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PERSPECTIVE

Understanding NF- κ B signaling via mathematical modeling

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Mammalian inflammatory signaling, for which NF- κ B is a principal transcription factor, is an exquisite example of how cellular signaling pathways can be regulated to produce different yet specific responses to different inflammatory insults. Mathematical models, tightly linked to experiment, have been instrumental in unraveling the forms of regulation in NF- κ B signaling and their underlying molecular mechanisms. Our initial model of the I κ B–NF- κ B signaling module highlighted the role of negative feedback in the control of NF- κ B temporal dynamics and gene expression. Subsequent studies sparked by this work have helped to characterize additional feedback loops, the input–output behavior of the module, crosstalk between multiple NF- κ B-activating pathways, and NF- κ B oscillations. We anticipate that computational techniques will enable further progress in the NF- κ B field, and the signal transduction field in general, and we discuss potential upcoming developments.

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Introduction

The transcription factor NF- κ B is a central inflammatory mediator, as it is essential for the majority of gene induction events in response to inflammatory cytokines as well as pathogen-derived substances. In unstimulated cells, NF- κ B is bound to I κ B proteins which hold it latent in the cytoplasm. Cellular stimulation with inflammatory agents results in

IKK-mediated phosphorylation of I κ B proteins, their ubiquitination, and proteasome-mediated proteolysis, allowing free NF- κ B to accumulate in the nucleus and bind the cognate κ B elements in target gene promoters (Box 1; reviewed in Hayden and Ghosh, 2008). Regulation of NF- κ B is important for the physiology of inflammation and immune activation, and misregulation of NF- κ B activity has been identified as a major culprit of chronic inflammatory diseases and cancer. As such understanding NF- κ B regulation has been a major focus of biochemical, mouse genetic, and human disease studies since its discovery more than 20 years ago (Sen and Baltimore, 1986).

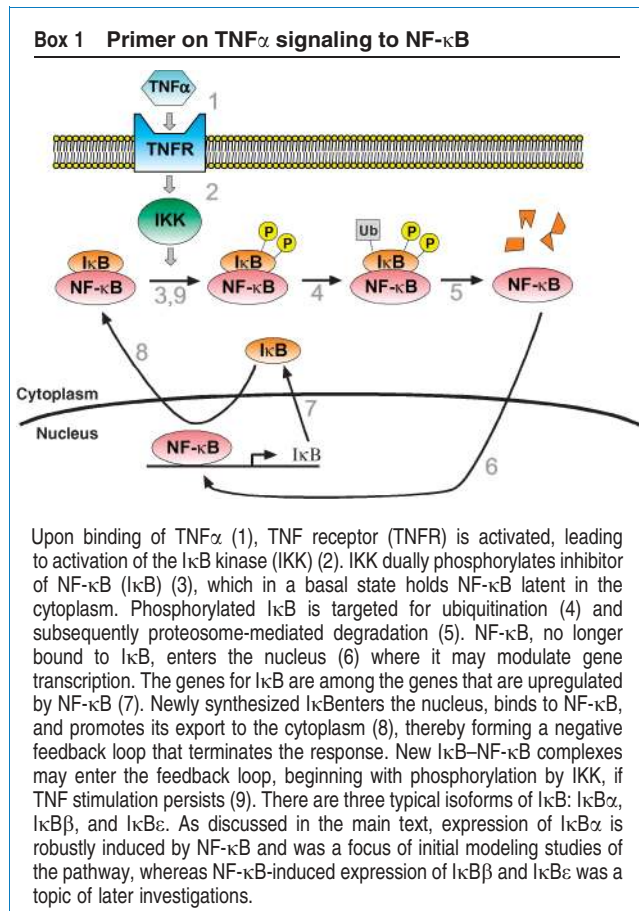
Major components of many signaling pathways that activate NF- κ B have been mapped, and this information is often summarized in pathway diagrams (e.g. Box 1). However, the dynamics of molecular level regulation are insufficiently captured by the static representation inherent in such diagrams. Mathematical models, on the other hand, can quantitatively describe how changes in signaling occur in space and time, enabling exploration of signaling pathways *in silico* (Box 2). The resulting insights can provide a theoretical framework and generate testable predictions for subsequent experimental studies. Experimental results likewise inform the development and refinement of mathematical models with predictive power. In this way, our understanding of cell signaling processes can be progressively advanced (Kearns and Hoffmann, 2008).

Here, we review how mathematical modeling has impacted our understanding of signaling through NF- κ B pathways. First, we summarize our original mathematical model, which is the predecessor of many models used to study the regulation of NF- κ B dynamics (Table I). Then, we describe how mathematical and computational models have been instrumental in increasing our understanding of the control of NF- κ B signaling. We also discuss the emerging areas of research in which mathematical models may shed light.

