role in v-Rel-Mediated Transformation
Regulators of apoptosis	Bcl-xL	complements weakly transforming mutants
	Bcl-2	complements weakly transforming mutants
	Bfl-1/A1	
	c-IAP2	c-IAP1 - essential
	c-FLIP	
	GADD45β	
	A20	
	CD95	
	TRAF1	
	IEX1	
Cell cycle regulators	Cyclin D1	
	Cyclin D2	
Transcription factors	JunB	c-Jun - essential
	IRF1	
	IRF4	IRF4 - essential
	c-Myc	
	Stat5a	
Tumor suppressors	p53	
Chemokines/cytokines/ growth factors	Interleukin 1	
	Interleukin 6	
	Interleukin 8	
	Interleukin 13	
	MIP-1α	
	GM-CSF	
	TNFα	
	VEGF	
Cell surface receptors	CD40	
	CD44	
	CD86	
	CCR7	
	CXCR4	
Cell adhesion molecules	ICAM-1	
	VCAM-1	
Metalloproteinases	MMP-9	

the canonical and noncanonical NF-κB pathways.[42] Another virus that contributes to human cancer via NF-κB is the Epstein-Barr virus (EBV) implicated in Burkitt's and Hodgkin's lymphomas. The EBV nuclear antigen (EBNA)-2 and latent membrane protein (LMP)-1 enhance NF-κB activity thereby preventing apoptosis in EBV-transformed B cells.[41,43] This is consistent with the ability of LMP-1 to induce expression of NF-κB-dependent anti-apoptotic proteins such as Bfl-1/A1.[44,45] Akin to Tax, LMP-1 induces proteolytic processing of p100/ NF-κB2 to its p52 form, consistent with the high levels of p52 found in Hodgkin's lymphoma and nasopharyngeal carcinoma from EBV-infected patients.[46,47]

iκb Gene Mutations

Although much less frequent than upstream activation of the NF-κB pathway, there have been a few reports of *iκb* gene mutations implicated in constitutively activating NF-κB in human tumors. Mutations that suppress the inhibitory activity of IκBα or IκBε were observed

in some Hodgkin's lymphomas and a large B-cell lymphoma cell line (Table 1).[48-53] The fact that bi-allelic mutation was needed for IκBα loss-of-function in Hodgkin's lymphoma raised the suggestion that it may act as a tumor suppressor.

nf-κb Gene Rearrangement, Amplification and/or Overexpression in Human Cancer

While sustained activation of NF-κB signaling is the most common mode of NF-κB activation in human tumors, there are a number of cases in which *rel/nf-κb* gene amplification, rearrangement and/or overexpression was documented (Table 1).[1] The majority of human *rel* and *nf-κb* genes (i.e., c-*rel*, *relA*, *nf-κb1* and *nf-κb2*) have been targeted in this fashion, although *nf-κb2* and c-*rel* are the most commonly affected.

Chromosomal rearrangements disrupting the 3' coding region of the *nf-κb2* gene are frequently observed in cutaneous T-cell lymphoma and also in a small number of B-cell non-Hodgkin lymphoma, chronic lymphocytic leukemia and multiple myeloma.[54-58] The resulting C-terminally truncated p100/NF-κB2 proteins primarily localize to nuclei and bind to NF-κB DNA motifs. However, how tumor-derived truncated p100 proteins contribute to oncogenesis remains to be clarified. Loss of the C-terminal ankyrin motifs in tumor-derived p100 mutants was proposed to abolish the IκB-like function of p100, resulting in abnormal NF-κB activity. A more recent study suggested another mechanism for oncogenic activation, i.e., that loss of a putative C-terminal death domain in tumor-derived p100 mutants might abrogate a proapoptotic effect of p100,[59] although this has been a subject of debate.[60] It is interesting to note that homozygous deletion of the C-terminal ankyrin repeats of p100 leads to gastric and lymph node hyperplasia in mice, suggesting that overexpression of p52/NF-κB2 contributes to oncogenesis.[61-63] In support of this hypothesis, tumor-derived rearranged p100 proteins undergo constitutive processing to produce functional p52, due to deletion of a C-terminal processing-inhibitory domain (PID).[64] Moreover, overexpression of p52 was detected in several malignancies including T-cell leukemia and breast and colon carcinoma.[9,65,66] The ability of p52 homodimers to function as transcriptional activators in combination with RelB or the IκB-related Bcl-3 transcription factor to promote expression of antiapoptotic and proproliferative genes such as *bcl-2* and *cyclin D1* is consistent with this model.[9,67]

The human c-*rel* locus is amplified in a significant proportion of diffuse lymphoma with a large cell component (DLLC; 23%) and also in primary mediastinal (thymic) B-cell lymphoma, classical Hodgkin's lymphoma and certain follicular large cell lymphoma.[1] However, the extent to which c-*rel* gene amplification causes elevated nuclear c-Rel protein levels is unclear. While some found a correlation between amplification of the c-*rel* locus and nuclear c-Rel protein accumulation in Hodgkin's lymphoma and mediastinal large B-cell lymphoma (MLBCL),[68,69] others found no close association between the two or with NF-κB target gene expression profiles in diffuse large B cell lymphoma (DLBCL).[70,71] These findings suggest that if c-*rel* plays a role, its function may be heterogeneous in different lymphomas or that it might play a role early in the history of some of these tumors that is no longer required later on. Although c-*rel*'s contribution to some of these tumors remains a point of contention,[72] future studies will undoubtedly provide important information on the subject.

Molecular Basis for Oncogenesis by Rel/NF-κB

Studies with the retroviral NF-κB oncoprotein v-Rel and its cellular Rel/NF-κB homologues have provided important insights into the oncogenic properties of Rel/NF-κB factors and the functional mechanisms involved. These are reviewed in this section.

The Viral NF-κB Oncoprotein v-Rel: A Potent Transforming Factor

Evidence pointing to a role for Rel/NF-κB in cancer came about long before the discovery of the *rel/nf-κb* gene family, with the isolation in 1958 of the Rev-T retrovirus from the liver of a diseased turkey.[73,74] The culprit Rev-T-encoded oncogene was identified many years later as v-*rel*,

the first member of the Rel/NF-κB family.[75] v-*rel* immortalizes and transforms immature and mature B and T lymphoid, myeloid and dendritic cells from chicken spleen and bone marrow and induces aggressive and fatal leukemia/lymphoma in infected young birds.[76-79] v-Rel can also transform chicken embryo fibroblasts that induce tumors in immunocompetent young chicks.[80]

The oncogenic activity of v-*rel* was believed for some time to be restricted to avian species, as efforts to stably express it in rodent fibroblasts or lymphoid B cells resulted in apparent cytotoxicity.[81-83] Although the molecular basis for this effect remains to be clarified, stable expression was recently achieved in mouse fibroblasts using a mouse stem cell virus (MSCV),[84] but it remains to be seen if MSCV-driven v-*rel* will be transforming in mouse lymphoid cells. Yet, the discovery that transgenic mice expressing v-*rel* under the control of the *lck* promoter developed aggressive T-cell leukemia/lymphoma provided unambiguous proof of its oncogenic potential in mammals.[85] It is noteworthy however that the onset of tumor development in transgenic mice is remarkably slower than in infected chickens, with mice succumbing between 6 to 10 months of age compared to 7 to 10 days in young chicks. Another distinction between the avian and mammalian systems is the fact that tumors arising in v-*rel* transgenic mice are oligoclonal, rather than polyclonal in nature, and that they fail to transplant in syngeneic animals.[85] This suggests that additional cytogenetic alterations are necessary for manifestation of v-*rel*'s tumorigenic potential in mammals. In this regard, chickens lack $p16^{INK4a}$ and express a truncated but functional ARF protein.[86] This raises the possibility that tumor suppressors such as those encoded by the *ink4b-arf-ink4a* locus perhaps contribute to the increased susceptibility of chickens to v-*rel*-induced transformation. Nevertheless, in light of the rapidly increasing number of studies implicating Rel/NF-κB activity in human tumors, v-*rel* is a highly prized tool to unravel the molecular basis for the oncogenic activity of cellular Rel/NF-κB factors.

A Role for c-Rel in Cell Transformation and Tumorigenesis: Lessons from Birds and Mice

Since v-*rel* arose by recombination of the non-transforming Rev-A retrovirus with the turkey c-*rel* proto-oncogene, it is not surprising to find that overexpression of the chicken, mouse or human c-*rel* genes transforms primary chicken cells in culture that induced tumor development in animal models, albeit at a lower efficiency than v-*rel*.[87-91] However when tested under similar conditions, other mammalian Rel/NF-κB subunits namely RelA, RelB, p50/NF-κB1 or p52/NF-κB2 failed to transform lymphoid cells.[87] Together, these findings suggest that overexpression of the c-Rel protein in some tumors showing c-*rel* gene amplification and/or constitutive activation of c-Rel-containing NF-κB complexes might contribute to certain human leukemia/lymphoma.

Importantly, c-*rel* was recently shown to also exhibit an oncogenic capacity in a mammalian system. Indeed, 31% of transgenic mice expressing the mouse c-*rel* gene under the control of the mouse mammary tumor virus (MMTV) promoter developed mammary tumors at an average age of 19.9 months.[92] Tumor development coincided with nuclear localization of NF-κB subunits and upregulation of many NF-κB-target genes including *cyclin D1, c-myc,* and *bcl-xl* (Table 2; see below). The significance of these findings is highlighted by the fact that many human breast cancer specimens show elevated NF-κB activity.[8,9,93-95]

Rel/NF-κB Functions Necessary for Cell Transformation

As a result of its acquisition and evolution in the context of the Rev-T retrovirus, v-*rel* encodes a truncated and mutated version of the turkey c-Rel protein fused to remnants of the Rev-A retroviral *env* gene. v-Rel carries a number of deletions and point mutations compared to c-Rel, including the loss of 118 C-terminal amino acids that correspond to a strong transactivation domain (TAD) in c-Rel.[74] Many of these differences contribute to the increased oncogenicity of v-Rel compared to c-Rel. For example selection for C-terminal truncation of c-Rel, reminiscent of that seen in v-Rel, was observed in tumors that arose following retroviral-mediated delivery

of c-*rel* into young chickens.[96] Recent work from our group indicates that c-*rel* gene deletion or mutation is not necessary for lymphoid cell transformation "per se", but that it may rather be selected for during tumor progression to confer enhanced tumorigenicity, enable escape from immune surveillance and/or facilitate cell adaptation to growth in culture.[87]

A model for v-Rel-mediated oncogenesis has emerged that invokes its ability to transactivate κB site-dependent gene transcription as being critical for cell transformation. Mutations that decrease its DNA-binding or transactivation functions are detrimental to cell transformation, whereas those that increase these activities enhance its transforming potential.[97-106] Consistent with this model, v-Rel shuttles between the nucleus and the cytoplasm, and a threshold of nuclear v-Rel is necessary to transform cells.[107] Other factors also contribute to the enhanced oncogenicity of v-Rel compared to c-Rel.[74] These include the fact that: (1) v-Rel is less susceptible than c-Rel to inhibition by IκBα.[108,109] This agrees with the partial nuclear distribution of v-Rel/IκBα complexes compared to predominantly cytoplasmic NF-κB/IκBα complexes in unstimulated cells.[110,111] Despite its reduced susceptibility to inhibition by IκBα, v-Rel is nevertheless subject to IκBα control, as overexpression of IκBα in v-*rel* transgenic mice attenuated its tumorigenic phenotype.[112] (2) v-Rel binds to a broader range of NF-κB DNA sites compared to c-Rel and other NF-κB subunits. Nehyba et al identified mutation clusters in v-Rel responsible for this difference, and Phelps and Ghosh recently pinpointed amino acid differences between the Rel-homology domains (RHDs) of v-Rel and c-Rel in this effect.[109,113] (3) The particular dimers in which v-Rel participates also dictate its oncogenic potential. Mutational analysis revealed a critical role for v-Rel homodimers in cell transformation.[114] Although v-Rel/p50 heterodimers and v-Rel homodimers are the major DNA-binding complexes in v-*rel* transgenic mice, transgenic expression of v-*rel* in a p50 knockout background led to a more aggressive tumor phenotype.[85] (4) Recent work from our group indicated that critical determinants for the different oncogenic potentials of individual Rel/NF-κB subunits reside within their divergent TADs.[87] While RelA fails to transform primary chicken spleen cells, substitution of its TAD by that of the transforming v-Rel or c-Rel proteins conferred a strong transforming phenotype both in vitro and in vivo. Intrinsic differences between individual Rel/NF-κB TADs might confer distinct oncogenic potentials owing to differences in the repertoire of genes that they activate, as suggested by preliminary microarray analyses (Gupta, Fan, Delrow and Gélinas, unpublished data). Furthermore, the strength of individual Rel/NF-κB TADs is inversely correlated with their transforming potential, indicating that the magnitude of gene activation must be within a suitable range. For example, deletion of either of the two human c-Rel TADs reduced its transcriptional activity and increased its transforming efficiency.[115] Since strong TADs such as that of RelA perhaps activate gene expression to a level that is incompatible with cell transformation, it is tempting to speculate that RelA mutants with decreased transactivation potency might be capable of transformation. Preliminary data from our group suggest that this may indeed be the case (Fan and Gélinas, unpublished data). Overall, these findings underscore a fundamental role for gene transactivation in the transforming ability of Rel/NF-κB.

Functional Consequences of Rel/NF-κB-Mediated Gene Activation in Oncogenesis

The Rel/NF-κB transcription factors activate a wide variety of target genes that influence its oncogenicity. These include cell death inhibitors, cell cycle regulators, transcription factors and oncoproteins, cytokines and receptors, and cell surface and adhesion molecules. This section reviews how these contribute to the transformation process by affecting the regulation of apoptosis, cell proliferation, angiogenesis and metastasis.

Suppression of Apoptosis

Escape from apoptosis is a major factor in oncogenesis and in the resistance of tumor cells to therapy. It is therefore not surprising that NF-κB's anti-apoptotic activity has been linked to

many different cancers and that it impedes effective treatment.[116,117] Consistent with the fact that Rel/NF-κB inhibits cell death by activating expression of antiapoptotic genes that can at least partially substitute for NF-κB to suppress cell death, many tumor-derived cell lines display elevated expression of NF-κB-dependent antiapoptotic factors (Table 2).[4] For instance, therapy-resistant DLBCL tumors and malignant H/RS cells have elevated levels of *bfl-1/a1* transcripts compared to controls, and *FLIP* is upregulated in DLBCL while *c-iap2* is induced in H/RS cells.[11,19,71,118] The important role for NF-κB in these cancers is highlighted by the fact that many tumor-derived cell lines, including those derived from HD, DLBCL and breast cancer undergo spontaneous apoptosis, or are sensitized to death-inducing stimuli, following NF-κB inhibition (for example see refs. 6,8,12,13,119-121). Although ectopic expression of Bcl-xL could rescue apoptosis of H/RS cells in which NF-κB activity was suppressed,[19] it should be noted that in other cases multiple apoptosis inhibitors appear to act in concert to promote survival in NF-κB-associated tumors.[12] These findings agree with the observation that sustained expression of v-*rel* is necessary to maintain the viability of transformed lymphoid cells and that v-*rel*-mediated transformation requires expression of specific apoptosis inhibitors (Table 2).[100,106,122-127] Though a majority of studies emphasize a fundamental role for the cytoprotective activity of NF-κB in oncogenesis, there are exceptions. For instance, survival in Bcr-Abl-induced leukemia was reported to be independent of NF-κB's antiapoptotic activity.[31]

Alternative mechanisms have emerged in which NF-κB promotes cell viability by interacting with other factors. For example, NF-κB interferes with the transcriptional function of the pro-apoptotic tumor suppressor p53 by competing for coactivators.[128-130] Similarly, p65/RelA sequesters coactivator p300 to inhibit expression of tumor suppressor PTEN and allow cell survival in lung and thyroid cancer cells.[131] The recently discovered capacity of IKKβ to increase expression of Mdm2 and decrease p53 stability to suppress chemotherapy-induced cell death is another example.[132] Lastly, NF-κB-mediated suppression of the p53-related p73 factor antagonizes apoptosis in antigen-stimulated naïve T cells.[133] This raises the possibility that a similar mechanism might operate in an oncogenic setting, although this remains to be established.

