Alexander Hoffmann
David Baltimore

Circuitry of nuclear factor κB signaling

Authors' addresses
Alexander Hoffmann[1], *David Baltimore*[2]
[1]Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA, USA.
[2]Division of Biology, California Institute of Technology, Pasadena, CA, USA.

Correspondence to:
Alexander Hoffmann
Signaling Systems Laboratory
Department of Chemistry and Biochemistry
9500 Gilman Dr
La Jolla, CA 92093
USA
Tel.: +1 858 822 4670
Fax: +1 858 822 4671
E-mail: ahoffmann@ucsd.edu

Acknowledgements
We apologize to the many researchers whose work we have not been able to discuss in this limited review. A. H. thanks X.-F. Qin and T. H. Leung for longstanding collaboration and communication of unpublished results, G. Ghosh for figures of protein structures, and E. A. Komives, G. Ghosh, and V. Sivakumar for insightful discussions.

Immunological Reviews 2006
Vol. 210: 171–186

Immunological Reviews
0105-2896

Summary: Over the past few years, the transcription factor nuclear factor (NF)-κB and the proteins that regulate it have emerged as a signaling system of pre-eminent importance in human physiology and in an increasing number of pathologies. While NF-κB is present in all differentiated cell types, its discovery and early characterization were rooted in understanding B-cell biology. Significant research efforts over two decades have yielded a large body of literature devoted to understanding NF-κB's functioning in the immune system. NF-κB has been found to play roles in many different compartments of the immune system during differentiation of immune cells and development of lymphoid organs and during immune activation. NF-κB is the nuclear effector of signaling pathways emanating from many receptors, including those of the inflammatory tumor necrosis factor and Toll-like receptor superfamilies. With this review, we hope to provide historical context and summarize the diverse physiological functions of NF-κB in the immune system before focusing on recent advances in elucidating the molecular mechanisms that mediate cell type-specific and stimulus-specific functions of this pleiotropic signaling system. Understanding the genetic regulatory circuitry of NF-κB functionalities involves system-wide measurements, biophysical studies, and computational modeling.

Introduction: a short historical perspective

Nuclear factor (NF)-κB was discovered biochemically as a DNA-binding activity in activated B cells, with affinity for the transcriptional enhancer of the immunoglobulin κ light-chain gene (1). Induction of the activity was correlated with expression of antibody, suggesting that NF-κB would be an important regulator of antibody production. However, recent knockout and knock-in mouse studies have not substantiated this notion: B cells derived from a variety of NF-κB knockout mice are able to produce κ light-chain-containing antibodies, as are mice in which the κB consensus sequence site in the κ light-chain enhancer has been mutated. In fact, while the name NF-κB remains, none of the information it implies is fully correct. As mentioned, NF-κB is neither a critical regulator of the κ light-chain gene, nor is it B-cell specific, nor truly a nuclear factor. In fact, NF-κB is present in all cell types,

its nuclear-cytoplasmic localization is intricately controlled, and it regulates a large number of genes that control diverse cellular responses. NF-κB turned out to be a stimulus-responsive pleiotropic regulator of gene control (2).

Induction of NF-κB activity was found not to require protein synthesis (3, 4), thereby inaugurating a research field devoted to the elucidation of the pathways that allow for receptor-specific signal transduction that culminates in nuclear NF-κB activity. First, experiments distinguished between two mechanisms: a precursor processing mechanism or regulation by a separate inhibitor protein. The detergent deoxycholate was shown to liberate fully active κB-site DNA-binding activity in unstimulated cells (5), suggesting the existence of inhibitor proteins, termed IκB (6). Biochemical purification using the same chromatographic media first in the absence and then in the presence of deoxycholate led to the identification of the NF-κB polypeptides p65 and p50 (7, 8), and IκBα and IκBβ (9). The activation mechanism in response to inflammatory stimuli was soon shown to involve the signal-induced proteolysis of NF-κB-bound IκB proteins (10). Following the identification of signal-induced phosphorylation of specific serines in IκB proteins as a necessary step in Iκβ proteolysis NF-κB activation (11), the responsible IκB kinase (IKK) was purified (12) and shown to be the primary regulator of NF-κB activity (13). Indeed, based on a large body of literature, the activation mechanism of NF-κB is sufficiently well understood such that a mathematical model recapitulates the temporal regulation of canonical NF-κB signaling (14).

Other recent work has uncovered a second NF-κB activation pathway (often called the non-canonical pathway) in mammals and *Drosophila* that is currently thought not to involve inhibitor degradation but rather protein precursor processing (15). While this signaling pathway shares many of the molecular mediators with the canonical pathway, the mechanism of stimulus-responsive partial proteolysis remains the focus of active research.

Biochemistry 101 of NF-κB signaling

The molecular constituents

Current knowledge describes the NF-κB-signaling system as consisting of about a dozen different dimers comprising five homologous proteins (*Fig 1*): the classical and ubiquitously expressed p50 and p65/RelA proteins, which together constitute the primary inflammatory mediators; the cRel (also termed Rel) protein, which is triggered in the hematopoietic compartment, and the p52 and RelB polypeptides, which, as a heterodimer function in the non-canonical NF-κB pathway that is responsive to non-inflammatory stimuli (15) and plays a role in activation chemokine genes involved in

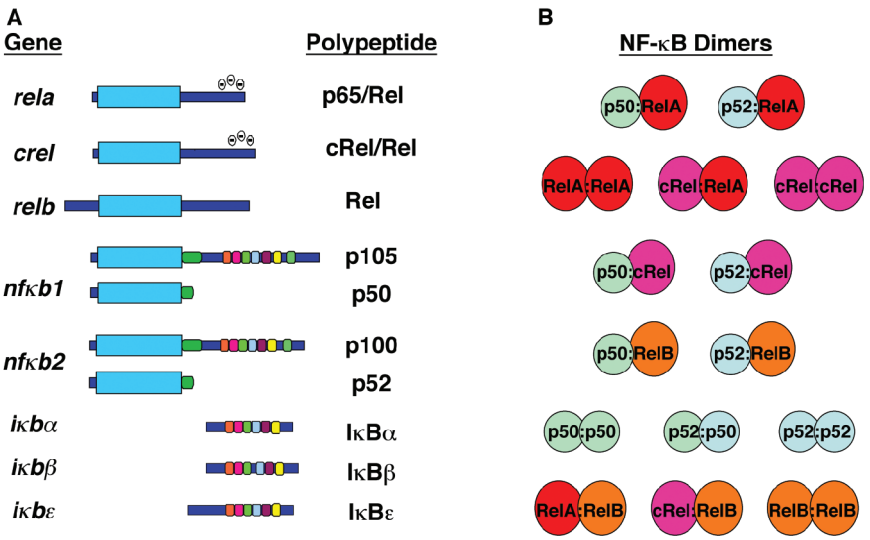

Fig. 1. Nuclear factor (NF)-κB and IκB genes, polypeptides, and dimers. (Left) Five mammalian NF-κB genes give rise to five transcription factor proteins, RelA, cRel, RelB, p50, and p52, that share the Rel-homology domain (pale blue box), which is responsible for DNA binding, dimerization, and association with IκB proteins. RelA and cRel contain acidic transcriptional activation domains. The proteins p50 and p52 derive from a proteolytic processing mechanism of precursor proteins p100 and p105. Their C-terminal portions contain ankyrin repeat domains that are the hallmark of the three IκB inhibitor proteins IκBα, IκBβ, and IκBε. (Right) Five NF-κB polypeptides can form 15 transcription factors through homo- and hetero-dimerization. The top four rows show nine dimers that can function as transcriptional activators, the fifth row indicates dimers lacking transcriptional activation domains, and the bottom row shows dimers that are not able to bind DNA. In a given cell, a subset of dimers may be present, depending on the cell type, stage, and conditioning by environmental cues. Generally, RelA dimers are ubiquitously expressed, but cRel-containing dimers are more highly expressed in mature lymphoid cells.

lymphoid organogenesis. All dimers share the ability to bind to the κB-site consensus sequence and may therefore be referred to as NF-κB. How different NF-κB isoforms differ in regards to their biochemical functions and their physiological roles continues to be an active focus of current research and is addressed in more detail below.

Five homologous proteins are known to play functionally inhibitory roles on the DNA-binding activity of NF-κB and have been termed IκB proteins (*Fig. 1*). This family includes the canonical IκBs IκBα, IκBβ, and IκBε, as well as the precursor proteins p105 and p100, whose C-terminal portions have also been termed IκBγ and IκBδ, respectively, and whose N-terminal portions encode p50 and p52. It remains unclear whether the precursor proteins p100 and p105 and/or the C-terminal portions actually function as *bona fide* IκB proteins (inhibit the activity of a pre-existing dimer and allow for its activation in response to cellular stimulation). Current research also continues to address the regulation of synthesis and degradation of the canonical IκB inhibitors of NF-κB activity, as well as IκB isoform-specific functions in regard to subsets of NF-κB dimers or NF-κB-inducing stimuli.