The original mathematical model of the I κ B–NF- κ B signaling module

NF- κ B activation involves stimulus-induced degradation of its inhibitor I κ B, which allows for its translocation to the nucleus. The earliest attempt to capture the dynamics of these events in mathematical equations was aimed at understanding how NF- κ B translocation and I κ B association/dissociation rate constants keep the majority of NF- κ B in an inactive state in resting cells (Carlotti *et al*, 2000). However, this work did not result in a model that allowed for computational simulations of the full NF- κ B activation and attenuation process.

Our interest was to understand the differential functions, if any, of the three I κ B isoforms (I κ B α , I κ B β , and I κ B ϵ) that modulate inflammatory activation of NF- κ B. Biochemical



studies had shown that all three sequester p65–p50, the predominant NF- κ B dimer, are degraded in response to stimulation with tumor necrosis factor alpha (TNF α) (Ghosh *et al*, 1998). Nevertheless, mice deficient in any one of these three I κ B proteins have distinct phenotypes, indicating that the I κ Bs have different and non-overlapping functions (Beg *et al*, 1995; Klement *et al*, 1996; Memet *et al*, 1999; Mizgerd *et al*, 2002). As time-course data, derived from electrophoretic mobility shift assays (EMSAs), indicated that the three I κ B proteins had differential dynamic control, we set out to construct a mathematical model of NF- κ B signaling to study the specific roles of each I κ B isoform in regulating the temporal control of NF- κ B (Hoffmann *et al*, 2002).

We defined the scope of the model to be that of the I κ B–NF- κ B signaling module, in which the IKK activity as an input to the model determines the NF- κ B activity over time. The model consisted of a system of differential equations based on mass action kinetics of the association/dissociation, synthesis/degradation, and translocation of IKK, I κ B, and NF- κ B species. Of the 34 independent model parameters, about one-third were derived from the extensive biochemical literature on NF- κ B, especially for the parameters of the Michaelis–Menten reactions of IKK-mediated I κ B phosphorylation. A further third, especially those parameters relating to species half-lives, transport rates, and I κ B–NF- κ B affinities, was constrained by published time-course data. We used a genetic approach to reduce the complexity of the signaling module to obtain the data used to fit the remaining

parameters (primarily mRNA and protein synthesis). By mouse reverse genetics, we obtained cells deficient in any two of the three I κ B isoforms, thereby enabling us to parameter fit three reduced models each containing only one I κ B isoform that were then combined into a wild-type cell model.

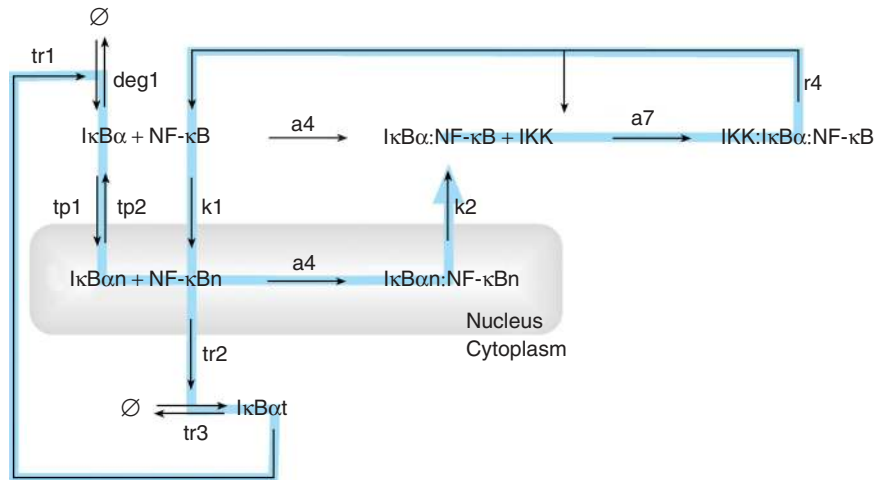
Exploration of the model with computational simulations resulted in two major insights. First, it described how differential functions of the I κ B isoforms could give rise to strikingly different NF- κ B dynamics in genetically reduced cells. The role of I κ B α , whose expression is induced by NF- κ B, was to provide negative feedback. This was aptly demonstrated by pronounced oscillations in NF- κ B activity in cells lacking the other isoforms (Figure 1A). The role of I κ B β and I κ B ϵ was to dampen these oscillations. When all three isoforms were present, the NF- κ B response was biphasic, with an initial NF- κ B activity rising and falling within \sim 1 h, followed by a late activation phase characterized by a steady intermediate level of activity (Figure 1B). Second, we explored the ‘temporal dose–response’ characteristics of the NF- κ B signaling module by simulating the NF- κ B response duration for different stimulus durations. The model predicted that the module would generate the initial phase of 60 min of NF- κ B activity even with much shorter stimuli, while only for longer lasting stimuli ($>$ 1 h) did the responses have durations proportional to the input duration. This prediction was confirmed by using EMSA on wild-type cells. Moreover, we found experimentally that the initial phase of NF- κ B activity is sufficient to drive the expression of a subset of inflammatory genes, while others require longer lasting NF- κ B activity. Hence, the functions of I κ B α , I κ B β , and I κ B ϵ combine to allow the signaling module to distinguish between short and longer lasting stimuli. A subsequent study of gene expression in single cells also found that some target genes require longer lasting TNF α stimulation than others (Nelson *et al*, 2004).