Cell Proliferation

Independent studies highlight a link between Rel/NF-κB's effects on cell proliferation and oncogenesis. Consistent with the critical role of the c-Rel subunit in B cell proliferation,[134,135] lymphoid cells transformed by a temperature-sensitive mutant of v-Rel fail to proliferate at the restrictive temperature under conditions where apoptosis is rescued by cell death inhibitor Bcl-2.[127] Aside from generating autocrine loops to constitutively activate the NF-κB pathway in tumor cells (see above), NF-κB activates expression of factors that influence cell cycle entry such as cyclins D1, D2 and D3 (Table 2).[19,135-137] These findings concur with the elevated levels of cyclin D2 in malignant H/RS cells and cyclin D1 in mantle cell lymphoma and breast cancers that display sustained NF-κB activity, as well as in MMTV-c-*rel*-induced mouse mammary carcinoma.[19,92,138]

NF-κB can also enhance proliferation by activating other transcription factors. Some of them directly contribute to v-Rel-mediated transformation of lymphoid cells (Table 2). For example transcription factor AP-1(c-Jun) is essential for v-Rel's transforming activity in primary lymphoid cells and fibroblasts.[139] Both c-Jun and JunB are aberrantly expressed in malignant H/RS cells of HD, where upregulation of JunB is NF-κB-dependent.[140] These factors act together with NF-κB to stimulate H/RS cell proliferation and expression of cyclin D2, Bcl-xL, c-*met* and chemokine receptor CCR7. Other examples are interferon regulatory factor 4 (IRF 4) that decreases induction of the anti-proliferative IFN pathway and facilitates v-Rel-mediated transformation[141] and c-Myc, a target of p50/c-Rel dimers that is induced in MMTV-c-*rel* mouse mammary tumors.[92,142] Stat5a recently joined this group as an NF-κB target that is activated constitutively in HD and is linked to cell growth regulation.[10]

Angiogenesis and Metastasis

The ability of tumor cells to acquire sustained angiogenesis, invade surrounding tissues and metastasize to remote sites is one of the most significant factors contributing to cancer patient mortality. Here too NF-κB makes an important contribution by inducing expression of factors that promote angiogenesis (Table 2). Elevated NF-κB activity in cancer cells enables deregulated production of chemokines and chemokine receptors, like IL-8 and CXCR4, which increase migratory activity and promote angiogenesis.[143,144] NF-κB-mediated induction of vascular endothelial growth factor (VEGF) is another important contributing factor.[144,145] NF-κB also promotes invasion of surrounding tissues by inducing various cell adhesion molecules and matrix metalloproteinases.[7,145] Together, these factors contribute to the pathogenesis of NF-κB in cancer.

Other Means for NF-κB to Participate in Oncogenesis

Several protein interactions and post-translational modifications modulate the transcriptional activity of NF-κB, and in some cases its contribution to oncogenesis.

Interaction with Transcription Factors and Coactivators

v-Rel and its cellular homologues c-Rel and RelA functionally interact with basal transcription factors and transcriptional coactivators to synergistically enhance gene transcription (for example see refs. 146-148). In some instances, interactions were confirmed in transformed lymphoid cells.[146] Although various coactivators like CBP/p300, TAFII105 or TAFII250 mediate NF-κB dependent transcription of anti-apoptotic genes, their implication in a tumor context is awaiting.[149-153] However PARP (Poly-ADP ribose polymerase-1) that behaves as a coactivator for NF-κB was recently implicated in NF-κB-mediated susceptibility to skin cancer induced by DMBA and TPA in mice but their coordinate action in human carcinogenesis remains to be verified.[154,155]

Post-Translational Modifications

Phosphorylation, acetylation and ubiquitination of NF-κB subunits influence their activity, although in many cases evidence of their role in NF-κB-associated tumors has yet to be obtained.

Various kinases including IKK, casein kinase II and AKT enhance the transcriptional activities of p65/RelA and c-Rel by phosphorylating their TAD.[156-162] Moreover, mutation of certain serines in the v-Rel TAD decreases its transcriptional activity and impairs transformation of lymphoid cells.[106] In addition, the catalytic subunit of PKA (PKAc), MSK1 and PKCζ phosphorylate the RHD of p65/RelA and modification by PKAc is necessary for p65 interaction with coactivator p300.[163-166] Of interest, serine phosphorylation in the C-terminal domain of Bcl-3 by GSK3 affects its interaction with HDACs and is correlated with attenuation of its transforming potential in a mouse model.[167] Acetylation bestows another level of regulation, as exemplified by reversible p300/CBP- and P/CAF-mediated acetylation of p65/RelA that blocks association with IκBα and promotes p65 nuclear localization, DNA binding, and transactivation.[168-171]

A role for the ubiquitin-proteasome pathway in directly regulating the stability of NF-κB subunits came to light in work showing that C-terminally truncated c-Rel mutants and v-Rel display reduced proteasome-mediated turnover coincident with oncogenic transformation.[172] Since then, poly-ubiquitination of p65/RelA was implicated in terminating the NF-κB response.[173] In this regard, the peptidyl-prolyl isomerase Pin1 was recently described to enhance NF-κB activity by associating with nuclear p65 to prevent its SOCS-1-mediated ubiquitination and degradation.[174] The fact that Pin1 is highly overexpressed in human breast cancer suggests a possible role for Pin1 in enhancing the oncogenic activity of NF-κB in certain tumors.

A Tumor Suppressor Role for NF-κB

Despite a large body of evidence supporting a positive role for Rel/NF-κB in oncogenesis, a growing number of studies indicate that NF-κB can behave as a tumor suppressor in some circumstances.[175,176] Indeed RelA opposes the action of TNFR1 and JNK to curb epidermal cell growth, and suppression of NF-κB in skin cooperates with oncogenic lesions such as oncogenic Ras to favor development of squamous cell carcinoma.[177-181] Consistent with this, immortalized *relA-/-* fibroblasts induce tumors in SCID mice.[182] Moreover, RelA actively represses transcription of anti-apoptotic genes in response to certain stimuli such as UV-C and chemotherapeutic agents doxorubicin or daunorubicin,[183] although others found NF-κB to be protective in this context.[184,185]

The interaction of NF-κB with tumor suppressors to downregulate proproliferative or antiapoptotic genes, or to induce expression of pro-death factors further supports the notion that NF-κB can inhibit tumor growth in certain settings (Table 2). ARF, best known for its role in activating p53 via inhibition of Mdm2, inhibits p65/RelA-mediated transcription by inducing p65 association with histone deacetylase HDAC1.[186] This effect is promoter specific, as it leads to downregulation of anti-apoptotic NF-κB target Bcl-xL but not IκBα. Similarly, tumor suppressor p53 converts transcriptionally active Bcl-3/p52 complexes into transcriptionally inactive p52/HDAC1 complexes that inhibit cyclin D1 expression to induce cell cycle arrest.[187] The significance of these findings is highlighted by the observation that c-Rel, p52 and Bcl-3 are activated in human breast cancer.[9,95] Interestingly, tumor suppressor BRCA1 physically interacts with p65/RelA to enhance NF-κB-mediated transcription of proapoptotic gene Fas, while ING4 controls brain tumor angiogenesis by negatively regulating RelA's transcriptional activity.[188,189] Lastly, NF-κB was reported to be required for p53-dependent apoptosis,[190] and recent work implicated the serine/threonine kinase ribosomal S6 kinase 1 (RSK1) in its p53-mediated activation.[191] While one study found that NF-κB activation by doxorubicin decreases p53 stability,[132] another showed that NF-κB stabilizes p53 to provoke apoptosis in response to genotoxic stress.[192] Regardless of the mechanisms involved, the capacity of NF-κB to sometimes behave as a tumor suppressor has obvious implications for its role in oncogenesis and for therapeutic approaches aimed at inhibiting its activity (see below).

Conclusions and Perspectives for Therapy

While additional work is needed to pinpoint the precise role of Rel/NF-κB in human cancer and the mechanisms involved, a parallel has emerged between activities needed for lymphoid cell transformation and lymphomagenesis induced by the v-Rel and c-Rel proteins and those observed in many human tumors displaying constitutive NF-κB activity. These include inappropriate activation of cellular gene expression and aberrant expression of proproliferative and antiapoptotic genes. While the Rel proteins are potently oncogenic in avian species, the delayed onset of tumors in transgenic mice expressing v-*rel* or an MMTV-c-*rel* transgene suggests that additional cytogenetic alterations are necessary for NF-κB to manifest its oncogenic phenotype in mammals.[85,92] Recent studies uncovered a crucial role for interaction between inflammatory cells and precancerous cells in tumor promotion and showed that NF-κB is critical in this process.[193-195] Thus, identification of the genes and pathways that act cooperatively with NF-κB in human cancer is an important goal in the field.

The NF-κB pathway constitutes an important therapeutic target.[145] NF-κB is implicated in the intrinsic resistance of cancer cells to apoptosis and the induced chemoresistance of many tumors to anti-cancer drugs, and its inhibition often enhances the effectiveness of treatment (for example see refs. 119,121,184). However recent advances uncovered a more complex scenario, by revealing that NF-κB can sometimes repress transcription of anti-apoptotic genes in response to "atypical" activators including several chemotherapeutic agents.[175,183] Its interaction with tumor suppressors such as p53, ARF and BRCA1 is another potentially important factor in the outcome of cancer therapy. Careful attention should

thus be given to the tumor cell type, the death-inducing agent and perhaps other cytogenetic alterations in these tumors. Ongoing studies in the field promise to further advance our understanding of the transcriptional, anti-/pro-proliferative and anti/pro-apoptotic functions of NF-κB, the modifications and factors that modulate its activity and their impact on the oncogenic process. Together these will help to develop and improve appropriate strategies for cancer therapy tailored to particular contexts.

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CHAPTER 10

NF-κB in Human Cancers

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Introduction

Clinical oncology is increasingly based in science, such that physicians are routinely administering drugs designed to inhibit specific growth and survival pathways in cancer cells. Perhaps the best-established targeted therapy is Imatinib Mesylate (Gleevec, STI571), a tyrosine kinase inhibitor that impedes signaling through the hybrid enzyme generated by genetic translocation of the *c-abl* protooncogene in chronic myelogenous leukemia (CML). This drug was promoted based on its capacity to inhibit the abnormal tyrosine kinase activity resulting from the translocation, and is now a standard treatment for patients with CML.[1-3] Of interest to the reader, it was investigators in David Baltimore's group who originally described *c-abl* and recognized its likely role in CML oncogenesis, ultimately leading to targeted therapy for patients with this disease.[4,5]

Nuclear Factor kappa B (NF-κB) proteins were initially reported in the mid-1980s as regulators of immunoglobulin (Ig) kappa gene transcription in B lymphocytes, also based on work in Baltimore's laboratory.[6,7] As a group, NF-κB and related proteins exert a profound influence on the transcriptional response to immune stimuli, thereby controlling both healthy and pathologic inflammatory reactions.[8-10] Over the past two decades, five mature mammalian NF-κB family members and their structural precursors have been elucidated. These are p50, p52, p65 (RelA), c-rel and RelB. NF-κB proteins are distinguished by a conserved region at the N-terminus of approximately 300 amino acids, termed the Rel Homology Domain (RHD). Like other transcriptional regulators, NF-κB proteins control gene expression by binding DNA at consensus sequences for which they have structural affinity.[11,12] It is through the RHDs that NF-κB molecules form dimers, interact with inhibitor of κB (IκB) proteins, enter the nucleus upon activation and, ultimately, bind DNA (Fig. 1).

Whereas in most cell types NF-κB enters the nucleus and induces transcription of pro-inflammatory genes upon activation of an IκB kinase (IKK), in mature B cells, macrophages and neurons some NF-κB proteins reside constitutively in the nucleus.[13-15] The human IκB molecules, including IκBα, IκBβ, IκBγ and IκBε contain characteristic ankyrin-repeat domains which afford their capacity to interact with other cytoplasmic proteins. As they bind and render NF-κB proteins inactive, IκB proteins are also key regulators of gene transcription, cell survival, and in some circumstances, tumor growth and potential therapy. Bcl-3, an atypical and inducible member of the IκB family, can directly regulate gene transcription by its interaction in the nucleus with p52.[13,16] It is noteworthy that the *bcl-3* locus was identified and cloned based on its translocation and over-expression in some cases of chronic B cell leukemia (CLL),[17,18] although the precise relevance of bcl-3 to CLL pathogenesis remains elusive.

The IKK complex includes IKKα and -β, as well as a protein that regulates activation of the complex by binding its other elements, termed NF-kappa B essential modulator (NEMO,

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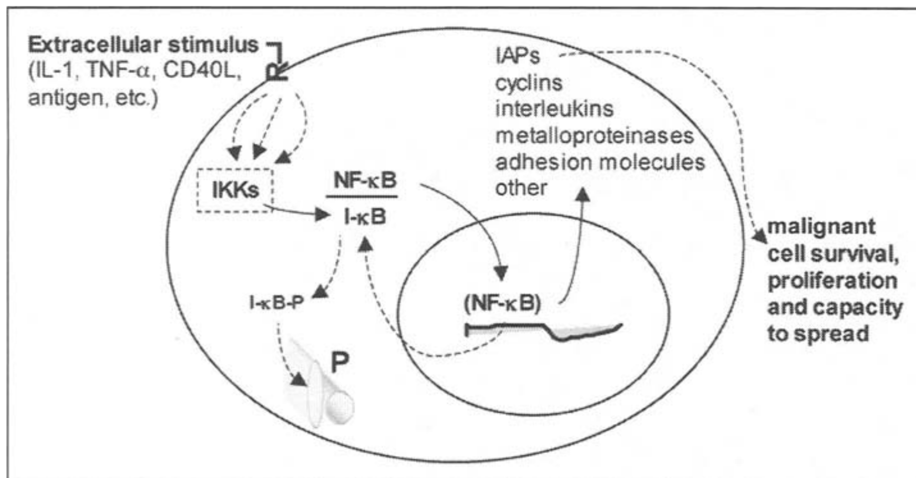

Figure 1. Extracellular stimuli and intracellular signals can lead to NF-κB activation, consequent gene transcription, cellular proliferation and survival. Inactive NF-κB is generated and cleaved in the cytosol, where it is lies bound to inhibitor, IκB. Upon triggering of cell surface receptors such as that for interleukin-1 (IL-1), CD40, the T cell receptor (TcR) or the B cell receptor (BCR) for antigen, cytoplasmic kinases activate the IκB kinase (IKK) complex. Upon specific phosphorylation of IκB, NF-κB is released from its inhibitor to enter the nucleus, and IκB is degraded along the ubiquitin-proteosome pathway (P). In the nucleus, NF-κB complexes regulate transcription of genes that promote cellular proliferation, adhesion and survival.

IKKγ).[19,20] NEMO in itself can regulate transcription by its capacity to enter the nucleus and interact there with p65 and cAMP-responsive element-binding protein-binding protein (CBP).[21] Because NEMO regulates NF-κB activity directly and indirectly through its "scaffolding" relationship with IKK-α and -β, NEMO-IKK interactions are important potential targets for pharmacology. For instance, the inflammatory responses engendered by IKKβ activation can be prevented by application of a small molecule designed precisely to block the IKK-NEMO binding site, termed NEMO-binding domain (NBD) peptide.[20] NEMO is subject to ubiquitination by bcl-10,[22] a molecule implicated in lymphoma pathogenesis,[23] and thereby may be vulnerable to some proteosome inhibitors as are now used in some B cell tumors including multiple myeloma (see below).

The link between NF-κB and cancer was established along several lines, including the early recognition of p50 and p65 as mammalian homologs of v-*rel*, the transforming gene of the avian reticuloendotheliosis virus (REV-T) and its cellular counterpart, c-*rel*.[24-28] In the 1960s, the c-*rel* locus was postulated as a potential factor in oncogenesis based on observed genetic abnormalities at this locus in chickens, turkeys and quail with lymphoid malignancies.[29,30] Over time it was determined that c-*rel* can transform hematopoietic cells in vitro and induce tumors in chickens.[31,32] In humans, c-*rel* sequences were localized to chromosome 2p1, the site of some chromosomal translocations in cancer.[33]

NF-κB Controls Cellular Proliferation, Adhesion and Survival

The significance of NF-κB activity in tumor cell growth and survival is now widely appreciated.[12,34-38] This is largely a function of the capacity of NF-κB to confer a proliferative and survival advantage through induction of cell-cycle and apoptosis regulatory genes. For oncologists, knowing how and when distinct NF-κB components are generated, activated such that they can enter the nucleus and bind DNA, and degraded, affords insight regarding current and future therapies based on NF-κB inhibition.