Biophysical studies and X-ray crystallography have provided insights about the structure of the NF-κB dimerization and DNA-binding domains and about the interactions with IκB proteins and with DNA bearing κB-binding sites (*Fig. 2*). These initial studies have revealed subtle but functionally important differences between NF-κB and IκB isoforms and in the interaction between NF-κB and different κB-site sequences. Each interaction is characterized by allostery that suggests molecular specificity mechanisms that are not merely governed by bivalent affinity rules. For example, the RelB protein can exist in two very distinct conformational states

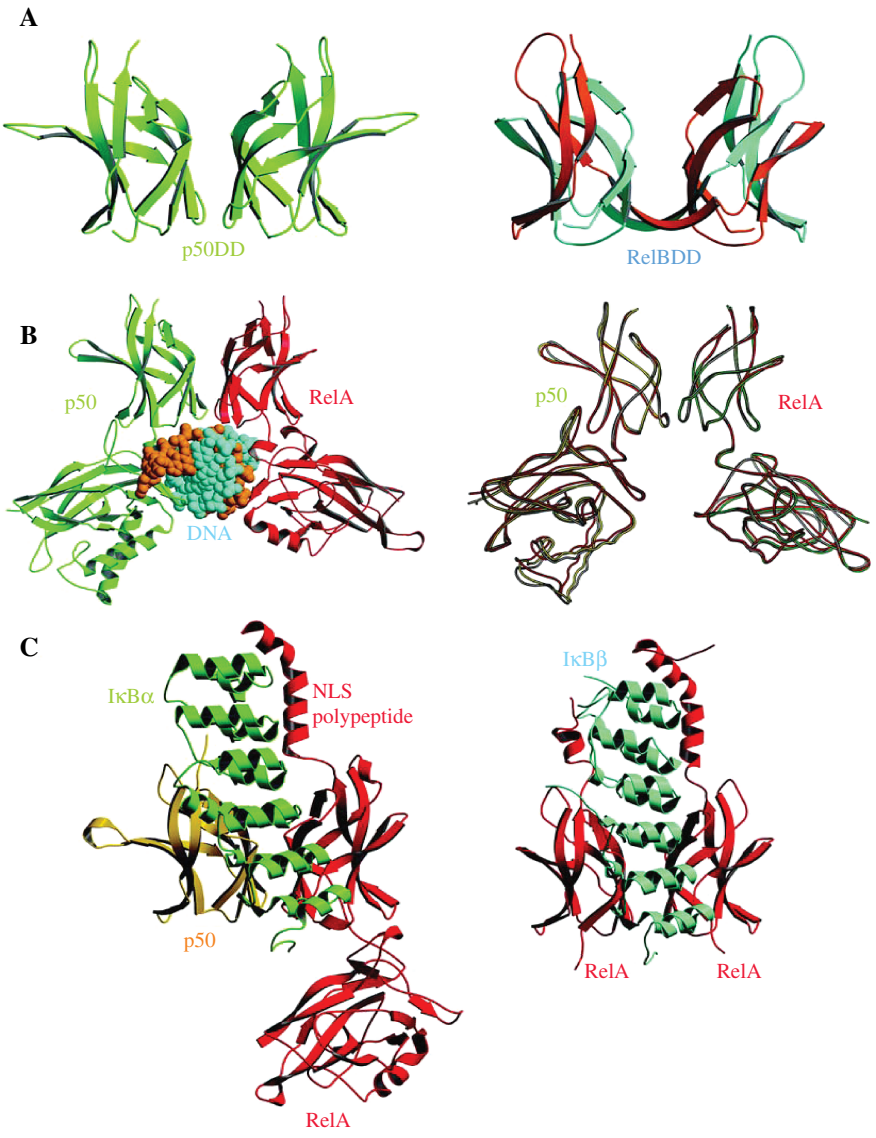

***Fig. 2.* Allostery within the nuclear factor (NF-κB)-signaling system.** (A) NF-κB dimer conformation is determined by the binding partner. Ribbon representations of the dimerization domains of the p50:p50 (left) and RelB:RelB (right) homodimers. The structures are different; a large conformational change determined by the binding partner renders an 'open' dimer (left) that can bind DNA or an intertwined dimer (right) that is unable to bind DNA. (B) The conformation of NF-κB bound to DNA is determined by the sequence of the κB site. The structure of the rel homology regions (RHR) of p50:RelA bound to Ig-κB DNA sequence (left). Superimposition of the α-carbon trace of three structures determined of the p50:RelA RHR heterodimer bound to three different κB sites [right; Ig-κB, interferon (IFN)β-κB, and uPA-κB, located in the enhancer/promoters of the immunoglobulin κ light chain, IFN-β and urokinase plasminogen activator genes, respectively]. This comparison reveals conformational changes in the protein interaction surfaces of the dimer induced by single base differences in the sequence of κB-site-binding element. (C) NF-κB bound to IκB proteins. The structure of IκBα bound to the p50:RelA heterodimer (left). The NLS of RelA, which is unstructured in the absence of IκB proteins, folds into a helical structure upon binding to IκBα. The NLS of p50 does not contact IκBα. The structure of IκBβ bound to the RelA homodimer (right). The NLS of one RelA subunit interacts with IκBβ as that in IκBα-p50:RelA complex. The second NLS also makes weak contacts with IκBβ. While unbound IκBα is incompletely folded, within the complex with NF-κB it is fully folded.

depending on its dimeric interaction partner (16) (*Fig. 2A*). Second, the same NF-κB dimer bound to DNA exhibits sequence-specific conformations that are distant from the DNA interaction surface (17) (*Fig. 2B*). Third, the folding states of IκB proteins and NF-κB dimers are critically regulated by their interaction with each other (18). These observations emphasize the importance of comprehensive biophysical characterizations of trapped conformations revealed by X-ray crystallography and of the dynamics of interactions and concomitant conformational changes to understand the molecular basis of specific, distinct, and overlapping functionalities of the molecular constituents of the NF-κB-signaling system.

Two NF-κB signaling pathways

Two distinct and evolutionarily conserved NF-κB-signaling pathways have been described (*Fig. 3*). They are distinguished by two multiprotein IKK complexes that regulate the stimulus-responsive degradation of IκB proteins. The so-called 'canonical' IKK complex contains the IKK2 (IKKβ) protein and is regulated by the scaffold protein NF-κB essential modulator (NEMO, also known as IKKγ), whereas the 'non-canonical' IKK complex appears to consist solely of an IKK1 (IKKα) homodimer. Both the so-called canonical and the non-canonical NF-κB-signaling pathways play important roles in the functioning of the immune system, yet their roles seem surprisingly distinct. While the canonical pathway is largely responsible for regulating inflammation as well as the control of proliferation and apoptosis of lymphoid cells during the immune response, the non-canonical pathway is associated with the development of lymphoid organs that ensure the mounting of an effective immune response. As described below, the biochemical characteristics of these pathways echo this functional distinction: the canonical pathway is fast acting (responds within minutes) and is reversible due to the presence of multiple negative feedback mechanisms, whereas the non-canonical pathway responds more slowly (over hours and days), providing long-lasting nuclear NF-κB activity.

The canonical NF-κB-signaling pathway

Pro-inflammatory stimuli, such as pathogen-derived lipopolysaccharide (LPS) and cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1, are strong inducers of NF-κB activity in many cell types (*Fig. 4*). The primary NF-κB isoform induced by these stimuli is the p50:RelA dimer (also known as 'classical NF-κB'), a potent transcriptional activator of genes regulated by the κB sequence element. The p50:RelA dimer is present in unstimulated cells, but its DNA-binding activity is inhibited by IκB. Upon inflammatory stimulation,

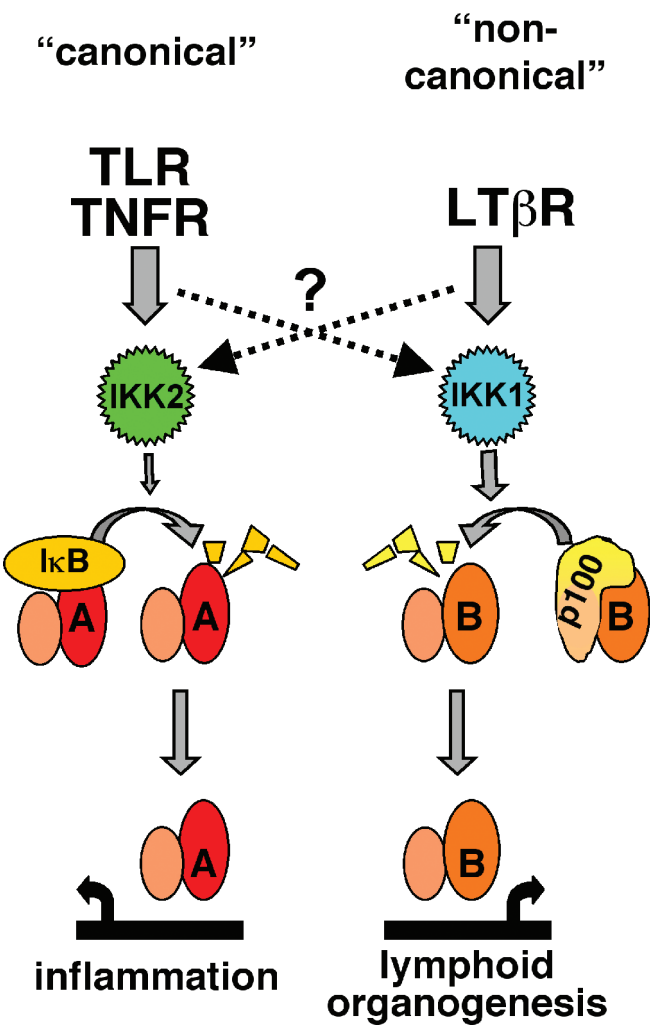

Fig. 3. Two nuclear factor (NF)-κB activation pathways. The so-called canonical NF-κB pathway is triggered by many inflammatory stimuli to induce IKK2-containing IKK complexes that specifically phosphorylate the three canonical IκB proteins, thereby marking them for ubiquitination and proteasome-mediated proteolysis. RelA as well as cRel-containing dimers are thereby released to translocate to the nucleus and activate genes. The non-canonical pathway is mediated by IKK1 and induces the processing of p100 to p52 to release RelB-containing dimers to the nucleus. Crosstalk between canonical and non-canonical signaling pathways is of current research interest.