Two of the more significant advances provided by our study were that temporal dynamics of NF- κ B help control the expression of inflammatory genes, and that mathematical modeling could be extremely useful in understanding the molecular mechanisms that regulate NF- κ B dynamics. This spurred a number of subsequent modeling studies designed to further understand the regulation of NF- κ B dynamics, which we review below. Some of these studies were primarily theoretical in nature and pointed to interesting potential dynamical properties of NF- κ B signaling, whereas in others, modeling was tightly integrated with experiment leading to a plethora of unexpected insights into the mechanisms that control NF- κ B dynamics.

Mechanisms that control NF- κ B dynamics revealed by mathematical models

In this section, we highlight how mathematical and computational models have been applied with impressive success to direct or illuminate experimental studies to characterize additional feedback loops involving NF- κ B, IKK dynamics, crosstalk between inflammatory and non-inflammatory inducers of NF- κ B activity, and NF- κ B oscillations.

Box 2 Primer on modeling NF- κ B pathways using differential equations



The core of our original model of NF- κ B signaling is depicted below as a set of linked biochemical reactions. The diagram omits reactions (e.g. dissociation, reactions involving I κ B β and I κ B ϵ) that are present in the full model but are not essential to oscillatory behavior. Complexes are denoted by ':' and generic sources and sinks for synthesis and degradation are denoted by ' \emptyset .' Rate parameters are shown above their respective reactions, named according to the convention of the original model (Hoffmann *et al*, 2002). The input into the model is a step increase in IKK, which is a surrogate for TNF α stimulation. This allows the first reaction, IKK binding to I κ B α -NF- κ B complex (a7), to proceed. The steps of phosphorylation, ubiquitination, and proteosomal degradation of I κ B α within this complex are lumped into a single reaction whose products are free IKK and free NF- κ B (r4). NF- κ B enters the nucleus, denoted by the suffix 'n' (k1). This leads to synthesis of I κ B mRNA transcript, denoted by the suffix 't' (tr2). The half-life of the transcript is determined by tr3. Translation leads to synthesis of new I κ B α (tr1), whose half-life is determined by deg1. I κ B α can enter (tp1) and leave (tp2) the nucleus, and in the nucleus, I κ B α is also denoted with the suffix 'n.' Nuclear I κ B α and NF- κ B associate (a4), and together are exported to the cytoplasm (k2). In all, these steps form a negative feedback loop (also described in Box 1), whose overall sequence is shown by the blue arrow. Mass action kinetics are used to convert these biochemical reactions into a system of ordinary differential equations. For example, the equation for the time rate of change of cytoplasmic I κ B α -NF- κ B complex is given by

$$\frac{d[\text{I}\kappa\text{B}\alpha : \text{NF-}\kappa\text{B}]}{dt} = a4[\text{I}\kappa\text{B}\alpha][\text{NF-}\kappa\text{B}] + k2[\text{I}\kappa\text{B}\alpha\text{n} : \text{NF-}\kappa\text{Bn}] - a7[\text{I}\kappa\text{B}\alpha : \text{NF-}\kappa\text{B}][\text{IKK}]$$

where the terms show increases in the amount of complex due to association of I κ B α and NF- κ B (a4) and export of nuclear complex (k2), and decreases in the amount of complex due to association with IKK (a7). Equations are written in this way for each chemical species. In the full version of the original model, similar reactions govern the behavior of I κ B β and I κ B ϵ , resulting in additional differential equations. In this model formulation, the parameters are biochemical rates of association, dissociation, catalysis, transport, synthesis, and degradation. Thus, their values may be quantitatively measured or constrained by biochemical experiments. The procedure we used is summarized in the main text. Finally, to run the model, the initial concentrations of each species must be specified. (Running the model means to numerically solve the differential equations, e.g. with Mathematica's NDSolve function, to determine time courses of the concentrations of each species.) We initialized the model with a biologically plausible total level of NF- κ B (0.1 μ M) with all other concentrations set to zero. The basal state of the cell (non-stimulated) is simulated by running the model starting from this initial state until it reaches steady state. At steady state, NF- κ B is found in the cytoplasm and nucleus, as well as free or complexed with I κ B, but is predominantly found complexed in the cytoplasm in accordance with experimental observations. Following a step increase in IKK, the model can be further run to simulate the effects of TNF stimulation.