A number of cell cycle regulatory elements are NF-κB target genes (Fig. 1). One of the best-studied of these is cyclin D1.[16,39,40] Over-expression of cyclin D1 is implicated in mantle cell lymphoma, some forms of breast cancer, and other tumors.[41-47] Of interest, NF-κB-dependent cyclin D1 expression and consequent proliferation of mammary epithelial cells appears to be a physiologic response to NF-κB activation in pregnancy.[48,49] In other cell types, interleukins such as IL-6 and IL-8 are induced upon NF-κB activation, resulting in increased cell turnover and inflammation.[19,50-52] In B cells, c-rel is essential to cell cycle progression upon stimulation through surface IgM (sIgM).[53] Upon sIgM crosslinking, NF-κB triggers c-*myc* expression, fostering the proliferative response to antigen for which the B cell has affinity.[54] Induction of c-*myc* via NF-κB pathway activation is implicated in diverse cell types, including fibroblasts.[55] In theory, NF-κB-dependent induction of c-*myc* would be most relevant to pathogenesis and potential therapy of B-cell tumors in which malignant cell growth and survival is enhanced by engagement of the B cell receptor (BCR) by antigen for which the tumor-specific Ig has affinity, such as Burkitt's lymphoma and CLL.[56-59]

The capacity of NF-κB activity to inhibit apoptosis is central to its role in tumor development and resistance of cells to cancer treatment (Fig. 1). In this regard, one of the earliest observations about NF-κB function was that Rel A (p65) protects cells from apoptosis upon exposure to TNF-α, ionizing radiation, and the chemotherapeutic reagent daunorubicin.[60,61] Several anti-apoptotic bcl-2 family members, including bcl-x_L and bfl-1 (A1), are well-established NF-κB target genes;[62-66] through induction of these potent survival factors, stimulation of the NF-κB system enhances the propensity of cells to survive under stress. The gene encoding Bcl-2, the prototypic member of this key family of apoptosis regulatory molecules, contains several NF-κB binding motifs in its promotor. Some evidence suggests that bcl-2 transcription is turned on in the nucleus by p50 or p52 homodimers upon their association with bcl-3.[67] Similarly, the cellular inhibitor of apoptosis-1 (c-IAP-1) and -2, and X-linked inhibitor of apoptosis (XIAP) are induced by NF-κB.[68-70] In addition, both viral and cellular inhibitors of Fas-mediated apoptosis, v-*FLIP* (FLICE, Fas-associated death domain (FADD)-like interleukin-1 β-converting enzyme, caspase 8) Inhibitory Protein, and the cellular homolog, c-*FLIP*, are NF-κB target genes which promote survival of tumor cells by inhibition of caspase 8.[19] Overall, these observations reflect that NF-κB induces a powerful cell survival program, conferring apoptosis resistance in cells through induction of bcl-2 family members, IAPs, and other molecules that inhibit death of malignant cells.

NF-κB stimulates expression of adhesion molecules, metalloproteinases and other inflammatory mediators that play a role in tumor cell growth and dissemination. Adhesion molecules that are thought to facilitate metastatic spread and are NF-κB-dependent include intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule 1 (ELAM-1)[71-74] Among the inflammatory molecules, such powerful cytokines as Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), IL-1, IL-8, and beta interferon (β-IFN) are induced by NF-κB.[75-77] Cyclooxygenase 2 (COX-2), implicated in pathologic inflammatory states and, in some cancer models, tumorogenesis, is also an NF-κB target gene.[78] The metalloproteinase-9 (MMP-9), which is involved in tumor metastatic potential and invasiveness, is also NF-κB -responsive.[79]

Taken together, these responses support that NF-κB activity has a role in formation of numerous tumor types by promoting cell survival, proliferation in some circumstances, and by generation of adhesion molecules rendering cells hardier and more aggressive in phenotype.

Tumors in Which NF-κB Is Implicated in Pathogenesis

Aberrant and increased NF-κB activity are implicated in a variety of human malignancies, including tumor types derived from cells of immune origin such as lymphocytes, monocytes, and myelocytes, as well as tumors arising from cells of nonimmune origin (Table 1). Among the solid tumors, abundant evidence links NF-κB to pathogenesis in breast cancer, hepatocellular carcinoma, renal cell carcinoma and other conditions. Several oncogenic viruses exert their effects, at least in part, by induction of NF-κB in malignant cells.

Table 1. Human tumors in which aberrant or increased NF-κB activity is implicated in pathogenesis

Leukemias and Lymphomas	
Adult T-cell leukemia lymphoma (ATLL)	80-82
Acute lymphoblastic leukemia (ALL)	83,84
Acute myeloid leukemia (AML)	85
Chronic lymphocytic leukemia (CLL)	86,87,123
Diffuse large B cell Lymphoma (B-DLCL)	88-90
Hodgkin's lymphoma	91-94
Mantle cell Lymphoma	95
Mucosa-associated lymphoid tissue (MALT) lymphoma	23,96-98
Multiple myeloma	99-101
Solid Tumors	
Brain cancer (glioma)	102
Breast cancer	48,103-105
Head and neck cancer	106
Gastric carcinoma	107
Hepatocellular carcinoma	108
Melanoma	109-111
Nasopharyngeal Ca	112
Pancreatic cancer	113-115
Prostate cancer	
Renal cell carcinoma	116
Thyroid cancer	117

B lymphocytes constitute the cell of origin in the majority of adult lymphomas, including both Hodgkin's and nonHodgkin's lymphomas, and in related tumors such as multiple myeloma and some forms of lymphocytic leukemia. Constitutive NF-κB activity is a distinctive feature of B lymphocytes and is due, at least in part, to constant degradation of IκB.[118] One physiologic stimulus of inducible NF-κB in B cells is CD40 ligand (CD154), a TNF-like molecule that is expressed in activated CD4+ T cells and, is itself, an NF-κB target gene.[119,120] Virtually all mature B cells, normal and malignant, express CD40, a cell surface receptor for CD40 ligand which promoted differentiation and survival of B cells.[121-123] Here, the pro-survival effects of CD40 ligation are mediated largely, but not completely, by NF-κB.[124-127]

Diffuse large cell lymphoma (DLCL) of B cell origin (B-DLCL) comprise approximately 30% of adult lymphomas. In B-DLCL tumor cells, autocrine expression and binding of the CD40 receptor and its ligand, CD154, can lead to continuous NF-κB activation.[89] Other lines of evidence point to activation of NF-κB in B-DLCL via alternative TNF receptors, such as the receptors for BAFF (B cell Activating Factor of the TNF Family) and APRIL (A Proliferation Induced Ligand). Ligation of BAFF and APRIL result in NF-κB induction and expression of NF-κB-dependent proliferation and survival factors such as *c-myc* and *bcl-x$_L$*, respectively.[90] In Hodgkin's lymphoma, the role of NF-κB is supported by the finding of p50 and p65 in the nuclei of Reed Sternberg cells in fresh tumor specimens.[128] Additional evidence stems from observations that constitutive NF-κB is necessary for growth of Reed Sternberg cell lines.[91] Aberrant, constitutive NF-κB activity in Hodgkin's lymphoma is associated with abnormal and mutated IκB-α.[92] In this tumor, signaling via CD30, another TNFR family member, is implicated.[93,94,129,130]

An interesting connection of NF-κB activity in inflammation and cancer occurs in MALT (Mucosal Associated Lymphoid Tumors) lymphomas, particularly in the stomach, where infection by the bacterium ***helicobactor pylori*** and subsequent inflammation play a role in tumor pathogenesis. Here, NF-κB is implicated at several levels, including the initial inflammatory response to the bacterium.[97,131] In the subset of MALT tumors containing the t(1;14) (p22;q32) involving the *bcl*-10 locus, a truncated form of bcl-10 is expressed that activates NF-κB and has transforming properties.[132] As considered above, bcl-10 functions as an adaptor protein targeting NEMO (IKK-γ) for ubiquitination;[22] this may result in high NF-κB activity and contribute to survival of these B lymphoma cells. Among MALT lymphomas, nuclear NF-κB activity appears to correlate with the t(1;14) translocation detection of, bcl-10 in the cytoplasm, and tumor aggressiveness.[133]

In chronic lymphocytic leukemia (CLL), another tumor of B lymphocytes, our group has demonstrated that the malignant B cells have high levels of active, nuclear NF-κB immediately upon isolation from patients' blood.[86] The most evident activity comprises p50, p65 and c-rel, consistent with the postulated responsiveness of CLL cells to extracellular, inducible stimuli in vivo.[58] Consistent with this hypothesis, NF-κB activity dissipates over a period of hours upon isolation of the cells ex vivo, but this activity and survival can be rescued by CD40 engagement of CLL cells in vitro. NF-κB in CLL cells is enhanced upon simultaneous stimulation of the BCR, which may in itself, be a modest inducer of NF-κB in CLL.[57] Based on these observations, we put forth a model for CLL pathogenesis based on chronic stimulation of the tumor cells in vivo by such factors as CD40 ligand and antigen for which the tumor cells have avidity.[123] In vivo, CLL cells maintain high levels of inducible NF-κB due to constant costimulatory and BCR-derived signals, leading to an intensely anti-apoptotic phenotype and drug resistance.[58] Among the NF-κB-target genes expressed in CLL are the anti-apoptotic bcl-2 family members bcl-x_L, bfl-1 (A1) and bcl-2,[57,67] cIAP-1 and -2, and XIAP (A. Bernal and E. Schattner, unpublished data). Other investigators have shown that in the microenvironment of CLL proliferation centers, CLL express survivin. This effect is located in the bone marrow and appears to be mediated by tumor infiltrating CD4+ T cells expressing CD40L.[134]

One of the first tumors for which NF-κB was considered as a therapeutic target is multiple myeloma, a cancer of malignant plasma cells.[99] In multiple myeloma, NF-κB dependent IL-6 is a potent growth factor, and additional NF-κB-mediated transcripts confer resistance to apoptotic stimuli such as TNF-α.[100] Bortezomib (PS341) was the first FDA-approved drug targeting the proteosome, a large, cytosolic multi-component protein complex that degrades proteins marked for degradation by a ubiquitin ligase. Among the proteins degraded by the proteosome is IκB. Based on a vast amount of in vitro data supporting the capacity of PS341 to promote cell death of myeloma cells in vitro, Bortezomib was tested in humans and determined to be effective in patients with refractory cases of multiple myeloma.[101,135] Subsequent studies of Bortezomib have demonstrated activity in numerous malignancies including solid tumors.[136] However, one problem that applies to the interpretation of these studies is that the proteosome degrades many cytoplasmic proteins, including some with pro-apoptotic functions. It remains to be formally demonstrated that altered NF-κB is responsible for the efficacy of Bortezomib in patients with myeloma.

Virus-Associated Tumors

Viruses can turn on NF-κB activity in tumor cells by coopting normal cellular activation pathways. One of the best-delineated NF-κB-activation pathways occurs in adult human T cell leukemia lymphoma (ATLL). In this rare neoplasm, the malignant CD4+ T cells are infected by human T-cell lymphotrophic virus-1 (HTLV-1), of which the *tax* gene product induces NF-κB by activating IKK.[80-82,137] The viral tax protein effects T-cell survival and proliferation through a variety of pathways, including NF-κB-dependent induction of c-*myc*.[138]

Viral-mediated NF-κB activation contributes to pathogenesis in B cell tumors infected with Epstein Barr Virus (EBV). Here, the EBV Latent Membrane Protein (LMP)-1 interacts directly with the cytosolic domain of CD40, so as to activate IKKβ, induce expression of bfl-1,

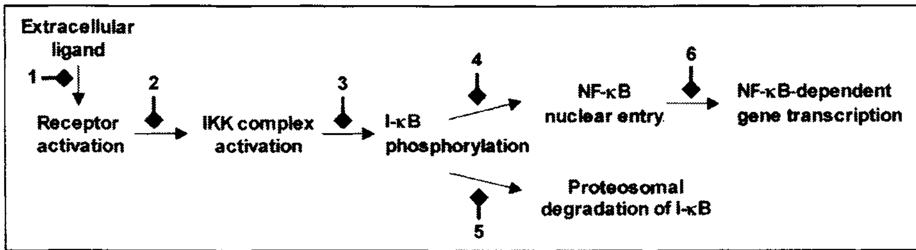

Figure 2. Blocking NF-κB in cancer. Potential sites for disruption of NF-κB activation and downstream effects include 1) impeding ligand-receptor interaction at the cell surface, such as by application of antibody to the ligand or to the receptor; 2) dampening activation of the IKK, such as by use of a small peptide that binds NEMO (NF-κB essential modifier, IKKγ) where it associates with IKKα and IKKβ; 3) preventing phosphorylation of IκB, such as by introduction a "super-repressor" IκB molecule that lacks key phosphorylation sites; 4) preventing NF-κB nuclear entry, possibly through the identification and targeting of a putative NF-κB chaperone protein; 5) reducing IκB degradation in the proteosome; 6) targeting NF-κB activity in the nucleus, such as by interference with RHD domains or implementation of antisense to particular NF-κB target genes.

and enhance B-cell survival.[139] NF-κB activation along this route is thought to be an oncogenic factor in EBV-containing tumors including post-transplant lymphoproliferative disorders (PTLDs), some cases of Burkitt's lymphoma, Hodgkin's lymphoma, and primary effusion lymphomas (PELs). In human immunodeficiency virus (HIV)-associated PELs, infected with HHV-8 and in some cases also with EBV, NF-κB activity is high.[140] Exposure of PEL cells to Bay-11, an NF-κB inhibitor, results in PEL cell death in vitro and in vivo, further supporting the role of NF-κB in proliferation and survival of virally-infected malignant cells.[140,141]

Virus-mediated induction of NF-κB is relevant also in pathogenesis of some solid tumors. For example, in patients with hepatocellular carcinoma and hepatitis B, expression of the hepatitis X protein (HBx) is related to increased NF-κB activity, IL-8 production, and tumor aggressiveness.[108,142] In nasopharyngeal cancer associated with EBV, p50 homodimers are present in the nucleus bound to the transcriptional coactivator Bcl-3. Chromatin immunoprecipitation experiments suggest that the p50 homodimers are bound to NF-κB consensus motifs within the promoter for the endothelial growth factor receptor (EGFR).[112]

The role of NF-κB in breast cancer is evident from several lines of investigation. As considered above, in mammary epithelial cells cyclin D1 expression and cellular proliferation depend on NF-κB.[48] In breast cancer cells stimulated to proliferate in vitro, application of the IKK inhibitory peptide NBD blocked NF-κB induction and the cells died.[104] Some studies have established that high levels of nuclear p65 in biopsy specimens are most evident in tumors that express ErbB2 (Her2/neu), the molecule to which the commonly-used therapeutic antibody Trastuzumab (Herceptin) binds. In these cases, exposure of tumor cells to Trastuzumab resulted in loss of NF-κB activity and cell death. Studies of NF-κB in breast cancer by another group point to the p50 and p52 proteins, as well as bcl-3, rather than p65, in breast cancer pathogenesis.[143] Transfection of mammary tumor cell line with a dominant negative IκB, or with an NF-κB decoy, render cells vulnerable to apoptosis.

Taken together these data support that NF-κB activity contributes to chemotherapy resistance in a variety of tumor types.[34,103]

Inhibition of NF-κB in Cancer Treatment

In principle, interruption of NF-κB activity in tumor cells can be achieved at multiple levels (Fig. 2). Treatment might be aimed at the cell surface, where NF-κB can be activated by particular receptors that could be blocked using appropriate antibodies. For example, in a tumor in which CD40 engagement promotes survival largely via NF-κB-mediated effects, blocking that receptor might be sufficient to allow apoptosis of otherwise resistant tumor cells.