the canonical IκB proteins IκBα, IκBβ, and IκBε are phosphorylated by the canonical IKK complex on two specific N-terminal serines, which then act as a docking platform for the ubiquitin ligase β-TRCP. Subsequent ubiquitination (lysine 48-linked chains) induces proteasome-mediated proteolysis of the IκB protein without affecting the integrity of the bound NF-κB dimer. Instead, the liberated dimer is capable of binding DNA and activating genes. While early models suggested that IκB proteins also inhibit nuclear translocation of NF-κB dimers, it has become clear that dimers bound to IκBα and IκBε actively shuttle between nuclear and cytoplasmic compartments (19, 20). A key role of the IκB association

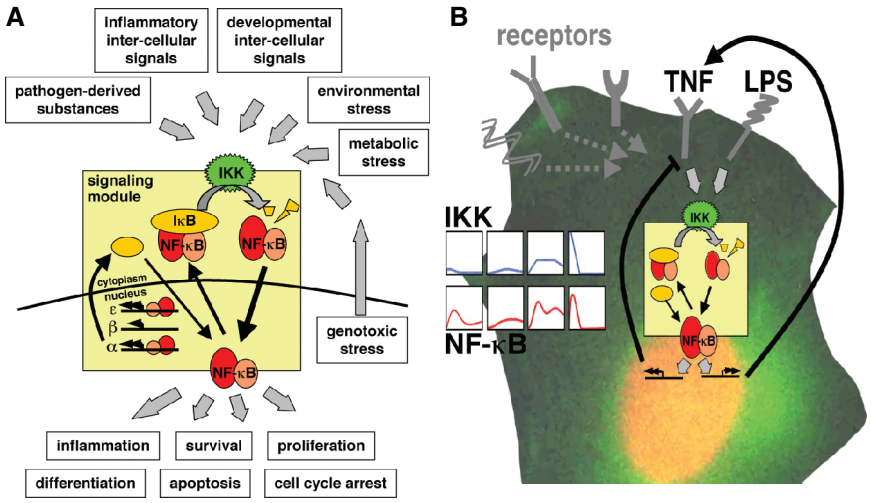

***Fig. 4.* The IKK-IκB-NF-κB-signaling module and temporal control.** (A) The canonical NF-κB pathway represented here by the so-called IKK-IκB-NF-κB-signaling module mediates a wide variety extracellular and intracellular signals to control a diverse set of cellular responses. Negative feedback control mediated by IκBα and IκBε allows for dynamic regulation of NF-κB. (B) The Signaling Module receives input signals in the form of IKK activity profiles. These are stimulus specifically regulated via negative feedback mechanisms and autocrine loops to result in specific dynamics. Each IKK dynamic profile is transduced by the signaling module to generate a stimulus-specific NF-κB activity profile that is critical for stimulus-specific gene expression.

with NF-κB is therefore its prevention of DNA binding. It has even been claimed that mutant NF-κB dimers, incapable of binding DNA, do not fully localize in the nucleus in the absence of IκB proteins (21). Together, these data point to a model in which association of IκB with NF-κB may shift the shuttling behavior of NF-κB quantitatively (i.e. ratio between nuclear import and export), rather than prevent its nuclear localization as originally proposed.

Initial activation of canonical NF-κB activity is typically rapid and does not require *de novo* protein synthesis. Upon cell stimulation, increases in nuclear NF-κB activity can be detected within 10 min, and some NF-κB-responsive promoters are induced almost immediately. One such early responding promoter is that of IκBα, which mediates a powerful negative feedback mechanism that is responsible for postinduction repression of NF-κB activity upon stimulus removal (14, 22) and may result in oscillatory NF-κB activity during chronic stimulation (14, 23). However, the oscillatory propensity in the signaling system caused by IκBα is counteracted by a second negative feedback mechanism mediated by IκBε, which is delayed and functions in anti-phase to IκBα (24). These insights indicate that cells have the capacity to intricately modulate the temporal activity profile of NF-κB and have provided the impetus for current interest in understanding the potential roles of dynamic control of NF-κB signaling in stimulus-specific cellular responses (25).

The non-canonical NF-κB-signaling pathway

Several non-inflammatory stimuli have been shown to engage the non-canonical NF-κB-signaling pathway in a variety of cell types. In B cells, the survival factor B-cell-activating factor belonging to the TNF family (BAFF) (26) and, in splenic stromal cells, lymphotoxin β signaling (27) were shown to activate NF-κB activity over a period of many hours or days. Depending on the stimulus and cell type, this activity consists of RelB as well as RelA complexed with the dimerization partners p50 or p52. However, the activation mechanism remains somewhat unclear. The *nfkb2* precursor protein p100, which is capable of dimerizing with RelB, was originally shown to undergo partial proteolysis or processing upon IKK1 stimulation. However, processing itself was shown to be cotranslational and is thereby restricted to newly synthesized p100 protein (28). In addition, the mechanism by which RelA dimers are induced remains unclear. Recent studies resulted in a simplified model, in which p100 functions as an IκB protein sequestering both p50 : RelA and p50 : RelB complexes in resting cells. In this model, stimulation of IKK1 activity leads to full degradation of p100, but newly synthesized p100 is preferentially processed to p52, which dimerizes with both RelB and RelA to ensure a long-lasting activity of both RelA- and RelB-containing dimers (S. Basak and A. H., unpublished observations).

It is important to note that non-canonical activation of NF-κB is significantly slower than canonical activation, and it appears to lack strong negative feedback mechanisms or highly dynamic control. This finding may in part be explained by the fact that RelB-containing dimers do not have high affinities for canonical IκB proteins and are therefore not subject to their highly dynamic regulation. Long-lasting steady NF-κB activity may indeed be indicative of the primary physiological functions of the non-canonical pathway in cellular differentiation, survival, and organogenesis.

Inducers and responsive genes

For largely complete lists of molecules, conditions, genes, and proteins related to NF-κB regulation, we refer the reader to www.nf-kb.org, which also indicates a reference for each. This website is maintained by Tom Gilmore (Boston University) and has become an increasingly valuable resource. Below, we characterize each category in broad thematic terms and provide a conceptual framework.

Hundreds of substances and physiological conditions are known to activate NF-κB (*Fig. 4A*). These include (i) bacterial, fungal, or viral products that are often recognized by the Toll-like receptor (TLR) pathways; (ii) intercellular signaling mediators often recognized by members of the TNF receptor superfamily; (iii) immunoglobulin domain-containing receptors that regulate the adaptive immune response through the recognition of free antigen or antigen on antigen-presenting cells or by mediating intercellular signaling; and (iv) metabolic or genotoxic stress conditions, often induced by environmental hazards. Such stress conditions may act through specific signaling mechanisms, such as the stress responsive kinases ATM/ATR (genotoxic stress), or pleiotropic mechanisms, such as translational inhibition (endoplasmic reticulum stress or the unfolded protein response). While cellular stress does not activate NF-κB exclusively, NF-κB activation is prominent due to an apparently sensitive balance in resting cells of synthesis and degradation of IκB proteins, for example. Such a dynamic equilibrium, or molecular homeostasis, deserves further study to better understand the molecular mechanisms and physiological functions of NF-κB activation by pleiotropic agonists.

Molecular biological studies over the past 20 years have led to identification of functional κB sites in about a hundred genes whose induction correlates with NF-κB activation. These are reflective of NF-κB's functions in regulating the communication between cells, regulating cell survival, and regulating proliferation. Genes encoding inflammatory mediators that control cell activation and chemotaxis, such as the pro-inflammatory cytokines TNF, IL-1, and IL-12 and chemokines such as monocyte chemotactic protein (MCP)-1, interferon (IFN)-inducible protein (IP)-10, and RANTES (regulated upon activation, normal T-cell expressed, and presumably secreted), are prominent examples whose NF-κB responsiveness was established long ago. Similarly, NF-κB's control of the expression of many of the cognate receptors is reflective of its major role in tuning the sensitivity of cells to such extracellular messengers. In addition, large numbers of NF-κB-dependent genes contribute to the innate immune response, such as the anti-microbial peptide β-defensin-2 or the C-reactive protein. Another functionally important category is genes that inhibit pro-apoptotic signaling pathways. They function by interfering with c-Jun N-terminal kinase signaling [e.g. GADD45β (29)], by preventing the accumulation of reactive oxygen species [e.g. MnSOD, FHC (30)], maintaining mitochondrial health (Bcl2 family members), or by targeting directly the caspase cascade [e.g. inhibitors of apoptosis (31), cellular FLICE inhibitory protein (32)]. In addition, enzymes involved in tissue remodeling [e.g. matrix-metalloproteinases (33)] and cell cycle regulatory proteins [e.g. cyclin D (34)] have been reported to be regulated by NF-κB. Misregulation of these genes due to elevated NF-κB activity in disease-associated cells appears to play critical roles in a variety of pathogeneses.

Several more recent studies have aimed at cataloging the genes that are regulated by NF-κB in a cell type- and stimulus-specific manner on a genome-wide scale using microarray technology (35–43). Genetic tools, such as cells derived from mice deficient in one or several NF-κB subunits or an ectopically expressed stimulus-unresponsive IκBα mutant protein (also known as the 'IκB super-repressor'), have identified more than 500 genes whose transcriptional regulation depends on NF-κB. Indeed, it appears that in all the cell types examined thus far, almost all genes induced by inflammatory stimuli are transcriptionally upregulated in an NF-κB-dependent manner. However, there is little information about the extent of NF-κB's role in the regulation of the large inducible gene expression programs in primary hematopoietic cells. As such, it remains unclear whether NF-κB dependence is stimulus- and/or cell type-specific, i.e. genes may be NF-κB-dependent when induced by inflammatory stimuli but independent when induced by non-inflammatory stimuli (or in the unstimulated cell) or when induced by the same stimulus in a different cell type. In addition, determination of whether NF-κB-dependent genes are direct or indirect targets of NF-κB is an important prerequisite to understanding the NF-κB regulatory circuitry that controls gene expression programs. Interestingly, decreases in the levels of specific mRNAs have also been observed to involve NF-κB, but in most cases, it remains unclear whether these are the result of transcriptional repression or enhanced degradation mechanisms.