Multiple feedback loops

The original mathematical model of the I κ B-NF- κ B signaling module revealed that NF- κ B-induced expression of I κ B α provides negative feedback and that this feedback is a major determinant of NF- κ B temporal dynamics. Subsequent studies, integrating experimental analysis and computational models, have shown that additional feedback mechanisms also control NF- κ B activity. One such loop involves I κ B ϵ , which like I κ B α , is expressed after TNF α stimulation in an NF- κ B-dependent manner (Tian *et al*, 2005). Unlike I κ B α , however, I κ B ϵ transcription is delayed by about 45 min relative to the onset of nuclear NF- κ B activity, as revealed by cells deficient in I κ B α and I κ B β (Kearns *et al*, 2006). Intuitively, delayed I κ B ϵ induction might provide oscillatory feedback in antiphase with I κ B α feedback, which combine to provide steady overall levels of I κ B with concomitant steady NF- κ B activity. A computational model derived from the original model encapsulating this idea predicted that the duration of

NF- κ B activity in response to a transient (45 min) TNF α stimulation would be prolonged in cells deficient in both I κ B α and I κ B ϵ , compared to cells deficient in only one of these isoforms, or to wild-type cells. This prediction, confirmed by EMSA, indicated that I κ B ϵ is capable of providing post-induction repression of NF- κ B. Likewise, the expression of inflammatory genes is prolonged in the I κ B α ^{-/-}I κ B ϵ ^{-/-} cells compared to I κ B α ^{-/-} and wild-type cells, providing functional evidence for the importance of I κ B ϵ in terminating the inflammatory response (Kearns *et al*, 2006). Overall, the negative feedbacks provided by I κ B α and I κ B ϵ appear to work in tandem to ensure rapid post-induction repression of NF- κ B, while suppressing sustained oscillations, thus solving a classic shortcoming of simple linear control systems (Coughanowr, 1991).

In addition to intracellular feedback due to I κ B α and I κ B ϵ , extracellular feedback might arise through autocrine signaling. A prime example of this phenomenon relative to the NF- κ B pathway was found while exploring cell responses to

Table I Comparison of published NF- κ B models

Model	Predecessor	Feedback	Major changes from predecessor
<i>The original mathematical model of NF-κB signaling</i>			
Hoffmann <i>et al</i> (2002)	Carlotti <i>et al</i> (2000)	Inducible I κ B α Constitutive I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • Responsive to IKK stimulus • IκBα negative feedback loop
<i>Direct descendants of the original model</i>			
Covert <i>et al</i> (2005)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α Constitutive I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • LPS stimulus modeled as two additive signals offset in time • Transcription and translation rates were re-fit
O'Dea <i>et al</i> (2007)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α Constitutive I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • IκB degradation rates were updated based on experimental measurements
Cheong <i>et al</i> (2006)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α , Constitutive I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • IKK time-course generator was added • Transcription, translation, and degradation rates were re-fit • Nuclear-cytoplasmic volume ratio was added
Kearns <i>et al</i> (2006)	O'Dea <i>et al</i> (2007)	Inducible I κ B α Delayed inducible I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • IκBβ and IκBϵ are inducible with a 45 min delay • IκB degradation rates were altered to fit new data
Werner <i>et al</i> (2005)	Kearns <i>et al</i> (2006)	Inducible I κ B α Delayed inducible I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • Cubic transcription rate • LPS modeled by using its IKK time course as an input
Moss <i>et al</i> (2008)	Identical to the model described in Werner <i>et al</i> (2005)		
O'Dea <i>et al</i> (2008)	Werner <i>et al</i> (2005)	Inducible I κ B α Delayed inducible I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • Some rate parameters were modified to model the effect of UV-induced NF-κB activity
Mathes <i>et al</i> (2008)	Werner <i>et al</i> (2005)	Inducible I κ B α Delayed inducible I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • Some rate parameters were modified to model the effect of IκBα mutants on NF-κB signaling
Basak <i>et al</i> (2007)	Werner <i>et al</i> (2005)	Inducible I κ B α , p100 Delayed inducible I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • Introduction of the IκB species p100 • LPS or TNF induces IKK2-mediated IκB degradation • LTβ induces IKK1-mediated p100 degradation
<i>Analysis of the original model by MR White and colleagues</i>			
Nelson <i>et al</i> (2004)	Identical to the model described in Hoffmann <i>et al</i> (2002)		
Ihekwa <i>et al</i> (2004)	Identical to the model described in Hoffmann <i>et al</i> (2002)		
Ihekwa <i>et al</i> (2005)	Identical to the model described in Hoffmann <i>et al</i> (2002)		
Ihekwa <i>et al</i> (2007)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α Constitutive I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • Identical to predecessor except some IKK-related parameters changed to match measurements based on experiments where cells were stimulated with IL-1
<i>NF-κB models by M Kimmel and colleagues</i>			
Lipniacki <i>et al</i> (2004)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α Inducible A20	<ul style="list-style-type: none"> • IκBβ and IκBϵ were removed from predecessor and A20 negative feedback loop was added • New assumptions about IKK activation and deactivation • Nuclear-cytoplasmic volume ratio was added • Transcription and translation rates were re-fit
Lipniacki <i>et al</i> (2006)	Lipniacki <i>et al</i> (2004)	Inducible I κ B α Inducible A20	<ul style="list-style-type: none"> • Stochastic translation and transcription • Some parameters were re-fit
Lipniacki <i>et al</i> (2007)	Lipniacki <i>et al</i> (2006)	Inducible I κ B α Inducible A20	<ul style="list-style-type: none"> • Introduction of TNF receptor and IKK kinase • Stochastic TNF receptor activation and IκBα/A20 transcription
Fujarewicz <i>et al</i> (2007)	Lipniacki <i>et al</i> (2004)	Inducible I κ B α Inducible A20	<ul style="list-style-type: none"> • Equations identical to predecessor but parameters were re-fit