Table 2. NF-κB inhibitors in humans

A. Current, FDA-approved pharmacologic agents that inhibit NF-κB activity:

Substance	Mechanism	Indication	Refs.
arsenic trioxide	inhibition of IKK	leukemia	146-148
Bortezomib	proteosome inhibition	multiple myeloma	149,150
butyrate	proteosome inhibition	investigational	151
cox-2 inhibitors	inhibition of IKK	anti-inflammatory	
	inhibition of DNA binding		152
glucocorticoids	increase IκB synthesis	anti-inflammatory	
	interference with DNA binding		153-158
N-acetylcysteine	oxygen radical scavenging	acetaminophen overdose	159
rapamycin	inhibition of IKK	immune suppressant	160,161
salicylates:	inhibition of IκB degradation	anti-inflammatory,	
including	inhibition of TNF signaling	inflammatory bowel disease	
sodium salicylate and acetylsalicylic acid	inhibition of IKK		
sulfasalazine	inhibition of IκB phosphorylation		
	inhibition of IKK		
mesalamine	inhibition of IκB phosphorylation		
	inhibition of RelA phosphorylation		
sulindac	inhibition of IKK		162-170
thalidomide	inhibition of IKK	leprosy	171
ursodeoxycholic acid (URSA)	interferes with p65-glucocorticoid receptor interaction	primary biliary cirrhosis	172

Table continued on next page

Alternatively, therapy may be based on the capacity of some compounds to prevent degradation of IκB, such as is now accomplished in a relatively nonspecific manner by means of proteosomal inhibition (Table 2). Other treatments might target enzymes of the IKK complex,[144] such as the NBD peptide and its effects on NEMO. Other therapies might block NF-κB nuclear entry, which may be feasible once more is understood about the requirements and possible chaperoning of specific NF-κB proteins when they enter the nucleus. Finally, treatments can be designed to impede NF-κB binding to consensus DNA sequences in the nucleus and subsequent gene transcription. For example, downregulation of p65 expression by RNA interference may enhance sensitivity of some tumor cells to chemotherapeutic drugs such as irinotecan.[145]

Conclusions

Twenty years since their discovery, our knowledge regarding the specific role of NF-κB proteins in particular tumor types, the interaction of these proteins with other transcriptional regulators in the nucleus, and our ability to tract and abrogate NF-κB activity, effectively and specifically, remain the subject of intense research efforts in cancer therapy. While NF-κB activity is the principle subject of this chapter and the target of several early clinical trials conceptually aimed at transcriptional regulation, other transcription factors also are potential targets for therapy. Understanding the relationship between NF-κB activity with other, parallel and interacting signaling pathways, is essential to development of effective and safe targeted therapies in cancer treatment.

Table 2. Continued

B. Natural compounds that inhibit NF-κB activity

Substance	Mechanism	Source	Refs.
anethole	inhibition of IκB degradation	fennel, anise	173,174
caffeic acid phenethyl (CAPE)	inhibition of NF-κB translocation	tree resin	175
capsaicin	Inhibition of IκB degradation	red chili peppers	176
curcumin	inhibition of IKK	curry	177,178
dicoumarol	inhibition of NF-κB activation	sweet clover	179
E330	inhibition of IκB degradation suppression of oxygen radical production interferes with DNA binding	quinone derivative	180,181
EGCG	inhibition of IKK	teas	182
epoxomicin	proteosome inhibitor	Actinomycetes	183
eugenol	inhibition of IκB degradation	cloves	174
flavonoids	inhibition of NF-κB	pomegranates	184
genistein	inhibition of IkB degradation	legumes	185,186
Substance	**Mechanism**	**Source**	**Refs.**
oleandrin	inhibition of IκB phosphorylation	plant leaves	187
resveratrol	inhibition of IKK	grapes	188,189
sesquiterpene lactones	inhibition of IκB degradation	plant remedies	190
sanguinarine	inhibition of IκB phosphorylation	root	191
sulforaphane	inhibition of DNA binding	cruciferous vegetables	192
silymarin	inhibition of IκB phosphorylation		193
s-allylcysteine	inhibition of NF-κB activation	garlic	194
theaflavins	inhibition of IKK	teas	182
ursolic acid	inhibition of IKK	berries, basil, rosemary, fruits	195

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CHAPTER 11

NF-κB in Neurons:

Behavioral and Physiologic Roles in Nervous System Function

Jonathan M. Levenson, Marina Pizzi and J. David Sweatt

Cells in general and neurons in particular display an amazing ability to respond to several different types of environmental stimuli and integrate this response physiologically. What is even more surprising is that some of these responses can outlive the original stimulus by days, weeks or even longer. Long-term changes in physiology that occur in response to an external stimulus are almost always mediated at least in part by changes in gene expression. To effect these changes, cells have developed an impressive repertoire of signaling systems designed to modulate the activity of numerous transcription factors.

Transduction of cytoplasmic signaling events from the plasma membrane to the nucleus in most cells is straightforward; the nucleus is surrounded by a relatively spherical cytoplasm (Fig. 1A). Thus, most cells consist of one extra-nuclear signaling compartment- the cytoplasm. The unique morphology of neurons however, presents an intriguing wrinkle to the problem of nuclear signal transduction. Neurons consist of three very different cytoplasmic compartments: soma, dendrite and axon (Fig. 1B). The soma is the central portion of the neuron, and contains the nucleus. The dendrites extend from the soma and consist of long, thin projections that form tree-like arborizations. A single axon projects from the soma and can connect to several hundred other neurons. Information flow in a neuron typically starts at the dendrite, travels to the soma and can pass down the axon if the signal is strong enough. When one considers that a majority of the neuronal cytoplasm resides in the dendrites, the question of how a neuron can translate distal signaling events into changes in nuclear transcription becomes very complex.

A Synaptic Messenger

A great deal of work has centered on characterizing a signaling system that translates synaptic activity to gene expression. This signaling system has been postulated to consist of a "retrograde" messenger that would travel from an activated synapse to the nucleus to regulate transcription.[1] A molecule must conform to five basic principles to be considered a synaptically activated "retrograde" messenger that signals the nucleus. (1) The putative messenger must be present in synapses in the inactive form. (2) Enzymes that activate the messenger must be present in synapses. (3) The enzymes that activate the messenger must be regulated by physiologically relevant stimuli. (4) Once activated, the messenger must be retrogradely transported to the nucleus. (5) Once inside the nucleus, the messenger must bind to DNA and regulate gene transcription. Researchers have identified several molecules that fulfill some of the criteria required of a synaptic retrograde messenger. In the following section, we will illustrate how the NF-κB signaling system meets all of the criteria required for a neuronal retrograde messenger whose function is to couple synaptic activity to gene expression.

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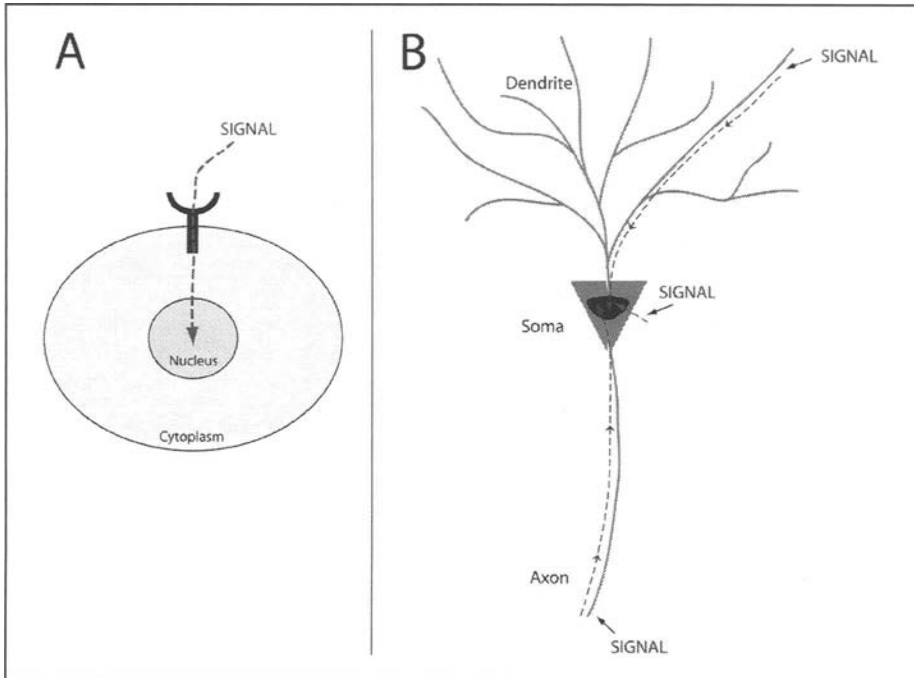

Figure 1. Neuronal morphology provides a unique challenge to retrograde nuclear signaling. A) Most cells have a simple morphology. There are only two signaling compartments: cytoplasm and nucleus. Therefore, movement of signals from the plasma membrane to the nucleus is straightforward. B) Neurons are highly specialized cells containing unique morphologies. This creates compartmentalization, complicating transport of signals from the plasma membrane to the nucleus.

The first evidence for the presence of NF-κB in the brain came in 1989, when NF-κB binding activity was observed in grey matter.[2] After this initial discovery, several labs have since localized NF-κB protein and binding activity in synapses located in several different regions of the brain.[3-5] Moreover, the synaptic NF-κB activity identified in these studies was only present if induced by exposure to a detergent, indicating that the native NF-κB proteins were present in an inhibited complex.[3-5] Therefore, *NF-κB exists in synaptic regions in an inactive form.*

As described in more detail elsewhere in this book, the NF-κB (also called NF-κB/Rel) family of dimeric transcription factors include p50, p52, p65 or RelA, RelB and c-Rel proteins, that use the Rel homology domain for dimerization and DNA binding.[6] In the cytoplasm NF-κB factors are bound to inhibitory proteins known as IκBs. Activation of signaling pathways that engage NF-κB signaling causes IκB to be phosphorylated by a protein complex known as IκB kinase (IKK). After it is phosphorylated, IκB is polyubiquitinated and degraded. The phosphorylation event releases NF-κB from IκB, allowing NF-κB to translocate to the nucleus and regulate transcription. The IKK complex consists of two catalytic (IKKα, IKKβ)[7] and one regulatory subunit (IKKγ).[8,9] To date, expression of only the α and β subunits of the IKK complex have been localized to synapses in the brain.[10,11] Despite the lack of information regarding the localization of IKKγ, the great deal of evidence regarding activation of NF-κB proteins by synaptic stimuli (see next section) coupled with the synaptic localization of the α and β subunits to the brain indicate that *the enzymes involved in activation of quiescent NF-κB protein are present in the brain, at synapses.*

Several lines of evidence indicate that activation of NF-κB can occur via physiologically relevant stimuli. Perhaps the most relevant physiologic stimulus in the brain is synaptic activity. NF-κB is regulated by both low- and high-frequency synaptic stimuli.[3] This observation likely explains why several researchers have observed constitutively active NF-κB DNA binding activity in the brain.[5,12-14] Glutamate, the primary excitatory neurotransmitter of the central nervous system, activates NF-κB by acting through a variety of glutamate receptors.[14-19] In addition, different cytokines, growth factors and lipopolysaccharide have been shown to modulate NF-κB activity in the brain. Therefore, *activation of NF-κB in the brain occurs in response to physiologically relevant stimuli.*

Recently, an additional level of regulation of NF-κB activity, that may involve post-translational modification of NF-κB subunits in the nuclear compartment and protein-protein interaction with other promoter-bound factors, has emerged. These new forms of regulation have been primarily characterized in nonneuronal cells, and include phosphorylation and acetylation of NF-κB factors.[20,21] One study has provided evidence that acetylation of NF-κB occurs in the brain during memory formation.[22] These results indicate that NF-κB activity is highly regulated, and that at least some of the pathways involved in fine-tuning NF-κB activity exist in the brain.

Activated NF-κB must translocate from the synapse to the nucleus to affect transcription. Therefore, stimuli that activate NF-κB must also promote its movement into the nucleus. Several methods have been employed to show that activation of NF-κB leads to increases in nuclear NF-κB amount and activity. Increases in the amount of active NF-κB have been measured immunocytochemically after activation with glutamate or glutamate receptor agonists.[15-17] NF-κB DNA binding activity in nuclear fractions has been shown to increase in response to several different types of synaptic stimuli.[3] Moreover, recent studies have utilized a chimeric protein containing both p65 and a protein isolated from jellyfish that fluoresces without excitation, known as green fluorescent protein, to visualize p65 subcellular localization in living neurons.[19] Upon stimulation of neurons with either glutamate, kainate, or high K^+ solutions, p65 was observed to rapidly redistribute from the synaptic regions of neurons to the nuclei.[19] These results indicate that *upon activation, synaptic NF-κB is retrogradely transported to the nucleus.*

The final step in demonstrating that a synaptic protein is a transcriptional retrograde messenger is to *show that transcription of genes regulated by the transcription factor is affected by the same stimuli that activate the messenger.* In this vein, the expression of NF-κB genes, including p50, p65 and IkBα, have been shown to be increased after stimulation of the NF-κB pathway.[23-25] Upon stimulation of neurons, expression of p50, p65 and IkBα have all been shown to increase.[3] Moreover, using reporter gene constructs several laboratories have shown that NF-κB-mediated gene transcription occurs in neurons and can be regulated by neuronal activity and levels of Ca^{++}.[5,12,13]

Signaling Pathways That Regulate Neuronal NF-κB

A great deal of work over the last decade has focused on characterizing the expression, regulation and function of NF-κB in the nervous system. NF-κB was the first transcription factor discovered in the brain that was present in synaptic regions, hinting that NF-κB might serve a crucial role in transducing synaptic events directly to the nucleus.[3,4] In the following sections we will discuss the regulation of NF-κB in the brain and its role in memory formation. While we will not discuss it here, it should be noted that a great deal of evidence exists indicating that NF-κB is also important in neuronal development and in neurodegeneration.[6]

To fully understand the role a transcription factor plays in any cell type, the signaling pathways that activate it must be identified. Neurons, arguably, have the most sophisticated complement of signaling systems known. Thus, the task of identifying the signaling pathways that play a role in regulation of NF-κB is not trivial. Ultimately, activation of these upstream signaling pathways must impinge on the IKKs to affect NF-κB function. Full characterization of the

upstream kinases and signaling molecules is still lacking in neurons, despite efforts by numerous laboratories. We will review what is currently known about the signaling pathways that modulate NF-κB activity in neurons.

Glutamate

Signaling events usually begin at the plasma membrane of a cell, and neurons are highly adapted to respond to various neurotransmitters present present at their cell surface. The primary excitatory neurotransmitter in the central nervous system is glutamate; most neurons in the brain either release glutamate as a neurotransmitter and/or possess receptors that are responsive to glutamate. Therefore, as an initial hypothesis one would expect neuronal NF-κB to be responsive to glutamate. In fact, several studies have shown that NF-κB is regulated by glutamate.[14,15,17,18,26] Further investigation has revealed that different subtypes of glutamate receptors contribute to the regulation of NF-κB by glutamate. Activation of NF-κB occurs upon stimulation of kainate receptors[16,17,19,27] or NMDA receptors,[5,14,15,18] and may be modulated by stimulation of metabotropic receptors.[28]

Growth Factors

In the brain, growth factors play important roles in regulation of synaptic plasticity, neuronal survival and differentiation. Therefore, growth factor signaling is extremely relevant to and vital for normal neuronal function. A great deal of evidence indicates that nerve growth factor (NGF) activates NF-κB through the p75 neurotrophin receptor.[29-35] Subsequent studies have shown that NF-κB is activated by a variety of growth factors including epidermal growth factor,[36] pigment epithelium-derived factor,[37] and brain-derived neurotrophic factor.[38] Interestingly, transforming growth factor β2 appears to inhibit NF-κB activity.[39] All of these observations suggest that NF-κB plays a critical role in neuronal growth factor signaling, and suggests that NF-κB coordinates a variety of transcriptional responses during synaptic plasticity and neuronal survival.