As an alternative to genetic/functional studies, chromatin immunoprecipitation (ChIP) has allowed determination of physical association of endogenous NF-κB proteins with promoter sequences during inflammatory stimulation. While single gene studies have found correlations between NF-κB dimer recruitment and inducible gene expression, large scale or genome-wide location analysis (also known as ChIP-chip),

which is currently in progress, appear to correlate less well with genetic NF-κB dependence. It remains unclear whether this discrepancy is due to technical challenges inherent in high throughput approaches or whether as yet unrecognized regulatory mechanisms may be the underlying cause. However, comprehensive location analysis for the RelA/p65 subunit on chromosome 22 (44) did suggest that NF-κB may be bound to many chromosomal locations that are not correlated with gene activity or stimulus responsiveness. Given the complexity of determining the regulatory networks that determine gene expression programs, NF-κB is an attractive model system for genome-wide expression and location analysis, due to its stimulus-responsiveness and importance to human health.

Inhibitors and interacting proteins

Hundreds of inhibitors have been described to interfere with NF-κB signaling (www.nf-kb.org). These include many natural products first characterized to have anti-inflammatory effects or environmental toxins. While these products have been shown to inhibit the induction of a κB-site transcriptional reporter plasmid, nuclear NF-κB DNA-binding activity, and/or IKK activity, the precise molecular targets of most of these inhibitors remain obscure. The primary pharmacological targets of the NF-κB activation pathway thus have been IKK and the 20S/26S proteasome. While the former is thought to act specifically on NF-κB (though other phosphorylation targets are being identified), the latter clearly has highly pleiotropic effects; in the presence of NF-κB-inducing stimulus, the effect on NF-κB may be functionally dominant. Other small molecules are known to act on diverse cellular mechanisms but also show an inhibitory effect on NF-κB. This effect may be due to a lack of molecular specificity or may be suggestive of signaling crosstalk. Antioxidants and a variety of mitogen-activated protein kinase (MAPK) inhibitors fall into this category.

Molecular specificity of pharmacologic inhibitors does not necessarily translate into specificity at the levels of physiological responses or stimulus-specific gene expression programs. It appears that molecular pleiotropic drugs such as sodium salicylate (aspirin) or PS-341 (bortezomib) can have useful physiologically specific effects. One might argue that partial inhibition of multiple molecular mechanisms may result in synergistic effects on a specific subset of physiological conditions, stimuli, or target genes. Therefore, to better understand the mode of action of successful pharmaceuticals and to identify further therapeutic strategies to regulate inflammation and NF-κB-dependent immune responses, quantitative dynamic models of the relevant signaling networks may prove critical.

The extent and challenge of such modeling efforts is underscored by the identification of a large number of cell- and pathogen-encoded proteins that physically and/or functionally interact with the NF-κB-signaling system (www.nf-kb.org). In addition to many single gene publications, recent systematic efforts by coimmunoprecipitation followed by mass spectrometry (45) and genome-wide yeast two hybrid approaches (46) have resulted in a long list of potential interactions whose functional roles remain to be explored. Interactions between NF-κB dimers and a variety of transcription factors [e.g. signal transducers and activators of transcription (STATs), IFN regulatory factors (IRFs), E2Fs, fos/jun, cAMP responsive element-binding proteins (CREB)] and coactivators/repressors [e.g. CREB-binding protein (CBP)/p300, histone deacetylases (HDACs), TAFs] are likely to play a role in stimulus- and promoter-specific transcriptional regulation. Interactions with upstream regulators, such as MAPKs, protein kinase A, and protein kinase C protein phosphatases, are indicative of the wider cellular network of signaling proteins that contribute to the regulation of NF-κB activity. In addition, a variety of pathogen-derived proteins have been shown to interfere with NF-κB signaling. Pathogens may benefit from downregulating NF-κB, as it may serve to evade immune surveillance by inhibiting inflammatory gene expression, while activation may prevent infected cells from undergoing apoptosis.

Immune functions of NF-κB

NF-κB has multiple roles in the immune system, both in the development, differentiation, and survival regulation of its effector cells, as well as in the dynamic regulation of local and systemic immune activity. Extensive discussions of each aspect may be found in several previous reviews (47–49). Below, we provide an overview of insights largely gained from the analysis of mouse knockouts. Many of the published and some unpublished phenotypes (A. H., X. F. Qin, D. B.) are listed in *Table 1*. It is increasingly recognized that interpretation of knockout phenotypes requires consideration of the fact that while only one polypeptide is targeted by a knockout, NF-κB is a dimeric transcription factor, and each of the several possible dimers functions within a homeostatic signaling network.

Lymphocyte biology

Given NF-κB's discovery in B cells, early analysis of mouse knockouts was focused on phenotypes in lymphocyte development and function. Indeed, the activation domain-containing

Table 1. **Phenotypes of NF-κB knockout mice**

Genotype	Lethality	Cause	Apoptotic sensitivity	Proliferation defects	Comments/other
rela$^{-/-}$	E14.5	Fetal liver apoptosis	To TNF		
tnfr$^{-/-}$rela$^{-/-}$	Alive			B and T cells	Lymph node organogenesis defect
tnf$^{-/-}$rela$^{-/-}$	Alive		To TNF	B and T cells	Lymph node organogenesis defect
crel$^{-/-}$	Alive		To TNF	B cells	Particularly with anti-IgM
				T cells	Partly correctable with IL2 or CD28 co-stimulation
nfkb1$^{-/-}$	Alive			B cells	At low LPS
nfkb2$^{-/-}$	Alive			B cells	Also maturation defect, Lymph node, Splenic architecture defect
relb$^{-/-}$	0–50% by 3 months	Multi-organ inflammation		B cells T cells	Also maturation defect Possibly due to anergy/exhaustion, Lymph node, splenic architecture and mammary gland development defects
rela$^{-/-}$nfkb1$^{-/-}$	E12.5	Fetal liver apoptosis	To TNF		
rela$^{-/-}$crel$^{-/-}$	E13.5	Fetal liver apoptosis	To TNF, B cells		
tnf$^{-/-}$crel$^{-/-}$rela$^{-/-}$	Perinatal	Unknown	To TNF		Epidermal development and homeostasis defects
rela$^{-/-}$nfkb2$^{-/-}$	E14.5	Fetal liver apoptosis	To TNF		
rela$^{-/-}$relb$^{-/-}$	E14.5	Fetal liver apoptosis	To TNF		
crel$^{-/-}$nfkb1$^{-/-}$	Alive		B and T cells DCs	B and T cells DCs	More severe B cell defects than single knockouts DC maturation
crel$^{-/-}$nfkb2$^{-/-}$	Alive		B and T cells	B and T cells	
crel$^{-/-}$relb$^{-/-}$	Alive		B and T cells	B and T cells	
nfkb1$^{-/-}$nfkb2$^{-/-}$	After weaning	No teeth	B cells	B and T cells	More severe defect than single knockouts Lymph node and splenic architecture defect Osteoclast differentiation defect
nfkb1$^{-/-}$nfkb2$^{-/-}$crel$^{-/-}$	After weaning	No teeth	B and T cells	B cells T cells	More severe than nfkb1$^{-/-}$nfkb2$^{-/-}$ As in nfkb1$^{-/-}$nfkb2$^{-/-}$ Lymph node, spleen and osteoclast phenotypes as in nfkb1$^{-/-}$nfkb2$^{-/-}$
nfkb1$^{-/-}$relb$^{-/-}$	80% by 3 months	Multi-organ inflammation	B and T cells	B and T cells	More severe defects than single knockouts Lymph node and splenic architecture defect
nfkb1$^{-/-}$relb$^{-/-}$crel$^{-/-}$	Alive		B and T cells	B and T cells	Severe proliferation and survival defects Lymph node and splenic architecture defect
nfkb1$^{-/-}$nfkb2$^{-/-}$relb$^{-/-}$	Perinatal	Multi-organ inflammation	B and T cells		
nfkb2$^{-/-}$relb$^{-/-}$	50% by 3 months	Multi-organ inflammation	B and T cells	B and T cells	More severe than single knockouts Lymph node and splenic architecture disrupted
nfkb1$^{-/-}$crel$^{-/-}$rela$^{+/-}$	Alive		B and T cells Macs to LPS	B and T cells	Most severe proliferation and survival defects Macrophage, neutrophil, DC sensitivity to TLR4 Hypersensitivity in chemotaxis
nfkb1$^{-/-}$crel$^{-/-}$rela$^{-/-}$	E14	Fetal liver	TNF, all cells		Canonical NF-κB knockout
nfkb1$^{+/-}$nfkb2$^{-/-}$rela$^{+/-}$	Alive				Skin: 10–30% incidence of hyperinflammation
nfkb1$^{-/-}$nfkb2$^{-/-}$rela$^{-/-}$	E12.5	Fetal liver			
nfkb1$^{-/-}$nfkb2$^{-/-}$ crel$^{-/-}$relb$^{-/-}$	Perinatal lethal	Unknown			
nfkb1$^{-/-}$crel$^{-/-}$relb$^{-/-}$nfkb2$^{+/-}$rela$^{+/-}$	50% by 3 months	Unknown			
nfkb1$^{+/-}$crel$^{-/-}$relb$^{-/-}$nfkb2$^{-/-}$rela$^{+/-}$	50% by 3 months	Unknown			

DC, dendritic cell; IL, interleukin; LPS, lipopolysaccharide; TLR, Toll-like receptor; TNF, tumor necrosis factor.
A very brief summary of phenotypes with emphasis on defects proliferation and regulation of apoptosis. Based on published (47–49) and unpublished observations (A. H., X.-F. Qin, D. B.).

proteins RelA and cRel were found to be critical for lymphocyte development and function. While cRel-deficient mice are viable and exhibit normal numbers of many lymphocyte subclasses, mature lymphocytes exhibit a number of activation defects, such as B- and T-cell proliferation and isotype switching (50). These defects are exacerbated in mice deficient for both cRel and its dimerization partner p50 (51). Absence of RelA, however, leads to embryonic lethality during embryonic day E14-15, due to massive apoptosis in the fetal liver brought about by the pro-apoptotic effects of TNF. Similarly, apoptotic sensitivity of hematopoietic precursors prevented the functional analysis of RelA deficiency in lymphocytes in fetal liver transplant experiments (52), although further analysis showed that RelA deficiency only causes a partial defect in lymphocyte proliferation (53). Further studies also indicate that canonical NF-κB provides survival and proliferative signals to lymphocytes and survival signals downstream of pre-TCR and pre-BCR signals, thus facilitating early B- and T-cell development (48).