Table I Continued

Model	Predecessor	Feedback	Major changes from predecessor
Joo <i>et al</i> (2007)	Identical to the model described in Lipniacki <i>et al</i> (2004)		
<i>Other descendants of the original model</i>			
Sung and Simon (2004)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α	<ul style="list-style-type: none"> • IκBβ and IκBϵ are removed from predecessor • NF-κB induction of IκBα has an explicit transcriptional time delay • Some parameters were re-fit • IκBβ and IκBϵ are removed, and IκBα has linear transcription rate • Whole model is stochastic • Reduces predecessor to a three-component system with five dimensionless parameters
Hayot and Jayaprakash (2006)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α	
Krishna <i>et al</i> (2006)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α	
Park <i>et al</i> (2006)	Hoffmann <i>et al</i> (2002)	Inducible I κ B α Constitutive I κ B β , I κ B ϵ	<ul style="list-style-type: none"> • Explicit TNF receptor to IKK pathway • IKK activity was affected by factors X and Y representing effects of HBV infection
<i>Other NF-κB models</i>			
Cho <i>et al</i> (2003)	None	No inducible factors	<ul style="list-style-type: none"> • Tree-like signaling pathway structure with no feedback loops • TNFα leads either to apoptosis (FADD) or proliferation (NF-κB)
Monk (2003)	None	Inducible I κ B α	<ul style="list-style-type: none"> • Proposes NF-κB oscillations derive from time delay of IκBα transcription
Janes <i>et al</i> (2005)	None		<ul style="list-style-type: none"> • Partial least-squares regression on a large compendium of cytokine signaling data
Janes <i>et al</i> (2006)	Identical to the model described in Janes <i>et al</i> (2005)		
Piotrowska <i>et al</i> (2006)	None	No inducible factors	<ul style="list-style-type: none"> • Two-component system with five dimensionless parameters • Negative correlation between IκBα and NF-κB is directly assumed • Proliferation rate is a function of NF-κB • Agent-based stochastic simulation • Incorporates events from receptor activation to NF-κB nuclear import
Pogson <i>et al</i> (2006)	None	No inducible factors	<ul style="list-style-type: none"> • TNFα leads either to apoptosis (caspase) or survival (NF-κB) • IκBβ and IκBϵ are not present • Parameters were taken from a variety of sources
Rangamani and Sirovich (2007)	None	Inducible I κ B α Inducible IAP	

lipopolysaccharide (LPS). LPS is a component of bacterial cell walls that serves as an important signal of infection activating two intracellular pathways that branch at the receptor level, respectively dependent on MyD88 and Trif. The NF- κ B activity in response to persistent LPS is normally steady over time, but it is oscillatory when either the Trif- or MyD88-dependent pathway is isolated by knockout of MyD88 and Trif, respectively (Covert *et al*, 2005). Reminiscent of I κ B α and I κ B ϵ , the Trif- and MyD88-dependent oscillations are out of phase. Computational modeling based on the original model indicated that the reason that the oscillations are out of phase is that the Trif- and MyD88-dependent pathways have similar activation kinetics, but the Trif-dependent pathway is activated 30 min after the MyD88-dependent pathway. A search for the biochemical mechanism underlying this delay uncovered an autocrine signaling loop. Specifically, the MyD88-dependent pathway was found to lead to fast, direct activation of NF- κ B, whereas the Trif-dependent pathway resulted in