Calcium

Synaptic activity and stimulation of glutamate receptors almost always results in changes in intracellular Ca^{++} concentrations. Given that dormant NF-κB is concentrated at synaptic terminals, one would expect NF-κB to be regulated by Ca^{++}. The NMDA receptor mediates many of the Ca^{++}-dependent signaling processes that occur in neurons, including activation of NF-κB.[5,14,15,18] In addition to the NMDA receptor, voltage-gated Ca^{++} channels and opening of In3P receptors associated with the intracellular stores of Ca^{++} also contribute to activation of NF-κB.[5,16,40]

Reactive Oxygen Species

Reactive oxygen species (ROS) have been implicated in both pathology and normal signaling in neurons.[41] ROS refers to any molecule derived from molecular oxygen. There is growing evidence indicating that generation of ROS is necessary for the formation of long-term forms of synaptic plasticity and memory.[42-45] One signal for the generation of ROS in neurons is the Ca^{++} influx caused by the activation of NMDA receptors.[46] Some evidence exists that suggests activation of NF-κB in neurons also requires ROS.[17]

Protein Kinases

Signaling events that begin at the membrane result in the generation of small second messenger signaling molecules, which ultimately activate downstream kinase pathways. Several different kinase cascades have been implicated in regulation of neuronal NF-κB. Influx of Ca^{++} activates the calcium-calmodulin dependent protein kinases (CaMK) via binding calmodulin and interacting with regulatory subunits on these kinases, and in an important recent study the

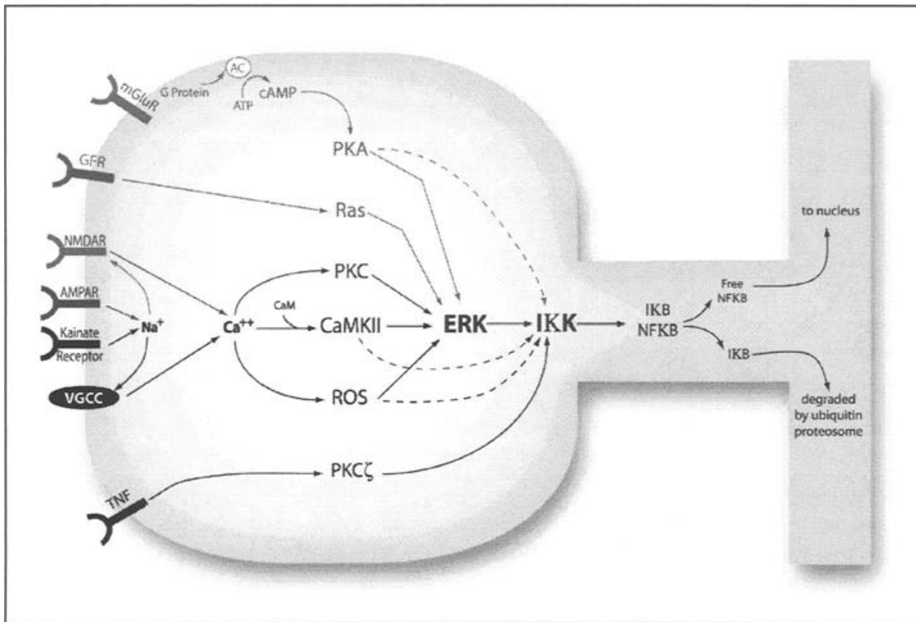

Figure 2. Regulation of NF-κB within neurons. Regulation of NF-κB in neurons can occur through multiple signaling pathways. Activation of various receptors at the plasma membrane engages many cytoplasmic signaling pathways that converge on the ras-MEK-ERK/MAP kinase system. ERK then activates IKK, either directly or indirectly, which leads to phosphorylation of IκB and release of NF-κB.

specific isoform CaMKII has been implicated in neuronal NF-κB signaling.[5] Many isoforms of protein kinase C (PKC) are expressed in the brain, however only the atypical PKCζ has been directly implicated in regulation of NF-κB in the brain.[16,47-50] The extracellular signal regulated kinase (ERK), which is a member of the MAP kinase superfamily, has also been shown to be involved in regulation of NF-κB activity.[16] Other kinases, such as the cAMP-dependent protein kinase and casein kinase, have been shown to phosphorylate IKK in vitro, however there is no convincing in vivo evidence for their importance in regulation of neuronal NF-κB.[51]

A Model for Activation of NF-κB in Neurons

From the previous sections, one can appreciate the variety and scope of cellular stimuli that can modulate NF-κB function. Drawing from what is known about basic signaling pathways in the brain, we can begin to assemble the puzzle of neuronal NF-κB regulation (Fig. 2). No comprehensive studies of the activation of NF-κB in neurons have been performed to date, so much of what we will discuss here is speculation. Activation of NF-κB begins at the plasma membrane, where activation of any number of receptors initiates second messenger signaling (Fig. 2). Key second messengers include Ca^{++} and cAMP. In addition to second messenger-mediated signaling, activation of growth factor receptors engages both ras and PKCζ[49] (Fig. 2). TNF activates NF-κB signaling via PKC.[52] In the immune system, TNF receptor-mediated arachidonic acid signaling activates NF-κB,[53] but data in neurons suggests that arachidonic acid signaling through TNFRs actually inhibits NF-κB.[49] Nevertheless, activation of all of these signaling cascades leads to phosphorylation of ERK MAP kinase[54] (Fig. 2). ERK activates IKK either directly or indirectly, which promotes dissociation of IκB from NF-κB, allowing NF-κB to move retrogradely to the nucleus to regulate transcription (Fig. 2).

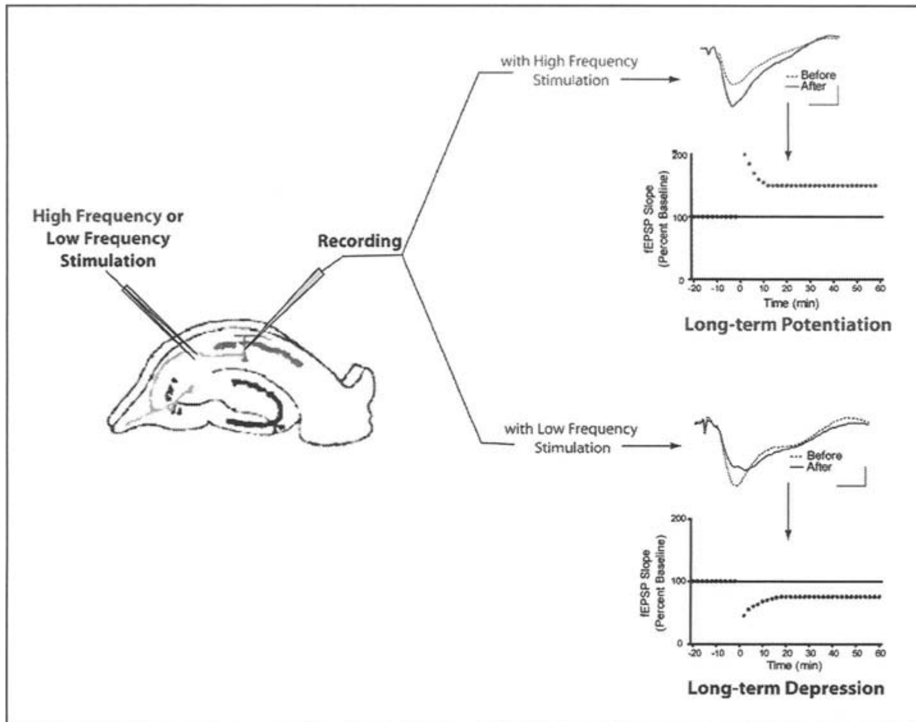

Figure 3. Synaptic plasticity. Synaptic plasticity refers to the ability of a synapse to change strength in response to synaptic stimuli. One preparation used to study the phenomenon of synaptic plasticity is the acute hippocampal slice. A stimulating electrode is placed along the presynaptic axon fibers and a recording electrode is placed in the dendritic field. Extracellular potentials provide an index of postsynaptic depolarization generated upon stimulation of the slice. High frequency stimulation leads to a lasting increase in the synaptic response, referred to as long-term potentiation. Conversely, low-frequency stimulation stably decreases the synaptic response, referred to as long-term depression.

Synaptic Plasticity

Neuron-to-neuron connections are referred to as synapses. There are two types of synapses: chemical and electrical. Chemical synapses are ones in which information from one neuron to another is transmitted by release of a specific chemical, referred to as a neurotransmitter. An electrical synapse is one where two neurons are directly connected, and changes in the electrical potential of one neuron are directly transmitted to the other neuron. Perhaps the biggest functional difference between these two types of synapses is the total inability of an electrical synapse to exhibit changes in strength in response to previous synaptic activity. These activity-dependent changes in synaptic strength, commonly referred to as synaptic plasticity, are thought mediate higher cognitive functions such as memory formation. Therefore, by studying synaptic plasticity, researchers can learn a great deal about the basic mechanisms that underlie very complex cognitive processes, such as memory formation, that occur in the brain.

Synaptic plasticity can take two basic forms: potentiation and depression (Fig. 3). Synaptic potentiation occurs when previous synaptic stimuli result in a larger, or enhanced synaptic response (Fig. 3). Conversely, depression occurs when previous synaptic stimuli result in a diminished synaptic response (Fig. 3). In general, high-frequency stimulation (≥0.2 Hz) usually leads to potentiation while low-frequency stimulation (~1 Hz) usually induces depression.

Changes in synaptic strength can exist for either very brief periods of time, lasting seconds to minutes, or persist for extended periods of time lasting several hours or longer. In the following paragraphs, we will focus specifically on two forms of synaptic plasticity referred to as long-term potentiation (LTP) and long-term depression (LTD). There are early and late phases of both LTP and LTD, which are distinguished by their reliance on protein synthesis. For our purposes, early-phases of plasticity refer to forms of plasticity that require only posttranslational modification, such as phosphorylation, but not new protein synthesis.[55] These early phases of plasticity generally do not persist for longer than 1-2 hours. Late-phase plasticity refers to forms of plasticity that require protein synthesis for their expression, and generally persist for at least several hours if not longer.[55]

NF-κB is a particularly attractive candidate transcription factor for the induction of synaptic plasticity because it is regulated by many of the same signaling pathways that are involved in induction of LTP and LTD (Fig. 2, see also ref. 54). Induction of LTP and some forms of LTD require activation of the NMDA class of glutamate receptors.[56-58] The small messenger molecules Ca^{++} and ROS are required for induction of LTP.[42,59,60] Moreover, the kinases PKA, PKC, CaMKII and ERK are all necessary for induction of LTP.[61-65] Induction of various forms of LTD requires PKC, CaMKII and ERK.[66-69] Therefore, induction of synaptic plasticity employs the same complement of signaling pathways that activate NF-κB, suggesting that long-term forms of synaptic plasticity may involve NF-κB-mediated gene transcription.

Initial studies into the regulation of NF-κB by synaptic activity focused on the expression of p50 and p65. Application of high-frequency stimulation to the perforant pathway input into the dentate gyrus induced LTP and increased expression of genes encoding p50, p65 and IkBα in dentate granule neurons.[3] The increases in all of these genes were time-dependent, with maximal increases occurring 1 h after induction of LTP.[3] Interestingly, low-frequency stimulation of the perforant pathway did not induce any form of synaptic plasticity, yet the expression of p50, p65 and IkBα were all increased to levels seen in after induction of LTP, suggesting that activation of the NF-κB pathway is sensitive to synaptic activity in general.[3] In concert with these findings, recent studies in cultures of hippocampal neurons demonstrated that NF-κB DNA binding activity was decreased by treatments that inhibited synaptic activity.[5] Moreover, both NF-κB DNA binding activity and NF-κB-mediated transcription were increased by treatments that increased synaptic activity.[5] All of these results demonstrate that the NF-κB pathway is activated not only by induction of synaptic plasticity, but also by basal levels of activity.

The activation of the NF-κB signaling pathway may not be ***sufficient*** to trigger lasting changes in synaptic strength, however several lines of evidence indicate that proper NF-κB function is ***necessary*** for induction of synaptic plasticity. One technique used to assess the involvement of NF-κB in induction of synaptic plasticity utilized a response element decoy technique. Acute brain slices were exposed to DNA oligonucleotides containing the NF-κB responsive element for several hours prior to induction of LTP.[70,71] The decoy DNA acts to competitively inhibit binding of NF-κB transcription factors to the nuclear DNA, effectively blocking the ability of NF-κB to regulate gene expression. Exposure of slices to the NF-κB decoy DNA attenuated induction of LTP in the hippocampus, and blocked induction of late-phase LTP in the amygdala.[70,71] These results suggest that NF-κB-mediated gene expression is necessary for induction of transcription-dependent synaptic plasticity in the amygdala and hippocampus.[70,71]

A second class of experiments investigating the role of NF-κB in synaptic plasticity focused on the cytokine tumor necrosis factor (TNF). TNF is expressed in the brain, and its production appears to be regulated by synaptic stimulation.[72,73] TNF normally binds to either the p55 or p75 TNF-receptors (TNFR), which results in activation of the NF-κB signaling pathway.[74-78] Early studies of the possible effects of TNF on synaptic physiology revealed that very brief treatments of hippocampal slices with exogenous TNFα increased synaptic strength, and inhibiting the normal action endogenous TNFα with exogenous TNF receptor fragments decreased synaptic strength.[79] The effects of TNFα were attributed to regulation of glutamate receptor

expression.[79] Subsequent studies of the long-term effects of TNFα on synaptic physiology revealed that TNFα actually downregulated AMPA and NMDA receptor-mediated current, and increased voltage-gated Ca^{++} currents at the plasma membrane.[27] These results indicate that TNF can exert a variety of effects on synaptic physiology depending on the temporal kinetics of TNF signaling.

Treatment of neurons with TNF has profound effects on the ability to induce synaptic plasticity. Exposure of acute hippocampal slices to TNFα inhibits induction of LTP in area CA1 and the dentate gyrus.[80,81] Moreover, genetic knockout of both TNF receptor (TNFR) isoforms has no effect on induction of LTP, however, induction of LTD is severely impaired.[70] To fully understand the effects that TNF might have on synaptic plasticity, recall that induction of both LTP and LTD are dependent upon influx of Ca^{++} and the frequency of synaptic activity,[82] and exposure of neurons to TNF enhances their ability to maintain Ca^{++} homeostasis via NF-κB-dependent transcription.[27,83,84] Therefore, TNF may inhibit the induction of LTP by upregulating the expression of Ca^{++}-buffering enzymes while causing the loss of TNFRs and thus, may lead to erroneous induction of LTP by diminished expression of Ca^{++}-buffering enzymes. Alternatively, exposure of slices to TNF might result in regulation of glutamate receptor trafficking that prevents or occludes expression of LTP. All of these effects are presumably mediated by TNF-induced activation of NF-κB. Therefore, all of the studies above indicate that interference with the normal function of NF-κB inhibits proper induction of synaptic plasticity. Thus, NF-κB-mediated transcription appears to be necessary for proper induction or expression of synaptic plasticity.

Memory Formation

Synaptic plasticity is a candidate mechanism that may contribute to the formation of memory in vivo. As outlined above, several lines of evidence indicate that NF-κB plays a role in induction of various forms of synaptic plasticity. This suggests the intriguing possibility that NF-κB might be involved in the formation of memory in the behaving animal. Studies into the molecular mechanisms of memory formation have been greatly facilitated in the past decade with the advent of many genetic mouse model systems that mimic human disorders of memory formation.[85] Several experimental paradigms have been developed to probe several different kinds of memory. Researchers have just begun to develop mouse models suitable for the study of NF-κB in memory formation; therefore the number of experiments performed to date is relatively low. In the following sections we will review the behavioral paradigms used to investigate the role of NF-κB in memory formation, and the results.

Fear Motivated Learning: An Explanation

At its most basic level, learning involves exposure to an external stimuli that results in a lasting change in behavior. The study of learning and memory in animal model systems is difficult in that the animals cannot directly communicate whether they remember a certain event. Therefore, researchers have designed a number of simple behavioral tasks to test different forms of learning. A relatively popular paradigm currently in use is fear-motivated learning. Emotionally motivated learning paradigms evoke strong, easily quantifiable responses. Fear-motivated learning relies on the amygdala, an almond-shaped region of the brain that is part of the limbic system. Fear learning involves exposure of animals to a noxious stimulus, usually a mild electric shock to the paws, in conjunction with a previously innocuous stimulus such as a noise or light. When these 2 unrelated stimuli are presented together, the animal can then form an association between the noxious and the innocuous stimulus. Thus, an animal learns that an ***unconditioned stimulus*** (the footshock) that always evokes fear can be predicted by a ***conditioned stimulus*** (the light or noise) that never evoked a fearful response prior to training.

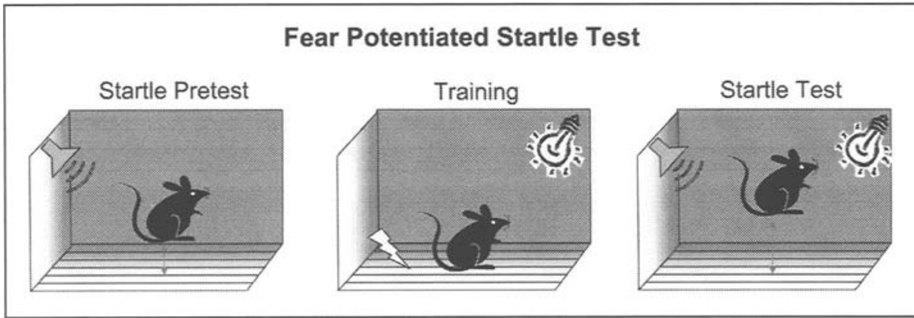

Figure 4. Fear potentiated startle. Fear potentiated startle is an associative learning paradigm that couples a noxious stimulus (electric shock) with an innocuous stimulus (light). A) Baseline startle responses induced by a brief, loud tone are measured. B) The subject is exposed to a series of training sessions that pair the presentation of a light cue with an electric shock. During the training period, the subject learns to associate the light with the shock. C) Startle responses are tested in the presence and absence of the light cue. Startle responses elicited in the presence of the light are usually 2-3 times greater than in the absence. This phenomenon is called fear potentiated startle.