It is tempting to equate cRel function with proliferation and RelA with pro-survival functions in lymphocytes. This model is clearly overly simplistic, as these two proteins also have overlapping functions in both processes. B lymphocytes deficient in cRel are prone to undergo mitogen-induced apoptosis, presumably due to a loss in upregulation of the Bcl2 homolog A1 (54). Indeed, it remains possible that the anti-apoptotic effects of RelA may in part be mediated by the RelA target genes *crel*, *nfkb1*, *nfkb2*, and *relb*. However, the regulation of these cellular processes by NF-κB transcription factors is of relevance to human health: inappropriate upregulation of NF-κB in B-cell lymphomas has been shown to provide essential pro-survival signals in the context of highly proliferating cells (55). These cells, in turn, exhibit gene expression signatures that are characteristic of elevated cRel levels (56).

While regulatory mechanisms controlling survival and proliferation may be difficult to separate, other studies have focused on differentiation processes during hematopoiesis. Combination knockout studies appear to have confirmed that NF-κB RelA and cRel protein are less likely to have a role in cell-intrinsic hematopoietic differentiation processes: its developmental roles are in the regulation of cell survival, proliferation, and cell-extrinsic factors present in the context of lymphoid organs. For example, recent reports indicate that the canonical NF-κB pathway is required in a cell-intrinsic manner for the efficient development of unique T-cell subpopulations, such as natural killer T cells (57) and $CD4^+CD25^+$ regulatory T cells (58). Their absence can cause a breakdown of peripheral tolerance (59). NF-κB's role in the development of these specialized cells is likely in regulating survival, maturation, and proliferation rather than in controlling differentiation or cell fate switch.

Secondary lymphoid organ development

Lymphocyte development requires the correct functioning of secondary lymphoid organs and their precise microarchitecture. Interestingly, both RelA- and RelB-deficient mice (60, 61) are deficient in peripheral lymph nodes, as are mice harboring p50 and p52 deficiencies (62). NF-κB activity is also required in stromal cells for the development of splenic microarchitectures. In fact, the developmental deficiencies in the B- and T-cell compartments of NF-κB knockout mice appear to be largely due to stromal, epithelial, or dendritic cell populations in thymus and spleen that are radiation resistant (63). Such deficiencies can also result in defective deletion of auto-reactive T cells and establishment of central tolerance. Interestingly, several gene knockout mouse models defective in components of the non-canonical pathway exhibit autoimmune symptoms and autoreactive T cells (64).

It remains unclear what the NF-κB target genes are which are critical for secondary lymphoid organ development. NF-κB target genes in the different cell types (stromal, dendritic, and lymphoid) are likely to play a role. The non-canonical signaling pathway, which is triggered by organogenic stimuli such as lymphotoxin β and regulates RelB-containing dimers, has been shown to activate a specific subclass of NF-κB-dependent genes, including those encoding the organogenic chemokines CCL21 and CXCL15 (65). As RelB is a known target gene of canonical NF-κB, it is of interest to determine whether RelA or p50 deficiency affects lymph node development by altering the activation of non-canonical NF-κB dimers. Indeed, signaling crosstalk between canonical and non-canonical pathways and characterization of the non-canonical gene expression program are important questions of current research.

Inflammation and innate immunity

A prominent function of NF-κB in the immune system is the regulation of inflammation, and much research has focused on the molecular mechanisms involved. Inflammatory stimuli are potent inducers of gene expression programs that are almost entirely NF-κB dependent, as determined by microarray experiments with murine embryonic fibroblasts, macrophages, dendritic cells, or B cells (35, 36, A. H. and D. B., data not shown). Many of these genes are also dependent on other transcription factors and regulatory mechanisms involving chromatin or post-translational modification events.

However, there are surprisingly few data on NF-κB's role in regulating inflammation during an actual immune response in the mouse. Cell type-specific knockouts or IκB super-repressor transgenes are likely to be instructive. Given the dependence of inflammatory gene expression on NF-κB, such genetic tools may also be useful in determining the role of different cell types in providing for inflammation that may be required for immune activation in response to different pathogens.

Recent commentaries have emphasized that the initiation of inflammation is intricately linked to its resolution (66). This physiological phenomenon is reflected also in the molecular mechanisms that regulate NF-κB activity. NF-κB is subject to a variety of negative feedback mechanisms, the most prominent being those mediated by IκBα, the ubiquitination enzyme A20, and more recently IκBε. Efforts to reconstruct these mechanisms in computational models (14, 24, 67) provide the tools to explore their overlapping and distinct functions in attenuating NF-κB activity. In addition, stimulus-induced degradation of promoter-bound RelA has been shown to be an important mechanism in macrophages for controlling inflammation, interestingly via the phosphorylation of RelA by IKK itself (68). Thus, IKK activation may be thought of as both inducing and limiting the activity of NF-κB.

Gene expression profiling suggests a major role of NF-κB in innate immunity via the production of anti-microbial peptides and serum proteins. These roles remain to be investigated in suitable experimental settings. However, macrophage and dendritic cell maturation and activation appear defective in the absence of NF-κB proteins (X. F. Qin, A. H., D. B., unpublished observations). More thorough analyses have revealed that activation of macrophages with TLR ligands can lead to apoptosis in monocytic cells defective in NF-κB activation, which suggests important survival functions for NF-κB (69). Interestingly, the NF-κB target genes responsible for mediating survival signals in macrophages in response to LPS are different from those required in hepatocytes or fibroblasts in response to TNF stimulation.

Emerging specificity mechanisms

NF-κB has emerged as a central player in a variety of physiological functions and in the transmission of diverse cellular signals. How do cells achieve cell type-specific, context-specific, and stimulus-specific responses by employing a single signaling pathway in so many functions?

Given a large number of diverse κB-binding site sequences, a family of NF-κB dimers, three IκB isoforms, and many different NF-κB-inducing stimuli, one might expect that each stimulus is capable of activating a specific gene expression program by inducing the degradation of a specific subset of IκB proteins, leading to the release of specific subset of dimers that is capable of binding only a subset of κB-binding sites. Despite significant efforts to characterize specificity at each of these mechanistic steps, little functional evidence has been presented for specificity at the level of the interaction between NF-κB isoforms and κB sites or between IκB isoforms and NF-κB isoforms; IκB isoform-specific degradation by a particular stimulus has also not been established.

However, recent work has begun to reveal several specificity mechanisms that are operating in unexpected and subtle ways. Some of these are occurring during the NF-κB activation process, and others are occurring on gene promoters. The former are determined by the receptors that activate specific signaling networks, and the latter are encoded in the regulatory code of each NF-κB target gene.

Specificity mechanisms in the regulation of NF-κB activity

Because NF-κB is constitutively present in resting cells in a latent form, the stimulus-responsive activation mechanisms that render it capable of binding DNA and activating genes comprise the first biochemical steps able to mediate stimulus-specific gene expression. Interestingly, NF-κB activation occurs with a stimulus-specific temporal profile. To study temporal regulation, we constructed a mathematical model based on differential equations that recapitulates the biochemical events that control nuclear NF-κB activity in response to TNF stimulation (14). Combined computational simulations and experimental analysis revealed that even very short transient TNF stimulations generate a complete hour of NF-κB activity, which is sufficient to drive the expression of many genes; however, longer stimulation was required for sustained activity, which is responsible for driving the expression of a second set of genes. Indeed, mutating the negative feedback mediated by IκBα resulted in aberrant gene expression in response to TNF pulse stimulation. These conclusions were further confirmed using single cell analysis (23).

In both single cell and biochemical analyses, using genetically engineered cells in which the IκBα-mediated negative feedback is dominant, oscillations of NF-κB activity were observed (14, 23). It is currently unclear whether such oscillations have important physiological functions in gene regulation or whether they are merely consequences of a potent postinduction attenuation mechanism triggered by a very strong, non-physiological stimulus in genetically altered cells (70). Interestingly, recent studies have identified a mechanism that dampens IκBα-mediated oscillations in normal cells.

IκBε expression was found to be NF-κB responsive, providing negative feedback for NF-κB but with a 45-min delay (24); the resulting anti-phase regulation of IκBα and IκBε allows for steadied NF-κB activity at late times without diminishing the ability to quickly terminate NF-κB upon stimulus removal.