slow indirect NF- κ B activation via TNF α production, secretion, and subsequent autocrine signaling (Figure 2). Interestingly, the same autocrine mechanism ensures that NF- κ B activity is steady not only in response to persistent LPS but also to transient LPS stimulation as well (Werner *et al*, 2005).

These discoveries suggest that mathematical modeling will be useful in understanding many other potential feedbacks involved in the regulation of NF- κ B. For example, the expression of the third inhibitor isoform, I κ B β , is weakly upregulated by TNF α (Kearns *et al*, 2006) and, although the removal of this inhibitor does not unmask oscillations, some more subtle signaling defects are likely to be present. The NF- κ B subunit RelB (Bren *et al*, 2001), the p50 subunit precursor p105 (Ten *et al*, 1992), and the p52 subunit precursor p100 (Lombardi *et al*, 1995), are all potentially expressed in response to TNF α , which could result in a change in NF- κ B dimer composition that could in turn affect all other transcriptionally mediated feedback loops. Likewise, NF- κ B

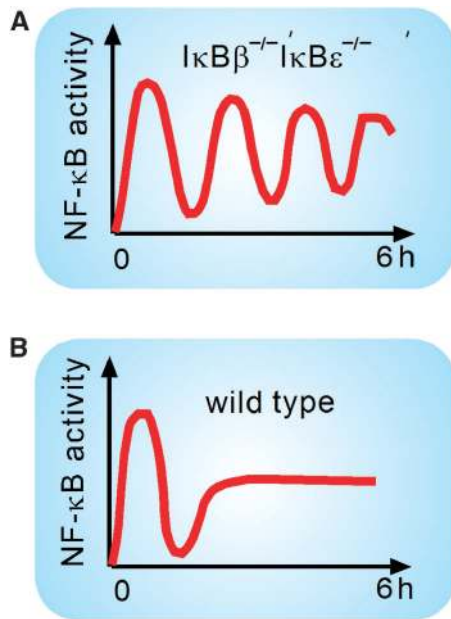


Figure 1 Schematic of NF- κ B dynamics in response to persistent TNF α . **(A)** Oscillatory time course of NF- κ B in response to TNF α in cells whose only classical I κ B is I κ B β (see also BioModels database <http://www.ebi.ac.uk/biomodels>, accession ID BIOMD0000000139). **(B)** Characteristic biphasic time course of NF- κ B signaling in response to TNF α in various wild-type cells. NF- κ B activity peaks around 30 min, drops to basal levels around 1 h, and rises to an intermediate level thereafter (see also BioModels accession ID BIOMD0000000140).

may be subject to a wide variety of extracellular feedback mechanisms, especially through autocrine signaling. NF- κ B target genes include the cytokines TNF α (Collart *et al*, 1990; Shakhov *et al*, 1990), many interleukins (Pahl, 1999), and lymphotoxin- β (LT β) (Kuprash *et al*, 1996), all of which are direct activators of NF- κ B. Well-defined computational models should prove useful in unraveling such complex feedback-rich signaling systems, addressing among other questions the roles of individual feedbacks and the need for all the feedbacks to be in place.

Control of NF- κ B dynamics through IKK

We defined the input of our original mathematical model of the I κ B–NF- κ B signaling module to be IKK, rather than TNF α or other extracellular ligands. This raised the question of how IKK dynamics control the downstream NF- κ B dynamics and how IKK activity itself is regulated.

It is apparent that IKK dynamics are important in controlling the timing of NF- κ B activity. Experimentally, we found that the initial phase of NF- κ B activity invariably lasted 60 min in response to different concentrations of TNF α (Cheong *et al*, 2006), paralleling the response to different durations of exposure to TNF α (Hoffmann *et al*, 2002). We found that the original pathway model failed to reproduce this behavior, despite an exhaustive attempt to refit the parameter values. This suggested that the model was incomplete, and perhaps omitted an important biochemical interaction needed to explain the observed dynamics. We surmised that IKK, whose regulation was not represented in detail in the original model,