Fear Potentiated Startle

Most animals exhibit defensive startle reflexes in response to sudden, unexpected environmental stimuli. These reflexes evolved to deal with the sudden presence of predators or other threats. Due to their nature, defensive reflexes are sensitive to the emotional state of an animal. For example, if an animal is frightened, then expression of defensive reflexes is enhanced. Researchers have utilized this phenomenon to measure fear-motivated learning in a learning paradigm known as fear potentiated startle (Fig. 4). In this learning paradigm, an animal's startle reflex is first assessed by presentation of a series of brief, but very loud audible tones (Fig. 4A). These unpredictable auditory stimuli cause the animal to flinch, which is measured with an accelerometer. Then, the animal is exposed to a series of training sessions that involve exposure to a light and an electric shock (Fig. 4B). During this training, the animal learns to associate the light with the electric shock. After training, the startle response is tested again, however in the presence and absence of the light cue (Fig. 4C). When the animal sees the light, the startle response is generally 2-3 times greater than pretraining values.

Some evidence exists for the involvement of NF-κB mediated gene transcription in fear potentiated startle memories. Fear potentiated startle training increases levels of NF-κB protein, acetylation of NF-κB and its DNA binding activity selectively in the amygdala.[22,71] These increases in DNA binding activity are accompanied by decreases in IκB and increases in the activity of IKK, indicating that the NF-κB signaling system has been activated.[71] Moreover, acquisition of fear potentiated startle memory is blocked by administration of NF-κB inhibitors or κB response element decoys directly to the amygdala.[71] Interestingly, some evidence exists that suggests fear potentiated startle memory can be enhanced by agents that increase acetylation of NF-κB.[22] Together, these data demonstrate that NF-κB is upregulated by fear potentiated startle, and that NF-κB function in the amygdala is necessary for the formation of fear potentiated startle memory.

Fear Conditioning

Fear conditioning is another fear-motivated learning paradigm currently in use. Fear conditioning also involves pairing a noxious unconditioned stimulus with a previously innocuous conditioned stimulus. When rodents become frightened they decrease their spontaneous activity, which is referred to as "freezing". Researchers quantify acquisition of fear conditioning by measuring freezing behavior (Fig. 5). There are two different forms of fear conditioning. Cued

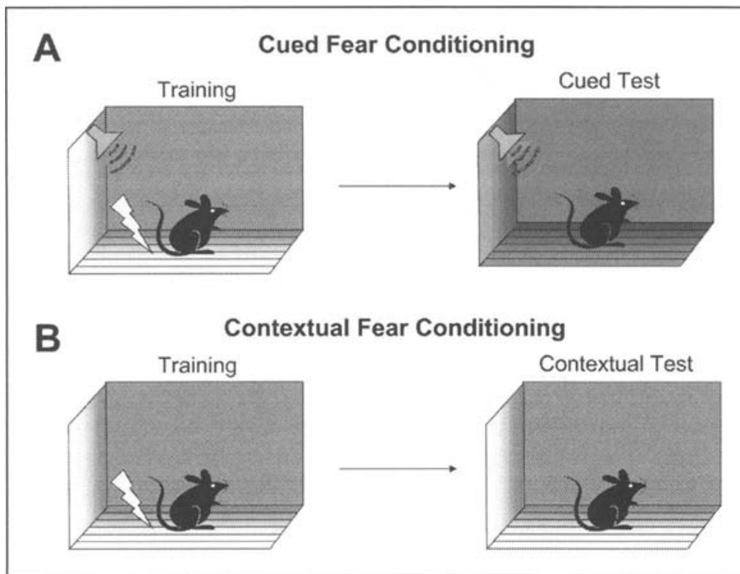

Figure 5. Associative fear conditioning. In the fear conditioning paradigm, subjects associate a previously innocuous stimulus with an aversive electric shock. A) In the cued fear conditioning paradigm, the subject is trained to associate a tone with an electric shock. Testing the subject in the presence of the tone elicits freezing behavior, indicating successful acquisition of the associative fear memory. B) In contextual fear conditioning, the animal is placed into a novel environment and receives a series of shocks. No other cues are provided. Therefore, the animal associates the aversive stimuli with the environment. Placement of the subject back into the environment where the shocks were administered elicits freezing behavior, indicating successful acquisition of the associative fear memory.

fear conditioning involves paired exposure of animals to a tone and electric shock (Fig. 5A). In the cued paradigm, the animals learn to associate the tone with the shock (Fig. 5A). Contextual fear conditioning involves placing the animal in a novel environment and administering a series of mild shocks (Fig. 5B). In the contextual paradigm, the animal learns to associate the environment with the shock (Fig. 5B). Formation of cued fear memories requires the amygdala.[86] Formation of contextual fear memory requires both the hippocampus and the amygdala.[86,87]

In a recent study the role of *c-rel* was ascertained in the formation of long-term associative fear memory.[88] Through a series of mRNA expression profiling and bioinformatics studies, it was discovered that c-Rel-mediated gene transcription is important for the formation of long-term contextual fear memory in the hippocampus.[88] To confirm the results, mice that lacked *c-rel* (*c-rel*$^{-/-}$) through genetic knockout[89] were tested in both cued and contextual fear conditioning paradigms. There were no differences between *c-rel*$^{-/-}$ and normal (*c-rel*$^{+/+}$) mice in the cued fear conditioning paradigm, indicating that *c-rel* was not necessary for the formation of long-term memory subserved exclusively by the amygdala. This is in agreement with other studies in the amygdala which have shown that c-Rel DNA binding activity is not upregulated in the amygdala after induction of LTP.[71] However, *c-rel*$^{-/-}$ animals exhibited significant deficits in contextual fear conditioning.[88] In addition to testing behavior, these experiments also provided some of the first characterization of nonmemory behaviors in any mouse model containing genetic alterations in the NF-κB system. *c-rel*$^{-/-}$ animals were less active overall than *c-rel*$^{+/+}$ animals.[88] However, *c-rel*$^{-/-}$ animals had similar levels of anxiety and nociception, indicating that genetic disruption of *c-rel* did not lead to gross abnormalities in sensory perception or emotional state.[88] Taken together, the above results indicate that *c-rel* is necessary for the formation of hippocampus-dependent long-term contextual fear memory.[88]

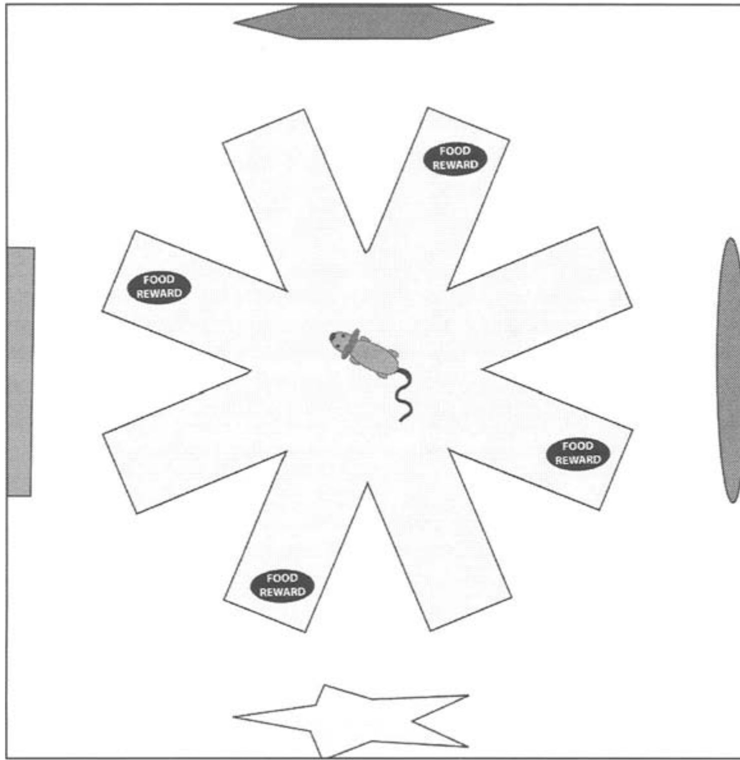

Figure 6. Radial arm maze. The radial arm maze tests the accuracy of an animal to navigate in a simple maze. Food rewards are placed in half of the arms. Animals begin in the center of the maze. Accuracy is scored based on either how many times an animal reenters arms of the maze, or how many errors the animal makes in the process of finding all of the rewards. Prominent spatial cues serve as visual landmarks, allowing the animal to form a mental map of the maze.

Radial Arm Maze

The radial arm maze test is a learning paradigm that can be used to investigate spatial and working memory. Remembering where your favorite restaurant is located is an example of spatial memory. Remembering at any given time where you have left your fork while you are eating there is an example of a working memory. The radial arm maze is used to model working memory and consists of at least 8 symmetric arms that protrude from a central platform (Fig. 6). Food is used to motivate the animals to learn the layout of the maze, so feeding is restricted in animals 1 week prior to the start of training and testing. In the spatial version of the radial arm maze, several distinct visual cues are placed such that the animal can use them as landmarks to navigate to specific areas of the maze (Fig. 6). In the cued version, the visual landmarks are removed and baited arms are indicated by either lights or flags (Fig. 6). The spatial version of the radial arm maze requires the hippocampus, while the cued version requires dorsal striatum.[90]

Trials are typically performed for over 2 weeks. Two different kinds of trials are performed each day; in one trial, several arms of the maze are blocked off. The arms that are not blocked are baited with food pellets. The animal is placed into the center of the maze and allowed 5 min to explore. For the second trial, all arms of the maze are open, and food pellets are placed only into the arms that were previously blocked. Animals are scored based on how many "mistakes"

they make as assessed by how many times animals enter an arm that has been previously visited. The number of correct arm entries out of the first few entries can also be scored as an index of whether the animal has successfully learned the task.

The radial arm maze task was recently utilized to assess the role of p65 in hippocampus and striatum-dependent memory formation.[5] Mice that lacked both p65 and TNFR1 ($p65^{-/-}$) through genetic knockout were tested in the spatial and cued versions of the radial arm maze. Initially, $p65^{-/-}$ mice performed worse than $p65^{+/+}$ in the spatial version of the radial arm maze.[5] Over time however, the $p65^{-/-}$ mice were able to learn the task and perform equally as well as $p65^{+/+}$ mice. In the cued version of the task, $p65^{-/-}$ mice performed equally as well as $p65^{+/+}$ mice. These results suggest that p65 is involved in facilitating the formation of hippocampus-dependent memory, but not striatal memory.[5] It should be noted that in these studies, all of the mice tested lacked TNFR1.[5] Recall that disruption of TNFRs leads to derangement of synaptic plasticity in in vitro preparations.[70] Therefore, the memory impairments observed in $p65^{-/-}$ mice may not be entirely due to loss of p65 function. Nevertheless, these experiments provide additional evidence supporting a role for the NF-κB signaling pathway in the formation of hippocampus-dependent memory.

Summary

The neuronal NF-κB signaling system represents a novel and potentially vital component involved in linking activity at the synapse to activity within the nucleus. NF-κB was the first transcription factor to be localized to the synapse. Since its initial characterization in the nervous system, NF-κB has been shown to be regulated by a variety of stimuli, suggesting that it may play a role in integration of numerous different types of information within the nervous system. Ample evidence exists demonstrating that NF-κB factors are engaged in and are necessary for formation of synaptic plasticity and long-term memory. Even though we are only beginning to understand the contribution of distinct NF-κB family members to the regulation of gene transcription in the brain, all of the evidence collected thus far indicates that NF-κB may represent a vital part of the molecular machinery involved in mammalian cognition.

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CHAPTER 12

Inhibitors of NF-κB Activity: Tools for Treatment of Human Ailments

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Summary

Apart from being a paradigm for understanding cellular signaling, the NF-κB pathway has been thoroughly investigated over the last two decades due to its involvement in a number of human diseases. In the post genomic era, improved knowledge and novel technologies have contributed immensely to the discovery of several hitherto unknown cellular processes that regulate NF-κB. Identification of covalent modifications of many NF-κB pathway components, both in the cytoplasm and the nucleus has shed light on novel mechanisms that regulate NF-κB activity. Similarly, study of a number of cellular and viral proteins that regulate this pathway has added to our understanding of the molecular mechanisms and molecular targets in the NF-κB pathway for drug development.

Introduction

NF-κB signaling plays a pivotal role in several cellular and developmental processes in metazoans.[1] Deregulation of this pathway is suspected in and also causally linked to the initiation and progression of many human pathologies.[2] Since its discovery in the B cells and the relatively greater understanding of its role in the cells of the immune system, uncontrolled NF-κB activity has most often been associated with inflammatory disorders. In recent years, hyperactivity of NF-κB has also been linked to insulin resistance,[3] cachexia,[4] Alzheimer's disease,[5] transplant tolerance/graft rejection,[6] organ ischemia/reperfusion injury,[7] incontinentia pigmenti[8-10] and several neoplastic malignancies.[11,12] These observations warrant the development of strategies for modulation and inhibition of NF-κB activity. A wealth of information regarding the efforts in this direction has been a topic of exhaustive reviews in the literature. The aim of this chapter is to update the progress made in the very recent years and to direct the reader towards much of the established facts for more detailed perusal.

NF-κB is a family of transcription factors and the term NF-κB usually refers to a dimer of two similar or heterologous subunits of the family. In resting cells, these proteins are found associated with IκB family of inhibitory proteins and are predominantly localized in the cytoplasm.[13] The common denominator for NF-κB activation is the removal of IκB proteins from the DNA binding subunits of NF-κB.[14] Except in response to select stimuli, this removal is mediated by proteasomal degradation and requires that the IκB molecules are phosphorylated at specific serine residues by the IκB kinases (IKK).[15] Ample biochemical and genetic evidence exists that βTrCP1 and βTrCP2 (vertebrate homologues of *Drosophila* Slimb; HOS)[16] form the receptor component

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of the SCF$^{\beta TrCP/HOS}$ E3 ubiquitin ligase along with Skp1, cullin1 and Roc1/Rbx1 components[17] and target IκB[18-21] and p100[22] molecules for ubiquitin-dependent proteolysis only when their conserved DSGXXS motif, is phosphorylated in a stimulus dependent manner.

Although, the rate-limiting step in the activation of NF-κB is the stimulus dependent degradation of IκB proteins, it is now apparent that mere release from IκB molecules is not enough for activation of NF-κB. Several modifications such as those of upstream molecules that activate the IKKs and NF-κB itself have been reported to be important for functional NF-κB activity.[23] Sub-cellularly, the targets of covalent modifications that regulate NF-κB activity range from adaptors such as TRAFs on the cell membrane, the IKKs in the cytoplasm to the p65 and p50 subunit in the nucleus.[23] Molecularly, the known post translational modifications include s-nitrosylation, addition/removal of phosphate, acetyl, ubiquitin, and sumo moieties and might include cis/trans isomerization by enzymes such as pin1.[24] While some of these modifications are crucial for NF-κB activity, others might play a subtler role in defining the duration and extent of NF-κB activity. The fact that NF-κB is activated by over 200 stimuli and it in turns activates an equally large subset of target genes in different cell types poses a major challenge in designing pathway specific inhibitors of NF-κB.[25] Knowledge of all relevant modifications of NF-κB pathway members is imperative to comprehend how specificity is generated in such a promiscuous pathway and this would be critical in designing inhibitors that do not have pleiotropic effects.