Subsequent studies revealed that the temporal profile of NF-κB activity is largely independent of the TNF dose (71) but determined by the type of stimulus (72) (*Fig. 4B*). Using a library of theoretical IKK profiles, computational simulations revealed that the IKK-IκB-NF-κB-signaling module is much more sensitive to the precise level of IKK activity at later times than at early times, leading to the prediction that stimulus-specific temporal profiles are likely to be generated by mechanisms that regulate the late activity of IKK (72). A major regulator of late IKK activity in response to TNF is the ubiquitination regulatory enzyme A20, which forms a negative feedback loop (67, 73). Examining LPS-induced IKK activity revealed the presence of autocrine regulation, which amplifies the late activity and constitutes a positive feedback loop (72) and results in stabilized NF-κB activity (74). Both negative and positive feedback mechanisms that control the stimulus-specific temporal profile of IKK activity were indeed shown to be critical for stimulus-specific gene expression (72).

The simple computational model recapitulates NF-κB activation in response to TNF- and LPS-specific IKK profiles remarkably well, suggesting that future studies ought to focus on the signaling pathways that emanate from each receptor to encode stimulus-specific IKK temporal profiles. Additional biochemical mechanisms may affect NF-κB activation in a stimulus- or cell type-specific manner. For example, RelA is known to be specifically phosphorylated on a variety of serines. As antibodies for specific phosphorylation events become available, their functional roles will become clearer. Stimulus-responsive phosphorylation, for example, has been observed in serine 536 and has been proposed to enhance the nuclear translocation rate of the RelA-containing dimer (75) and to regulate specific acetylation that is correlated with transcriptional activity (76). Phosphorylated serines may regulate DNA binding, possibly to specific κB-site sequences and/or the interaction with IκB molecules. Cytoplasmic RelA phosphorylation coincident with activation may also determine the ability of NF-κB to transactivate once bound to target gene promoters by regulating the interaction with coactivators and corepressors. Indeed, a phosphorylation code with stimulus and target gene specificity has been proposed (77).

Cytoplasmic signaling events may determine the distribution of NF-κB dimers that are activated. For example, the non-canonical signaling pathway is known to activate RelB-containing dimers in addition to the classical NF-κB p50 : RelA dimer (78). In B cells, RelB activation via the non-canonical pathway is correlated with the induction of a subset of NF-κB responsive genes (43). Within the canonical pathway, which controls activation of several different dimers, biochemical studies with recombinant proteins have not resulted in convincing evidence for interaction specificity. However, careful biochemical analysis of IκB and NF-κB/Rel gene knockouts may reveal stimulus-specific activation of subsets of dimers that play a role in determining stimulus-specific gene expression programs. In that regard, computational modeling of the entire NF-κB regulatory network will prove essential to understanding the dynamic behavior of the system and its ability to generate stimulus-specific temporal regulation of multiple dimers.

Specificity mechanisms on NF-κB-regulated promoters

Specificity in transcriptional regulation has long been proposed to involve the combinatorial control of several transcription factors. Generally, NF-κB may not be sufficient to activate gene expression but may function together with cooperating transcription factors and coactivators (*Fig. 5*) to effect chromatin remodeling, recruitment of general transcription factors, open initiation complex formation, transcriptional initiation, and/or elongation (79). The

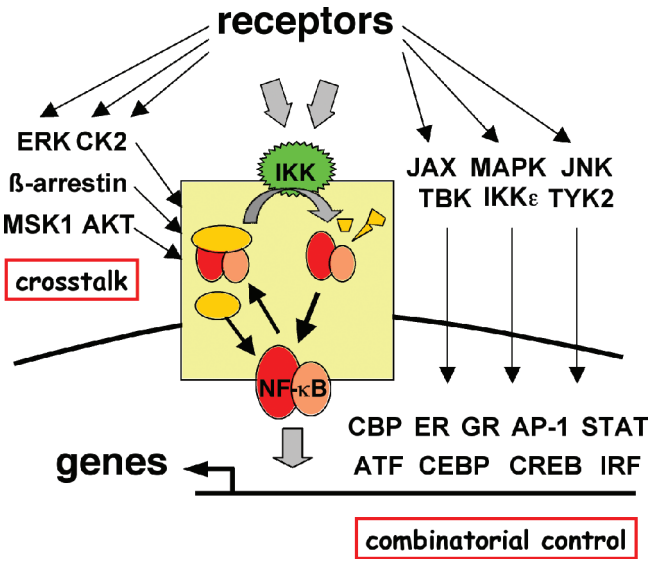

Fig. 5. Signaling crosstalk and combinatorial control. Stimuli that activate NF-κB also activate other signaling pathways that may modify the signal processing characteristics of the signaling module (crosstalk) or that may coordinately regulate the activity of other transcription factors to effect stimulus-specific gene expression and cellular responses.

ubiquitous histone acetyl transferase CBP/p300 binds RelA via two distinct interaction domains and is recruited to endogenous NF-κB target genes (80). However, a role in stimulus-specific gene activation has not been described, and recruitment can also be observed on promoters that are not induced. In contrast, the ankyrin repeat protein Bcl3, which is sometimes considered an IκB family member but has coactivator activity (81), appears to function stimulus-and promoter-specifically (82). Further studies are required to elucidate the regulatory mechanisms involving Bcl3.

NF-κB-responsive promoters contain consensus-binding sites for a variety of other transcription factors often clustered into enhancers. These factors include the constitutive transcription factor SP-1 and the inducible IRFs, STATs, ATFs, CEBPs, CREB, and AP-1, many of which have also been shown to interact with NF-κB directly. The enhancer of the IFN-β gene is best understood. This enhancer requires the assembly of an enhanceosome complex consisting of NF-κB, ATF2/c-Jun, and IRFs to recruit histone modification enzymes, effecting the repositioning of a core promoter nucleosome and recruitment of TFIID (83, 84). Interestingly, each of these transcription factors is inducible by different stimuli, but only the complete set, induced by viral infection, allows for IFN-β gene activation. The IL-2 gene promoter was shown to be regulated by cRel-containing dimers that effect nucleosome disassembly from regulatory sequences (85), while other transcription factors may regulate subsequent activation steps. Future studies on the combinatorial control of transcription factors on promoters ought to account for the dynamics by which each transcription factor is activated. Just as NF-κB activity is temporally regulated in a stimulus-specific manner, other, potentially cooperating factors are likely to exhibit their own stimulus-specific dynamics. Target promoters may require these to be temporally coincident or present in a particular sequence. Dynamic control of cooperating transcription factors is therefore likely to be a critical mechanism in stimulus-specific gene expression.

Investigating the molecular mechanism of the anti-inflammatory effect of steroid hormones has recently turned out to be a useful probe for stimulus- and promoter-specific coactivator function. The hormone-bound glucocorticoid receptor (GR) is recruited to many inflammatory promoters by binding RelA-containing dimers, thereby disrupting RelA's interaction with certain coactivators. The transcription factor IRF3, which is induced by a subset of TLRs, is an obligate coactivator (may not require its cognate DNA-binding site) for a subset of NF-κB-responsive promoters (86, 87). Interestingly, many of these IRF3-dependent promoters were found to be sensitive to repression by GR when activated by NF-κB-inducing stimuli (88). Similarly, the transcriptional elongation factor P-TEFb (an RNA polymerase CTD Ser2 kinase) was shown to be recruited upon cell stimulation to the NF-κB-responsive promoter of the IL-8 gene but not to the IκBα promoter. This coactivator requirement sensitizes the IL-8 gene to glucocorticoid-mediated inhibition (89). Whether a promoter requires a particular coactivator is determined by the promoter sequence, and as discussed below, that information may be encoded in the κB-site sequence itself.

Specificity mediated by NF-κB dimer isoforms

The fact that NF-κB comprises a family of homologous but different transcription factors suggests that specificity in the pathway may be achieved through their promoter-specific functions. As reviewed above, phenotypes of single and combination gene knockout mice indicate specific and overlapping molecular functions of the NF-κB proteins (47). A comprehensive comparison of single and double NF-κB knockouts with a panel of knockout murine embryonic fibroblasts did indeed reveal promoter-specific requirements for specific NF-κB proteins (90). In the case of the pro-inflammatory IL-12 p40 gene, whose induction in macrophages is largely cRel-dependent (91), the functional κB-site sequence exhibited lower affinity for RelA dimers than for cRel dimers, whose sequence preference appears to be broader (92). A similar working model of broader specificity for RelB-containing dimers is suggested by the observation that these dimers are able to activate promoters with unusual κB-site sequences (in addition to consensus sites) that are not bound by RelA dimers (65).

However, attempts to elucidate a general specificity code for NF-κB dimers *vis-à-vis* κB-site sequences have failed so far, both biochemically and genetically. In the comprehensive knockout study, the gene-specific requirement for specific NF-κB dimers did not neatly correlate with the specific sequence of the κB site (90), suggesting that other factors, most probably cooperating transcription factors, play a role in defining the specific NF-κB dimer requirement. Two additional observations support this view. First, promoters that are not activated by a specific NF-κB dimer may still show recruitment of the dimer, as determined by ChIP. Second, the specific dimer requirement of a gene was found to be stimulus-specific, i.e. the restriction imposed by a κB site for a subset of dimers pertains to one stimulus; it may be overcome by the coactivator or cooperating transcription factor induced by a second stimulus (82).

Despite the apparent involvement of cooperating factors, the stimulus specificity of two tandem κB sites was found to be transferable to a heterologous promoter. Interestingly, many NF-κB responsive promoters appear to have such a 'tandem κB site specificity module' that may functionally act like enhancer elements. By swapping κB sites from two such modules with differing NF-κB dimer specificities, it is possible to determine a code that is dependent on stimulus and cell type. Initial studies, focused on the ability to be activated by a RelA homodimer, revealed that specific κB-site sequences can be classified as being either restrictive (not allowing activation), permissive, or mediating the restriction when paired with a restrictive site (*Fig.* 6). These studies also suggested that the κB-site sequence determines the interaction repertoire of the bound NF-κB dimer with potential coactivators. In the case of the tandem κB site-specificity modules of the IP-10 and MCP-1 chemokine genes, a single base pair in the κB-site sequence appears to confer the requirement for the Bcl3 coactivator to allow for TNF-induced gene expression (*Fig.* 6). Transcription factor allostery affected by different binding sites has previously been proposed to account for functional specificity of the nuclear hormone transcription factors (93). In the case of NF-κB, an early comparison of NF-κB dimer bound to two different κB-site sequences revealed structural differences that correlated with functional differences (17).