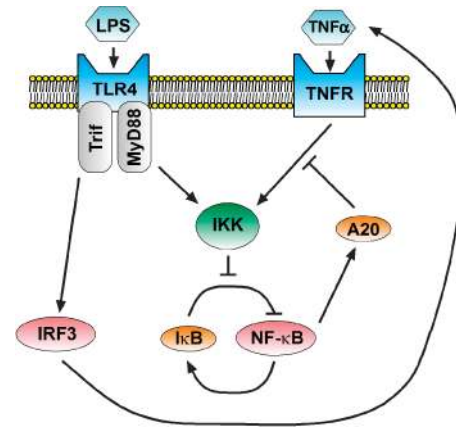


Figure 2 Feedback loops in NF- κ B signaling. IKK may be activated by the TNF α signaling pathway as well as the MyD88-dependent arm of the LPS signaling pathway. IKK leads to NF- κ B activity, which is regulated by a negative feedback loop involving I κ B (described in detail in Box 1), as depicted in the lower center. TNF α -induced NF- κ B activity also leads to A20 expression, and subsequent decrease in IKK activation. Also, the Trif-dependent arm of the LPS-signaling pathway activates the transcription factor interferon regulatory factor-3 (IRF3), leading to TNF α expression and subsequent autocrine signaling. Thus, A20 and TNF form feedback loops that regulate NF- κ B activity.

played an important role in determining the NF- κ B dynamics. By examining the model's responses to various IKK time courses, we found that the fixed duration of the initial phase of NF- κ B activity could be explained if the IKK activity was sharply attenuated. Specifically, the model predicted that at any TNF α dose, the IKK activity rises quickly upon exposure to TNF α , peaks after 5–10 min, and drops to a low but positive level after another 10–20 min. Experiments utilizing IKK assays validated this prediction (Cheong *et al*, 2006), indicating that the specific IKK dynamics are essential for maintaining a normal biphasic NF- κ B response. Importantly, this study showed how incongruities between models and experiments can be exploited to further understand the signaling system of interest.

More generally, the NF- κ B dynamics are sensitive to the timing and duration of the IKK activity. As discussed above, both in model and experiment, a peaked IKK profile, i.e. one that rises quickly then falls quickly, generates a transient NF- κ B response of fixed duration. In contrast, an IKK profile that plateaus, i.e. rises slowly to a sustained level, results in a delayed rise to a sustained level of NF- κ B activity. Importantly, these different IKK dynamics help enable stimulus-specific responses (Figure 3). For example, the peaked IKK profile results from transient TNF α stimulation, whereas the sustained IKK activity can result from transient LPS stimulation. Furthermore, these different IKK profiles, which in turn result in the different NF- κ B dynamics, allow for some genes to be specifically expressed in response to LPS and others to be specifically expressed in response to TNF α , even though the expression of these genes are all regulated by NF- κ B (Werner *et al*, 2005).

One important determinant of the IKK dynamics is A20, which inhibits IKK activation by modifying the ubiquitination pattern of a subunit of the TNF receptor complex (Wertz *et al*, 2004). The expression of A20 itself is induced by TNF α in an

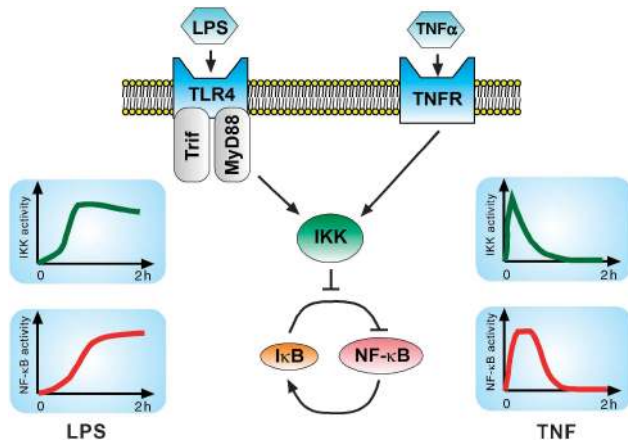


Figure 3 Schematic of stimulus-specific NF- κ B responses. Both TNF α and LPS activate NF- κ B through IKK, yet the NF- κ B responses to each are different. In response to a 45-min pulse of TNF α , NF- κ B activity rises quickly then terminates after approximately 60 min (bottom right). In contrast, in response to a 45-min pulse of LPS, NF- κ B activity rises slowly over 2 h (bottom left). The NF- κ B response correlates with the IKK activity profile, which is highly peaked in response to TNF α (upper right) but sustained in response to LPS (upper left). This illustrates how IKK helps to mediate stimulus-specific NF- κ B responses.