Inhibition of NF-κB Can Be Achieved at Multiple Points in the Pathway

Inhibition of NF-κB in various cellular compartments can be achieved by (a) inhibiting the receptors and the adaptors on the membrane, (b) the upstream kinases and IKKs, in the cytoplasm and (c) NF-κB DNA binding and transactivation in the nucleus. In this review, we describe the chemical and biological inhibitors of NF-κB known in the literature and then will classify them based on the specific molecular reaction they are known to inhibit. The biological inhibitors will be further classified based on their origin as cellular, viral, bacterial or natural products. Based on our knowledge of how NF-κB is activated in response to various stimuli, all cellular proteins essential for activation are targets for drug development. A common method to inhibit any signaling pathway is to make dominant negative versions of activators such that they now act as inhibitors. We will also discuss such biological molecules that do not exist in nature but which have been derived from proteins known to modulate the pathway. The chemical inhibitors will be classified as synthetic and natural. Given the relatively greater nonspecificity of chemicals inhibitors, we will describe the mode of action of only a few well-characterized class of compounds.

Biological Inhibitors

Cellular Inhibitors

IκB and Related Cellular Proteins Involved in Limiting NF-κB Activity

The most potent mechanism that inactivates NF-κB function is its association with IκB proteins. This process primarily prevents NF-κB DNA binding and also sequesters the complex in the cytoplasm.[1] Members of the IκB family have been proposed to play different roles in activation of NF-κB in response to different stimulation.[23] The knock-in of IκBβ in IκBα locus has clearly demonstrated that in the absence of IκBα, its biochemical function can be substituted by IκBβ and that the functional differences between these proteins might just be due to their differential expression patterns and/or association with other proteins.[26] Several viral proteins like HBX[27] and cellular proteins like G3BP2,[28] can associate with and regulate the subcellular localization and degradation of IκB proteins. It is conceivable that IκBα, IκBβ and IκBε proteins interact with different sets of cytoplasmic proteins and that this interaction,

in a cell type specific manner regulates the kinetics and the degree of their degradation. The κB-ras proteins are a case in point, since they specifically regulate the activation of IκBβ bound NF-κB complexes.[29] Recently β-arrestin2 has been shown to regulate the extent of IκBα phosphorylation and degradation.[30]

Although much of the attention in the field has focused on mechanisms that activate NF-κB, the events that regulate the degree of NF-κB activity and lead to efficient termination of the activity are only now beginning to be unraveled. At the level of IKK activation, inhibitory auto-phosphorylation of IKKs[31] and deubiquitination of adaptor molecules by enzymes such as CYLD[32,33] have been documented to prevent prolonged NF-κB activity. An abundant cellular protein, hnRNPU has been shown to regulate the $SCF^{\beta TrCp/HOS}$ complex and thereby impinge on NF-κB activation by limiting IκB degradation in the nucleus.[34] These cellular signaling molecules and the partners of IκB:NF-κB complex could be efficiently used to limit NF-κB activity.

Regulation of NF-κB by Transformation/Cell Cycle Related Proteins

Aberrant regulation of NF-κB is observed in several human diseases. Although the role of NF-κB in regulating molecules involved in inflammation might be the primary unifying mechanism underlying most of the diseases, other activities of NF-κB that aid in the development of diseases have now been uncovered. One prominent activity attributed to NF-κB that might contribute towards its role in some human disorders is its ability to protect cells from apoptosis[1] in response to a diverse set of physiological stimuli. Indeed the relevance of NF-κB in mitigating apoptosis is now becoming evident in the evolution of several human malignancies.[2] These malignancies include those of haematological origin, helicobacter pylori-associated carcinogenesis, and cancer of the breast, colon, liver and cervix.[12,35] Apart from the NF-κB mediated cytokines and adhesion molecules that play an important role in tumorigenesis,[36] NF-κB has also been described to interact with molecules involved in growth, differentiation and transformation. Inhibitors of NF-κB activity have been documented to augment existing chemotherapy regimens.[37,38] NF-κB mediated expression of Cyclin D1 has been shown to be important for growth and proliferation[39,40] and mammary gland development[41] and the p65 subunit of NF-κB is known to interact with cyclin E-CDK2 complex.[42] IKK2 mediated NF-κB activation can destabilize the levels of p53 in fibroblasts[43] and intestinal epithelial cells.[44] Similarly, NF-κB has been documented to play an important role in subverting p53 and p73 induced cell death in lymphocytes[45] and in MALT.[46] As evidence mounts that NF-κB mediated regulation of p53 is important in regulating apoptosis resistance and transformation, repression of NF-κB by tumor suppressor genes including p53 is also emerging as a potential mechanism of overcoming apoptosis resistance.

At least four tumors suppressors have now been documented to inhibit NF-κB function. The tumor suppressor ARF can inhibit NF-κB function by recruitment of the histone deacetylase, HDAC1 to the transcriptional activation domain of p65.[47] The candidate tumor suppressor gene ING4 involved in regulating brain tumor growth and angiogenesis, has also been demonstrated to physically interact with p65.[48] Infact, repression of p65 transactivation by ING is postulated as a potential mechanism of tumor suppressor function of ING.[48] Similarly, mutations of CYLD in familial cylindromatosis,[49] and consequent loss of its negative regulatory effects on NF-κB signaling might be linked to progression of this malignancy. Finally, transcriptional cross talk between p53 and NF-κB[50] has been documented and it is evident that p53 represses NF-κB activity,[51,52] probably as an autoregulatory mechanism. Similarly, the oncogenic protein *twist*, a known target gene of NF-κB, has now been documented to autoregulate its expression by negatively regulating NF-κB activity through repressing p65 function.[53]

Cellular Proteins Involved in Inhibiting Inflammation

Failure to downregulate NF-κB transcriptional activity leads to chronic inflammation and cell death. Hence, the factors that are responsible for preventing sustained NF-κB activity in

inflammatory diseases are important to understand. Mice deficient for the cellular zinc finger protein A20 display cachexia due to severe inflammation and die prematurely because they fail to terminate NF-κB activity.[54] Recently, a unique mechanism of downregulating NF-κB by A20 has been highlighted.[55] A20 mediated downregulation of NF-κB involves two sequential processes including deubiquitination and then polyubiquitination and proteasomal degradation of RIP, an essential component of TNF receptor 1 signaling complex.[55]

Repression of NF-κB by transforming growth factor-β1 (TGF-β1) is believed to be one of the mechanisms that is responsible for termination of production of pro-inflammatory cytokines in the gut.[56] Macrophages pretreated with the anti-inflammatory cytokine IL10 show reduced expression of TNFα but not IL-6 in response to LPS, due to reduced binding of p50:p65 complexes on the TNFα promoter.[57] Deciphering the mechanism of selective repression of certain NF-κB promoters would be crucial in designing strategies tailored towards inhibiting specific subsets of genes.

Mutations in cold-induced autoinflammatory syndrome 1 (CIAS1) genes are associated with chronic inflammatory disorders.[58] Multiple isoforms of CIAS1 protein inhibit p65 nuclear translocation,[58] suggesting a plausible mechanism for sustained inflammatory activity in patients with these mutations. Heparin-binding growth factor-like growth factor (HB-EGF), a member of the EGF family, is known to significantly decrease cytokine-induced NO production by preventing IκB degradation and thus NF-κB activation.[59] The anti-inflammatory effects of nitric oxide (NO), a free radical, involve repression of IKK2 kinase activity by S-nitrosylation of cysteine 179[60] and cysteine 62 of p50.[61] Cysteine residues of IKKs have been targets of many NF-κB inhibitors including arsenite,[62] H_2O_2,[63] 4-hydroxy-2-nonenal[64] and cyclopentenone prostaglandins.[65]

Cellular Proteins Involved in Inhibiting Viral Replication

Two cellular factors have recently been identified to regulate HIV1 replication by inhibiting NF-κB. While RelA-associated inhibitor (RAI),[66] identified by a two hybrid screen, is localized in the nucleus and prevents p65 DNA binding upon overexpression, Murr1, a gene previously known to be involved in copper metabolism was found to be essential to inhibit NF-κB activation and HIV replication in CD4+T cells.[67]

Miscellaneous Cellular Proteins Known to Repress NF-κB

Notch-1 proteins and their receptors are important for several developmental decisions including cell fate specifications.[68] The N terminal portion of human notch-1 ($Notch^{IC}$) was shown to inhibit NF-κB activity in the nucleus. Similarly, Fas-associated factor 1 (FAF1) has been documented to inhibit nuclear accumulation of NF-κB.[69] The inhibitor of Cdk4 (INK4), which contains ankyrin repeats, like IκB proteins has been reported to bind and modulate the activity of p65 in certain cell types.[70] IFNα and not IFNγ has been shown to inhibit HBx mediated NF-κB activation.[71] Expression of c-Myc has been shown to repress NF-κB activity by blocking the transactivation potential of p65.[72] Overexpression of manganese superoxide dismutase (MnSOD) can inhibit IκB degradation and NF-κB activation.[73] NRF, nuclear protein was found to inhibit NF-κB by steric hindrance, specifically on the IFNβ promoter.[74] Also, androgen receptor signaling represses NF-κB activity in the androgen sensitive LNCaP cells.[75] Understanding the molecular mechanisms underlying these repressions needs further experimentation.

Variants of Cellular Proteins That Inhibit NF-κB

Dominant Negative Molecules

One potential avenue of inhibiting NF-κB signaling is to make decoy receptors of ligands that activate this pathway. Indeed such approaches have been attempted and several TNF variants that could inhibit the endogenous TNF signaling identified by a structure-based rationally designed screen.[76] Similar efforts with other NF-κB pathway components could

yield more specific and potent inhibitors to specific stimuli. The most common and tested method of inhibiting NF-κB is to exogenously deliver IκB molecules that are not degraded following stimulation. The IκBαM[77] molecule is a prototype IκBα used in many studies, wherein the serine 32 and 36 which are sites of IKK phosphorylation are mutated to alanines. In addition, all the serines and threonines in the C terminal PEST domain of IκBα are mutated to alanines in IκBαM.[77] The advantage of using such molecules is that they bind and inhibit almost all NF-κB homo and heterodimers and thus achieve almost complete blockade of the pathway. Although all IκBαM overexpression results are interpreted to be because of inhibition of NF-κB activity, a cautionary note should be added since IκBαM has also been shown to bind and inhibit the function of cyclin dependent kinase-4.[78] In another variation of a similar strategy the lysine residues 21/22 (which are the site of ubiquitination) of IκBα are converted to alanines thus blocking its degradation.[79] Phosphorylation of tyrosine 42 of IκBα is another mechanism known to activate NF-κB which operates via removal of IκB from NF-κB.[80] The nonphosphorylatable IκBαY42F protein has also been used to block NF-κB activity and to prevent blockage of bone erosion associated with inflammatory arthritis.[81]

Cell Permeable Peptides

Recent advances in designing cell permeable peptides has led to the development of small peptide molecules that could interfere with protein-protein interactions essential for NF-κB signaling. These peptides can work at various subcellular locations. A peptide from interacting domain of TIRAP has been known to block its association to TLR4 and inhibit LPS-induced NF-κB activation.[82] Since NEMO or IKKγ is essential for IKK and NF-κB activation, another strategy used to inhibit NF-κB is to make a peptide derived from the extreme carboxy terminus of IKK2 and IKK1 that binds and squelches free NEMO in the cell.[83] In vivo studies with this peptide (NBD), are indeed showing promising results.[83,84] NBD peptide administered into mice prior to induction of inflammatory arthritis efficiently blocks in vivo osteoclast recruitment, inhibits focal bone erosion, and ameliorates inflammatory responses in the joints of arthritic mice.[85] Ben-Neriah and colleagues have used cell permeable phosphopeptides derived from IκBα as a means of competitively inhibiting the $SCF^{\beta TrCP}$ ligase.[86] Interestingly a range of cellular and viral proteins have recently been documented to inhibit the NF-κB pathway by competing for the $SCF^{\beta TrCP}$. It has been documented that a mutant of βTrCP1 that has been deleted of the F box (ΔF-βTrCP1) can function as a dominant negative in the process of IκBα degradation, since it can bind the substrate but cannot recruit it for efficient ubiquitination.[16] This method is also able to block processing of other IκB members like p100 and also prevent degradation of small amounts of phospho-IκBα generated by weak NF-κB activating stimuli such as DNA damage.[87] A twelve-residue-peptide from p65 covering serine residues 276, whose phosphorylation is required for NF-κB activation is fused to membrane permeable domain PTD was shown to selectively inhibit NF-κB activation induced by various inflammatory stimuli.[88] A forty-one-residue-peptide consisting the nuclear localization sequences of p50 can also effectively inhibit NF-κB.[89,90] However, since such an approach nonspecifically clogs the nuclear import machinery, it offers a very nonspecific method of NF-κB inhibition.[89,90] Theoretically peptides or phosphopeptides derived from all essential components of the IKK complex, such as ELKS[91] can also be used to make dominant negative inhibitors of IKK and NF-κB.

Bacterial Inhibitors

Many bacterial strains of the *Yersinia* species, known to colonize both plant and animal hosts, encode a set of effector proteins called the Yops (Yersinia outer proteins).[92] YopJ, a protein encoded by both plant and animal pathogens has been documented to block the NF-κB pathway.[93] It is now known that YopJ has a ubiquitin like protease (ULP) activity and may target several cellular proteins with ubiquitin domains,[93] thus explaining its inhibitory effect on many signaling pathways.[92] With the documented role of ubiquitination in activation of IKK[94,95] and thus NF-κB pathways, it is most likely that the YopJ mediated inhibition of

NF-κB activity operates by inhibition of IKK activation by upstream MAP kinase signaling. Peptides derived from YopJ might be useful tools to block IKK activity but this hasn't been tested yet. The periodontal pathogen *Porphyromonas gingivalis* has been documented to inhibit p65 DNA binding as a mode of inducing tolerance.[96]

Viral Inhibitors

Viruses have been known to evade the host immune system by a variety of mechanisms.[97] Since NF-κB is required for activation of many cytokines that are crucial to mount an effective immune response, inhibition of NF-κB activity seems a worthwhile strategy for viruses to utilize to escape immune surveillance.[98] Pichinde virus is an arenavirus which displays tropism towards macrophages and causes Lassa fever in humans.[99] Virulent, but not attenuated strains of this virus have been documented to cause repression of NF-κB activation which leads to decreased macrophage activation, possibly as a means to evade the host immune system.[99] In a similar vein, the African swine fever virus (ASFV), which replicates in macrophages, encodes a truncated version of IκB protein, which lacks the N terminal signal responsive regions but binds and inhibits NF-κB activity in response to all forms of activation.

The use of adenoviruses, which are large double strand DNA viruses in gene delivery is largely impeded by their immunogenicity. However these viruses are postulated to have evolved mechanisms to evade the immune system. Two adenoviral protein complexes, the E1A[100] and the E3-10.4/14.5[101] have been shown to inhibit IKK and thus NF-κB activation, possibly as a means to limit the action of interferon mediated immune action. Human cytomegalovirus (HCMV), a significant factor in infections of immune compromised patients, encodes the pp65 protein which inhibits NF-κB DNA binding.[102] Similarly, the NS1 protein of influenza A virus inhibits NF-κB activation in response to the double strand RNA generated during the course of infection as a means to inhibit IFN production.[103] The Epstein-Barr virus (EBV) ZEBRA protein can also inhibit NF-κB activation in T cells during acute EBV infections.[104] Vaccinia virus K1L gene product blocks IκB degradation as a mechanism of inhibiting NF-κB activity.[105]

Like the cellular protein hnRNPU,[34] two viral proteins, have also recently been documented to interact with $SCF^{\beta TrCp/HOS}$ complex and control the extent of NF-κB activation. The HIV-1 membrane protein, Vpu has been proposed to interact with βTrCP1,[106] while the latent membrane protein 1 (LMP1) encoded by EBV have been documented to interact with βTrCP2.[107] Since βTrCP1, unlike βTrCP2 is nuclear,[34,108] Vpu has been proposed to sequester βTrCP1 in the cytoplasm and thereby inhibit its function during viral infection.[106] Inhibition of NF-κB by Vpu and LMP1 could certainly represent a mechanism for HIV1 and EBV to counterbalance innate immunity pathways in response to viral infections.