These initial studies indicate that while the κB-site code is complex and determined at multiple levels of molecular interactions, genetic approaches complemented by extensive structural and biophysical studies will probably yield the important molecular mechanisms that determine the specificity of NF-κB regulation of gene expression. In addition, however, it is becoming clear that these mechanisms are operating in a dynamic manner. Early studies suggested that a p50 : p50 homodimer bound constitutively to the IFN-β promoter may give way to a p50 : RelA dimer upon cellular activation. More recently, time course studies by ChIP of NF–κB protein association with endogenous promoters revealed that the same promoter may be sampled by several different NF-κB dimers in a sequential manner following the activation. In particular, RelA-containing dimers initially (within 30 min) recruited to responsive promoters may be replaced at later times (during the second hour) by RelB-containing dimers, which are not susceptible to IκBα negative feedback inhibition. However, the functional consequence of this dynamic exchange remains unclear and may indeed be gene specific, as an RelB deficiency results in both decreases and increases in gene expression levels (A. H. and D. B., unpublished data). Transient association of RelA with promoters can in fact be regulated by proteasomal degradation that is critical in macrophages for limiting inflammatory responses. Interestingly, RelA phosphorylation of a specific serine by IKKα marks RelA for proteolysis (68). These observations emphasize the need to consider the dynamics of signaling by the family of NFκB dimers when studying the molecular mechanisms of NF-κB physiological function.

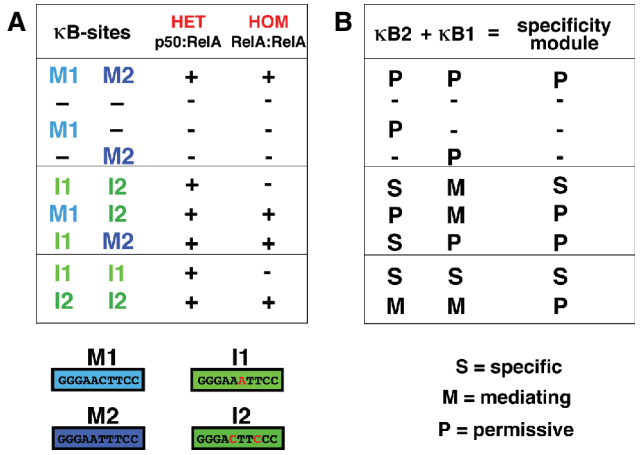

***Fig. 6.* Toward a nuclear factor (NF)-κB dimer specificity code.** (A) Two tandem κB sites are required for the expression of several NF-κB-responsive genes, and they are sufficient to confer a specific NF-κB dimer requirement to a heterologous promoter (82). Tandem combinations of κB sites derived from the MCP-1 or the IP-10 promoter (M1 and M2, and I1 and I2, respectively) show differential abilities to mediate gene activation by RelA homodimers (HOM) or only p50:RelA heterodimers (HET). (B) The above data suggest that each κB site within the tandem κB site specificity module can be classified as either imposing specificity for HETs (S = specific), allowing for homodimers function (P = permissive), or for mediating specificity imposed by an S site but not imposing it itself (M = mediating). As a result, a Boolean system of the three classes of κB sites can be constructed to specify whether a given promoter is permissive to the RelA:RelA homodimers (P) or specific for the p50:RelA HET (S).

Conclusion

NF-κB plays a central role in inflammation and in innate immunity by regulating the expression of numerous cytokines and chemokines, cell surface receptors, and adhesion molecules. By regulating the molecules that allow cells to communicate with each other, NF-κB plays a central role in the coordination of the multipronged pathogen-specific human immune response that culminates in development of adaptive immunity.

Extensive research efforts have already been devoted to describing NF-κB's role in many physiological and pathological processes. However, there is much more to learn. Given that NF-κB is so central and already a target of interest to the pharmaceutical industry, it is important to learn as

much as possible about its range of activities as a guide to developing agents that can affect some but not all of its activities. Important avenues in this regard are characterizing the accessory signaling pathways that impart specificity and the subtle biochemical but functionally relevant differences of isoforms within the NF-κB dimer family, the family of possible κB-site sequences, the IκB family, and the IKK family. Recent advances suggest that combining detailed genetic and biophysical studies will yield unexpected insights into the mechanisms that impart specificity.

Because the NF-κB activation process as well as its accessory signaling mechanisms are highly dynamic, mechanistic insights from genetic and biophysical studies ought to inform computational models. Recent advances already demonstrate that specificity in signaling can be imparted by dynamic control, suggesting that dynamic simulations with mathematical models are likely to provide important insights into specificity mechanism and to lead to identification of pharmacologic strategies that have stimulus-specific effects. In the longer term, *in silico* reconstruction of the NF-κB-signaling system and the molecular regulatory systems in which it is embedded will be critical to understanding how specific physiological signals not only control cellular behavior but also to the multiorgan process of immune development and immune function.

References

1. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 1986;**46**:705–716.
2. Lenardo MJ, Baltimore D. NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control. Cell 1989;**58**:227–229.
3. Sen R, Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein N-kappa B by a posttranslational mechanism. Cell 1986;**47**:921–928.
4. Baeuerle PA, Lenardo M, Pierce JW, Baltimore D. Phorbol-ester-induced activation of the NF-kappa B transcription factor involves dissociation of an apparently cytoplasmic NF-kappa B/inhibitor complex. Cold Spring Harb Symp Quant Biol 1988;**53**:789–798.
5. Baeuerle PA, Baltimore D. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. Cell 1988;**53**:211–217.
6. Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science 1988;**242**:540–546.
7. Baeuerle PA, Baltimore D. A 65-kappaD subunit of active NF-kappaB is required for inhibition of NF-kappaB by I kappaB. Genes Dev 1989;**3**:1689–1698.
8. Ghosh S, Gifford AM, Riviere LR, Tempst P, Nolan GP, Baltimore D. Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal. Cell 1990;**62**:1019–1029.
9. Zabel U, Baeuerle PA. Purified human I kappa B can rapidly dissociate the complex of the NF-kappa B transcription factor with its cognate DNA. Cell 1990;**61**:255–2653.
10. Traenckner EB, Wilk S, Baeuerle PA. A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. EMBO J 1994;**13**:5433–5441.
11. Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. Science 1995;**267**: 1485–1488.
12. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 1997;**388**: 548–554.
13. Rothwarf DM, Zandi E, Natoli G, Karin M. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature 1998;**395**:297–300.
14. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science 2002;**298**: 1241–1245.
15. Pomerantz JL, Baltimore D. Two pathways to NF-kappaB. Mol Cell 2002;**10**:693–695.
16. Huang DB, Vu D, Ghosh G. NF-kappaB RelB forms an intertwined homodimer. Structure (Camb) 2005;**13**:1365–1373.
17. Chen-Park FE, Huang DB, Noro B, Thanos D, Ghosh G. The kappa B DNA sequence from the HIV long terminal repeat functions as an allosteric regulator of HIV transcription. J Biol Chem 2002;**277**:24701–24708.
18. Croy CH, Bergqvist S, Huxford T, Ghosh G, Komives EA. Biophysical characterization of the free IkappaBalpha ankyrin repeat domain in solution. Protein Sci 2004;**13**:1767–1777.
19. Tam WF, Lee LH, Davis L, Sen R. Cytoplasmic sequestration of rel proteins by IkappaBalpha requires CRM1-dependent nuclear export. Mol Cell Biol 2000;**20**:2269–2284.
20. Lee SH, Hannink M. Characterization of the nuclear import and export functions of Ikappa B(epsilon). J Biol Chem 2002;**277**:23358–23366.
21. Tergaonkar V, Correa RG, Ikawa M, Verma IM. Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. Nat Cell Biol 2005;**7**:921–923.
22. Scott ML, Fujita T, Liou HC, Nolan GP, Baltimore D. The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. Genes Dev 1993;**7**:1266–1276.
23. Nelson DE, et al. Oscillations in NF-kappaB signaling control the dynamics of gene expression. Science 2004;**306**:704–708.
24. Kearns JD, Basak S, Werner SL, Huang CS, Hoffmann A. IkappaBepsilon provides negative feedback to control NF-kappaB oscillations, signaling dynamics, and inflammatory gene expression. J Cell Biol (in press).
25. Werner SL, Hoffmann A. What about dynamic control in signaling? The case of NF-kappaB. J Cell Sci (in press).
26. Claudio E, Brown K, Park S, Wang H, Siebenlist U. BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. Nat Immun 2002;**3**: 958–965.
27. Dejardin E, et al. The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. Immunity 2002;**17**:525–535.
28. Mordmuller B, Krappmann D, Esen M, Wegener E, Scheidereit C. Lymphotoxin and lipopolysaccharide induce NF-kappaB-p52 generation by a co-translational mechanism. EMBO Rep 2003;**4**:82–87.
29. De Smaele E, et al. Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. Nature 2001;**414**:308–313.