Natural Inhibitors

Dietary and natural products have long been used to reduce the risk for inflammatory disorders and cancers as complement regimens.[109] Molecular understanding of these natural NF-κB modulators may help us to design inhibitory small molecules compounds. Along with the rapid advances in our understanding of NF-κB signaling, large number of natural compounds are now being tested and reassessed for their actions in modulating NF-κB function. Phenolic compounds are one such group which contains a number of NF-κB inhibitory compounds such as EGCG, wogonin, oroxylin A, resveratrol, hepericin, curcumin, and silybin. The tea polyphenol EGCG is a potent inhibitor of TNFα-mediated NF-κB activity and it possibly functions by inhibiting IKK activity.[110] Curcumin found in the spice turmeric exhibits anti-inflammatory, anti-oxidant, and chemo preventive activities. It is suggested that curcumin most likely inhibits cell proliferation, cell-mediated cytotoxicity, and cytokine production through suppression of IKK-mediated NF-κB activation.[111] Some of other famous natural compound groups include isoprenoid compounds (such as kaurene diterpenes, kaurane diterpenes and cyclic diterpene) and class of sesquiterpene lactones (such as parthenolide and helenalin), triterpenoids (such as avicin, pristimerin, and oleandrin).[109] These compounds inhibit different steps in the NF-κB

activation cascade. Compounds like kaurene diterpeniods, parthenolide, oleandrin directly inhibit the IKK complex, while others such as avicin and helenalin suppress p65 function.[109]

Finally, cellular bioproducts of inflammatory reactions such as cyclopentenone prostaglandins inhibit IKK2[65] and NF-κB[112] by covalent modification as a mechanism of limiting prolonged NF-κB activity and this is important for the resolution of inflammation.

Synthetic Inhibitors

A number of conventionally used-inflammatory drugs and natural products have demonstrated NF-κB inhibitory activity by targeting various network components including IKK complex activity, IκB degradation, NF-κB nuclear localization, and NF-κB DNA binding and transcriptional capacity.[113] These agents include proteasome inhibitors glucocorticoids, nonsteroidal anti-inflammatory drugs, anti-inflammatory cytokines, and natural compounds. The finding of inhibition of NF-κB by multiple anti-inflammatory and anti-cancer pharmacologic agents reassures us of the involvement of NF-κB in pathogenesis of these diseases and its potential as drug target. Hence, development of pharmacologic inhibitors specific for NF-κB might provide safer and more efficacious drugs for the treatment of diseases ranging from inflammation to cancer.

Proteasome Inhibitors

Given the importance of the ubiquitination system in NF-κB activation, this system is a hot target for development of new anti-inflammatory therapies.[114,115] Proteasome inhibition is an effective way to block NF-κB activity generated by the canonical, noncanonical[114] and DNA damage pathways.[43] The proteasomal inhibitor Bortezomib (PS-341), is a potent inhibitor of NF-κB[116] activity, however like other proteasomal inhibitors, it is very toxic over a long period of time and each treatment lasts for a few days only.[117] Recently, PS-341 was approved by US food and Drug administration for the treatment of relapsed and refractory multiple myeloma.[118,119] It has demonstrated impressive antitumor activity in combination with other drugs. Although, evaluation of the mechanisms that underlie the antitumor effects of proteasome inhibitors reveals a considerable contribution of NF-κB blockade, it is evident that modulation of other cell cycle proteins and other pro- and antiapoptotic pathways also plays critical roles. In part, the nonspecific effects of proteasomal inhibition also stem from the fact the proteasome controls the physiological activity of several key cellular proteins, like p53[120] and β-catenin[18] to name a few.

Glucocorticoids

Glucocorticoids (GCs) such as synthetic steroid dexamethasome and hydrocortisone have been widely prescribed as anti-inflammatory and immunosuppressive drugs.[121] They inhibit expression of many genes including cytokines, chemokines, cell-surface receptors, adhesion molecules, tissue factor, degradative proteinases, and enzymes such as cyclooxygenase 2 (COX-2) and induced nitric oxide synthase (iNOS), which produce mediators of inflammation. Two mechanisms are proposed for the anti-inflammatory action of glucocorticoids. The first depends on its receptor binding activity directly to target transcription. And the second is dependent on its direct interaction and interference with other inflammatory transcription factors. Interfering with NF-κB signaling is thought as a major underlying mechanism for the anti-inflammatory capacity of GCs. Although the exact mechanism of GC mediated repression of NF-κB is not completely understood, several mechanisms have been proposed. GCs have been shown to increase the expression of IκBα, which retains NF-κB in cytoplasm and block its transcription activity.[122,123] GC-binding receptor also directly interacts with p65 in nucleus and inhibits activation of pro-inflammatory genes by blocking its transcriptional activity through modulating functions of RNA polymerase II and recruiting histone deacetylase 2.[124-126]

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are believed to operate by inhibiting cyclooxygenase activity to prevent pro-inflammatory prostaglandin (e.g., PGE2) synthesis. Interestingly, COX-2 is a NF-κB regulated gene and the inhibition of NF-κB further suppress the COX-2 expression. However interfering with NF-κB system is believed to contribute considerably to the anti-inflammatory effects of NSAIDs.[113] Several NSAIDs including aspirin and sodium salicylate, Ibuprofen, acetaminophen, sulindac, and tepoxalin are capable of inhibiting NF-κB activity. At a suprapharmacological dosage (mM range), both sodium salicylate and its semi-synthetic derivative, aspirin, bind and block the ATP binding site of IKK2.[127] Likewise, Sulindac and its metabolites, have been shown to inhibit the NF-κB-mediated signals through inhibition of IKK2 by direct interaction.[128,129] Celecoxib, a selective COX-2 inhibitor,[130] has recently been approved for the treatment of colon carcinogenesis, rheumatoid arthritis, and other inflammatory diseases and it can suppress NF-κB activation induced by various agents through inhibition of IKK and Akt.[35]

A recent estimate by Coussens and Werb[131] suggests that upwards of 15% of all cancers in humans are attributable to inflammation. The molecular mechanisms that sensitize sites of chronic inflammation to be malignant are virtually unknown. NF-κB is a key determinant of inflammatory responses and is known to be hyperactivated in many tumors. Use of nonsteroidal anti-inflammatory agents such as asprin and others that also block IKK2, reduce the risk of gastric, colon and lung cancers.[131] Taken together, these observations present a case that activation NF-κB could be the missing link between inflammation and cancer and that apart from their relevance in treating inflammatory disorders, NF-κB inhibitors could be useful means of preventing human malignancies.

Antioxidants

A number of NF-κB activating stimuli such LPS, H_2O_2, TNF, and IL-1 UV light and ionizing radiation increase cellular levels of reactive oxygen species ROS. Although the mechanistic details are still not completely clear, considerable evidence implicates ROS as common second messages in NF-κB activation. Introduction of various antioxidants such as ascorbic acid (vitamin C), vitamin E, NADPH, glutathione peroxidase, or MnSOD, along with these stimuli abolished NF-κB activation induced by these agents.[132] Interestingly, a direct linkage between the redox state of vitamin C and NF-κB signaling has been identified recently. It was shown that ascorbic acid quenches ROS intermediate involved in the activation of NF-κB and is oxidized to dehydroascorbic acid, which directly inhibit IKK2 and IKK1 kinase activity.[133] The list of anti-oxidant and inhibitors that prevent phosphorylation and degradation of IκBα in response to general and specific stimuli has been complied in a pervious review.[134]

IKK Inhibitors

Although a range of natural compounds and synthetic drugs are able to inhibit NF-κB activation pathway, they are not selective inhibitors of NF-κB activation pathway. Since undesirable side effects are often associated with such therapeutics, designing IKK selective inhibitors is a promising method to generate more NF-κB specific and safer therapeutics for the treatment of inflammatory diseases and cancer. Many drug companies are currently focusing on generating small molecules to specifically block IKK1 or IKK2 activities.

Several small molecule compounds have been identified which are highly selective, and orally bioavailable IKK2 inhibitors such as quinazoline analogues (SPC839), β-carboline derivatives (PS-1145), imidazoquinoxaline derivative (BMS-345541), amino-thiophenecar derivative (SC-514), ureido-thiophenecarboxamide derivative, diarylpyridine derivative.[135] The development of IKK1 specific inhibitor is also gaining momentum since IKK1 has a key role in the development of tooth,[136] B cells,[137] osteoclasts and breast epithelium.[41] Karin et al[135] and Bruke[138] summarize the efforts that are underway towards developing IKK inhibitors in recent reviews. Most of those inhibitors are still in preclinical stages of development and their safety and efficacy in the treatment of inflammatory disorder still remains to be determined.

Transcription Factor Decoy

Another approach towards inhibiting NF-κB signaling is to introduce double-stranded oligodeoxynucleotides (ODNs) into cells as decoy *cis*-elements that bind NF-κB and thus alter target gene expression. Decoy oligodeoxynucleotides to NF-κB have been designed and used in several studies[139-141] but they suffer from a number of limitations, including their solubility across membranes, their sensitivity to polymerases, lack of sequence specificity, and their tendency to activate cytokines production.[142] To overcome such problems, circular dumbbell decoys have also been designed which exhibit high resistance to nucleases, are easily taken up by cells, and have a nontoxic unmodified backbone that resembles natural DNA.[142]

Antisense Oligodeoxynucleotide

Antisense ODNs are short synthetic nucleotide sequences formulated to be complementary to a specific RNA message. Through binding of these ODNs to a target mRNA sequence, translation of the desired target genes can be selectively blocked, and the disease process generated by those genes can be halted.[143]

SiRNA As Inhibitors

Several recent reports have demonstrated the use of short hairpin RNAs (shRNAs) (for example against p65[144] and the upstream TAK1[145]) as an efficient means of inhibiting NF-κB pathway. Since mammalian cells are not known to make shRNA and although shRNAs could be delivered by a virus mediated route,[146] (or by direct transfection[147]) we have categorized them as synthetic inhibitors. However given that shRNAs have been reported to have several off-target effects,[148] the use of shRNA mediated inhibition should only be contingent upon rigorous evaluation of nonspecific effects. Also the use of regulated shRNAs[144] could be one way of limiting the off target effects of shRNAs.

Indirect Inhibitors

Several kinases including MEKK1, MEKK3, NIK, AKT, CKII, RSK, TAK1/T2K/NAK, GSK3β, MSK1 and MSK2 have been shown to be required for NF-κB activity.[1,149] Inhibitors that principally target these enzymes are also likely inhibitors of NF-κB, albeit with other side effects. Indeed wortmannin and other PI3K kinase inhibitors are potent inhibitors of NF-κB activity.[150] The involvement of AKT pathway in cancers has led to the development of several inhibitors of this pathway,[151] which could be used to inhibit NF-κB under certain situations for example in tumors with over active AKT. Similarly inhibitors of GSK3β have been shown to inhibit NF-κB activity.[152] Also, mitogen-activated protein kinase inhibitors as well as a mitogen and stress activated protein kinase (such as MSK1) inhibitors have been effective in blocking NF-κB activity in vitro.[153] Transforming growth factor beta 1 (TGFβ1) can stabilize the protein levels of IκBα by inhibiting CKII activity in hepatocytes.[154] Targeting of Hsp90, a component of the IKK complex,[1] by geldanamycin, a benzoquinone ansamycin isolated many years ago from Streptomyces, is known to inhibit NF-κB activity. However, these compounds also inhibit several other kinases due to the destabilizing affects they have on the general chaperone hsp90.[155]

Future Directions

The rapid advance in our understanding NF-κB signaling pathway has led us to a number of nodes where NF-κB activity could be regulated. Figure 1 summarizes the major inhibitors and the nodes of the pathway that they have been documented to operate upon.

Research on basic mechanisms of current pharmacological agents may provide potential for developing more specific and potent drugs with less toxicological side effects. Since blocking IκB degradation and its control by IKK complex are seminal steps in NF-κB activation, targeting this node for NF-κB specific blockade without a global inhibition is worth exploring further. This is particularly challenging since IKK1 and IKK2 that are highly similar have now

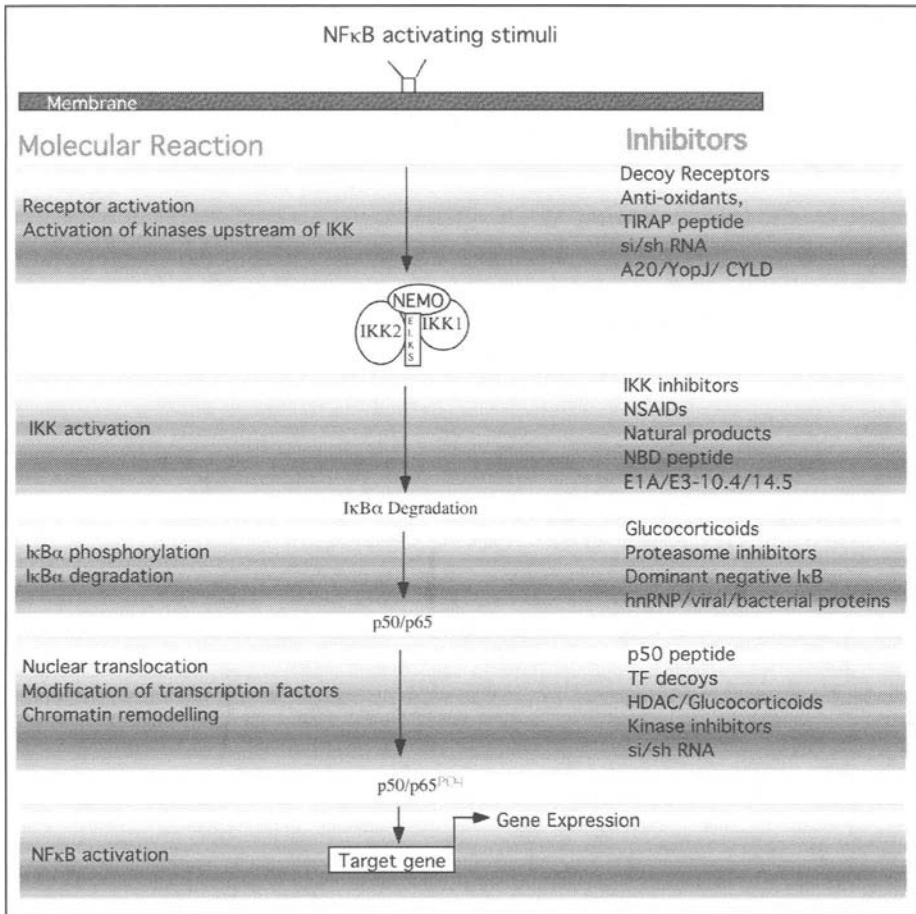

Figure 1. Major nodes in the NFκB pathway that have been inhibited experimentally. The molecular steps in the activation of NF-κB are depicted on the left and the inhibitors that have been documented to work have been shown on the right of the pathway. Only certain representative proteins or class of compounds have been shown. Please refer to text for details.

been shown to control distinct biological processes that may or may not work via NF-κB. Screening of bioactive substances from natural sources, against defined targets such as NF-κB and developing derivatization leads toward drugs should be a promising strategy for drug discovery. A large-scale genetic screen would be ideal towards rapidly understanding the distinct roles of IKK1 and IKK2 and in designing pathway specific inhibitors which do not have other systemic effects. Further, since it is becoming more evident that NF-κB is required for resolution of inflammation (since it is also critical for the activation of anti-inflammatory cytokines[156,157]), inhibitors of NF-κB will have to be designed to work efficaciously in a short period of time. Such inhibitors of NF-κB would also be beneficial to limit or block bystander effects on nontarget cells when systemic inhibition of NF-κB is called for.

Screening in Fish and Fly

The immense power of forward genetic screens have been very successful in identifying important signal transduction pathways involved in many biological processes in Drosophila.[158]

With the characterization of the NF-κB pathway in drosophila,[159] and now in zebrafish[160] these model systems represent an attractive tool for chemical and genetic screens to identify modulators and inhibitors of the NF-κB pathway. The rapid developmental stages and permeability of zebrafish embryos to small molecule inhibitors, and the strong homology between kinases from zebrafish and humans,[161,162] has made this system highly suited for high-throughput inhibitor screens. Zebrafish is now also used as a vertebrate model organism in immunologic research.[163] While present screening approaches target select candidate genes, recent organism-based screens have led to the discovery of chemicals that mitigate complex dysmorphic syndromes even without targeting the affected gene directly.[164,165]

Conclusion

In the last two decades we have learnt a lot about the basic mechanisms that activate NF-κB. With the advent of high throughput technologies, rational drug design techniques and novel model systems like drospohila and zebrafish, screening for specific modulators and inhibitors of NF-κB is sure to accelerate in the future. The challenge of the future would be to wade through a wealth of drug target hits found by such screens and to find the most effective and selective inhibitor of a given physiological process. The knowledge of how specific modifications regulate NF-κB activity in response to distinct stimuli would undoubtedly propel our efforts towards designing more specific inhibitors of NF-κB.

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