30. Pham CG, et al. Ferritin heavy chain upregulation by NF-kappaB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species. Cell 2004;**119**: 529–542.
31. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 1998;**17**:3247–3259.
32. Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF-kappaB signals induce the expression of c-FLIP. Mol Cell Biol 2001;**21**:5299–5305.
33. Okamoto T, Akuta T, Tamura F, van Der Vliet A, Akaike T. Molecular mechanism for activation and regulation of matrix metalloproteinases during bacterial infections and respiratory inflammation. Biol Chem 2004;**385**:997–1006.
34. Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M. NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Mol Cell Biol 1999;**19**:2690–2698.
35. Li J, et al. Novel NEMO/IkappaB kinase and NF-kappa B target genes at the pre-B to immature B cell transition. J Biol Chem 2001;**276**:18579–18590.
36. Li X, et al. IKKalpha, IKKbeta, and NEMO/IKKgamma are each required for the NF-kappa B-mediated inflammatory response program. J Biol Chem 2002;**277**:45129–45140.
37. Cardozo AK, et al. A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-dependent genes in primary rat pancreatic beta-cells. J Biol Chem 2001;**276**: 48879–48886.
38. Tian B, et al. Identification of NF-kappaB-dependent gene networks in respiratory syncytial virus-infected cells. J Virol 2002;**76**:6800–6814.
39. Tian B, Nowak DE, Jamaluddin M, Wang S, Brasier AR. Identification of direct genomic targets downstream of the nuclear factor-kappaB transcription factor mediating tumor necrosis factor signaling. J Biol Chem 2005;**280**:17435–17448.
40. Jiang X, Norman M, Li X. Use of an array technology for profiling and comparing transcription factors activated by TNFalpha and PMA in HeLa cells. Biochim Biophys Acta 2003;**1642**:1–8.
41. Zhao B, Stavchansky SA, Bowden RA, Bowman PD. Effect of interleukin-1beta and tumor necrosis factor-alpha on gene expression in human endothelial cells. Am J Physiol Cell Physiol 2003;**284**:C1577–C1583.
42. Zhao B, Bowden RA, Stavchansky SA, Bowman PD. Human endothelial cell response to gram-negative lipopolysaccharide assessed with cDNA microarrays. Am J Physiol Cell Physiol 2001;**281**: C1587–C1595.
43. Zarnegar B, He JQ, Oganesyan G, Hoffmann A, Baltimore D, Cheng G. Unique CD40-mediated biological program in B cell activation requires both type 1 and type 2 NF-kappaB activation pathways. Proc Natl Acad Sci USA 2004;**101**:8108–8113.
44. Martone R, et al. Distribution of NF-kappaB-binding sites across human chromosome 22. Proc Natl Acad Sci USA 2003;**100**:12247–12252.
45. Bouwmeester T, et al. A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. Nat Cell Biol 2004;**6**:97–105.
46. Rual JF, et al. Towards a proteome-scale map of the human protein-protein interaction network. Nature 2005;**437**:1173–1178.
47. Gerondakis S, Grossmann M, Nakamura Y, Pohl T, Grumont R. Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts. Oncogene 1999;**18**:6888–6895.
48. Siebenlist U, Brown K, Claudio E. Control of lymphocyte development by nuclear factor-kappaB. Nat Rev Immunol 2005;**5**:435–445.
49. Gerondakis S, Strasser A. The role of Rel/NF-kappaB transcription factors in B lymphocyte survival. Semin Immunol 2003;**15**:159–166.
50. Kontgen F, et al. Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. Genes Dev 1995;**9**: 1965–1977.
51. Pohl T, et al. The combined absence of NF-kappa B1 and c-Rel reveals that overlapping roles for these transcription factors in the B cell lineage are restricted to the activation and function of mature cells. Proc Natl Acad Sci USA 2002;**99**:4514–4519.
52. Horwitz BH, Scott ML, Cherry SR, Bronson RT, Baltimore D. Failure of lymphopoiesis after adoptive transfer of NF-kappaB-deficient fetal liver cells. Immunity 1997;**6**:765–772.
53. Horwitz BH, et al. The p65 subunit of NF-kappa B is redundant with p50 during B cell proliferative responses, and is required for germline CH transcription and class switching to IgG3. J Immunol 1999;**162**:1941–1946.
54. Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes Dev 1999;**13**:400–411.
55. Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med 2001;**194**:1861–1874.
56. Rosenwald A, Staudt LM. Gene expression profiling of diffuse large B-cell lymphoma. Leuk Lymphoma 2003;**44** (Suppl):S41–S47.
57. Sivakumar V, Hammond KJ, Howells N, Pfeffer K, Weih F. Differential requirement for Rel/nuclear factor kappa B family members in natural killer T cell development. J Exp Med 2003;**197**:1613–1621.
58. Schmidt-Supprian M, et al. Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. Proc Natl Acad Sci USA 2004;**101**:4566–4571.
59. Burkly L, et al. Expression of relB is required for the development of thymic medulla and dendritic cells. Nature 1995;**373**:531–536.
60. Yilmaz ZB, Weih DS, Sivakumar V, Weih F. RelB is required for Peyer's patch development: differential regulation of p52-RelB by lymphotoxin and TNF. EMBO J 2003;**22**:121–130.
61. Alcamo E, Hacohen N, Schulte LC, Rennert PD, Hynes RO, Baltimore D. Requirement for the NF-kappaB family member RelA in the development of secondary lymphoid organs. J Exp Med 2002;**195**:233–244.
62. Lo JC, et al. Coordination between NF-{kappa}B family members p50 and p52 is essential for mediating LT{beta}R signals in the development and organization of secondary lymphoid tissues. Blood (in press).
63. Weih F, Caamano J. Regulation of secondary lymphoid organ development by the nuclear factor-kappaB signal transduction pathway. Immunol Rev 2003;**195**:91–105.
64. Weih F, et al. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. Cell 1995;**80**: 331–340.
65. Bonizzi G, et al. Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB: p52 dimers. EMBO J 2004;**23**:4202–4210.
66. Han J, Ulevitch R. Limiting inflammatory responses during activation of innate immunity. Nat Immunol 2005;**12**: 1198–1205.
67. Lipniacki T, Paszek P, Brasier AR, Luxon B, Kimmel M. Mathematical model of NF-kappaB regulatory module. J Theor Biol 2004;**228**:195–215.
68. Lawrence T, Bebien M, Liu GY, Nizet V, Karin M. IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation. Nature 2005;**434**: 1138–1143.
69. Park JM, et al. Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis – CREB and NF-kappaB as key regulators. Immunity 2005;**23**:319–329.

70. Barken D, Wang CJ, Kearns J, Cheong R, Hoffmann A, Levchenko A. Comment on 'Oscillations in NF-kappaB signaling control the dynamics of gene expression'. Science 2005;**308**:52.
71. Cheong R, Bergmann A, Werner SL, Regal J, Hoffmann A, Levchenko A. Transient IKK activity mediates NF-kappa B temporal dynamics in response to a wide range of TNFalpha doses. J Biol Chem (in press).
72. Werner SL, Barken D, Hoffmann A. Stimulus specificity of gene expression programs determined by temporal control of IKK activity. Science 2005;**309**:1857–1861.
73. Wertz IE, et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature 2004;**430**:694–699.
74. Covert MW, Leung TH, Gaston JE, Baltimore D. Achieving stability of lipopolysaccharide-induced NF-kappaB activation. Science 2005;**309**:1854–1857.
75. Mattioli I, et al. Transient and selective NF-kappa B p65 serine 536 phosphorylation induced by T cell costimulation is mediated by I kappa B kinase beta and controls the kinetics of p65 nuclear import. J Immunol 2004;**172**:6336–6344.
76. Chen LF, et al. NF-kappaB RelA phosphorylation regulates RelA acetylation. Mol Cell Biol 2005;**25**:7966–7975.
77. Vermeulen L, De Wilde G, Notebaert S, Vanden Berghe W, Haegeman G. Regulation of the transcriptional activity of the nuclear factor-kappaB p65 subunit. Biochem Pharmacol 2002;**64**:963–970.
78. Muller JR, Siebenlist U. Lymphotoxin beta receptor induces sequential activation of distinct NF-kappa B factors via separate signaling pathways. J Biol Chem 2003;**278**:12006–12012.
79. Sheppard KA, et al. Transcriptional activation by NF-kappaB requires multiple coactivators. Mol Cell Biol 1999;**19**:6367–6378.
80. Zhong H, Voll RE, Ghosh S. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell 1998;**1**:661–671.
81. Fujita T, Nolan GP, Liou HC, Scott ML, Baltimore D. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev 1993;**7**:1354–1363.
82. Leung TH, Hoffmann A, Baltimore D. One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers. Cell 2004;**118**:453–464.
83. Agalioti T, Lomvardas S, Parekh B, Yie J, Maniatis T, Thanos D. Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell 2000;**103**:667–678.
84. Agalioti T, Chen G, Thanos D. Deciphering the transcriptional histone acetylation code for a human gene. Cell 2002;**111**:381–392.
85. Rao S, Gerondakis S, Woltring D, Shannon MF. c-Rel is required for chromatin remodeling across the IL-2 gene promoter. J Immunol 2003;**170**:3724–3731.
86. Sakaguchi S, et al. Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. Biochem Biophys Res Commun 2003;**306**:860–866.
87. Yamamoto M, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;**301**:640–643.
88. Ogawa S, et al. Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. Cell 2005;**122**:707–721.
89. Luecke HF, Yamamoto KR. The glucocorticoid receptor blocks P-TEFb recruitment by NFkappaB to effect promoter-specific transcriptional repression. Genes Dev 2005;**19**:1116–1127.
90. Hoffmann A, Leung TH, Baltimore D. Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. EMBO J 2003;**22**:5530–5539.
91. Sanjabi S, Hoffmann A, Liou HC, Baltimore D, Smale ST. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. Proc Natl Acad Sci USA 2000;**97**:12705–12710.
92. Sanjabi S, et al. A c-Rel subdomain responsible for enhanced DNA-binding affinity and selective gene activation. Genes Dev 2005;**19**:2138–2151.
93. Lefstin JA, Yamamoto KR. Allosteric effects of DNA on transcriptional regulators. Nature 1998;**392**:885–888.