NF- κ B-dependent manner (Figure 2). This fact led to the development of a version of the original model that suggested that A20-mediated negative feedback is sufficient to produce the sharply peaked IKK activity profile resulting from persistent TNF α stimulation (Lipniacki *et al*, 2004). However, initial experiments could not verify this prediction (Cheong *et al*, 2006) and the mechanism that leads to a rapid attenuation of TNF α -induced IKK activity remains an open question (Delhase *et al*, 1999; Cheong *et al*, 2006; Schomer-Miller *et al*, 2006). Nonetheless, A20 is clearly important in inhibiting late IKK activity and is required for the drop in NF- κ B activity that separates the early and late phases in response to TNF α (Lee *et al*, 2000; Werner *et al*, 2005). Computational models thus point to the gap of current knowledge about A20 and IKK regulation in general as a barrier to further understanding of NF- κ B dynamics, and modeling work in this area should prove fruitful for additional studies integrating models and experiments.

Crosstalk between the I κ B–NF- κ B module and other pathways

NF- κ B is activated by numerous inflammatory stimuli, such as TNF α and LPS as discussed above, and also by many non-inflammatory stimuli (Hayden and Ghosh, 2004). One such stimulus is LT β , a cytokine implicated in the normal development of lymph nodes. Unlike classical inflammatory stimuli, LT β -mediated activation of NF- κ B does not occur through the degradation of NF- κ B-bound I κ B (Beinke and Ley, 2004). Rather, it occurs through degradation of the inhibitory domain of NF- κ B-bound p100, an NF- κ B protein precursor that, as a homodimeric complex, has I κ B-like function. Furthermore, p100 is an NF- κ B target gene whose expression can be stimulated by TNF α , leading to potential crosstalk between the TNF α and LT β pathways. Specifically, an expanded version

of the original model that included the classical I κ Bs and p100 predicted that exposing cells to TNF α leads to a greater percentage of NF- κ B molecules bound to p100 instead of to the classical I κ Bs, thereby priming the cells to subsequent LT β exposure. Indeed, experimentally, LT β -induced NF- κ B activity can be increased \sim 3-fold in TNF-primed versus naïve cells, with concomitant increases in expression of NF- κ B-responsive genes (Basak *et al*, 2007).

Another non-inflammatory activator of NF- κ B is ultraviolet (UV) irradiation. One of the effects of UV irradiation is bulk arrest of translation in a dose-dependent manner, which inhibits basal and induced synthesis of I κ B. We recently showed, that although NF- κ B is liberated when free I κ B and NF- κ B-bound I κ B are gradually turned over, the NF- κ B signaling module is actually remarkably robust to such metabolic perturbations (O’Dea *et al*, 2008). However, UV can dramatically amplify the response to simultaneous inflammatory stimulation. This synergy has implications for how inflammation can enhance the effects of cancer-associated stresses (O’Dea *et al*, 2008).

NF- κ B can also be activated indirectly by signaling pathways that do not principally involve NF- κ B. For example, TNF α , through activation of IKK and NF- κ B, can induce the secretion of transforming growth factor- α (TGF α), leading to autocrine stimulation of the epidermal growth factor receptor. Taken together, TNF α and TGF α induce production of interleukin-1, providing an autocrine signal that can bring about a second episode of IKK and NF- κ B activity (Janes *et al*, 2006). This helps to explain why, for example, IKK activity can be better predicted computationally from the combination of growth factor and inflammatory signaling data versus inflammatory signaling data alone (Janes *et al*, 2005, 2006).

NF- κ B oscillations

Our analyses of the I κ B–NF- κ B signaling module concluded that oscillations in NF- κ B activity, primarily driven by negative feedback through I κ B α , underlie biphasic NF- κ B dynamics. These oscillations are largely hidden in wild-type cells by the effects of I κ B β and I κ B ϵ (Hoffmann *et al*, 2002), and oscillations do not seem to alter gene expression programs when compared to the biphasic response (Barken *et al*, 2005), raising doubts about the functional significance of oscillations. Nonetheless, the apparent mathematical and biochemical complexity underlying the existence and particular shape of these oscillations intrinsically begs the question of how to generate and control them. These questions have so far been primarily addressed through computational analysis.

Negative feedback is a common way to achieve oscillatory behavior. Indeed, a simple negative feedback system comprised of two components that interact linearly is sufficient to generate oscillations (Hoffmann *et al*, 2002), but it is important to note that this abstraction is fundamentally different from the I κ B–NF- κ B module. Linear systems do not require persistent stimulation to exhibit undamped oscillations whereas the module does. Also, the mathematical dependency between individual parameters and oscillation frequency (e.g. monotonic relationship versus existence of an optimum) does not translate even qualitatively from the linear system to the module (R Cheong and A Levchenko, unpublished

observations). Thus, components in the module and their nonlinear interactions play important roles in controlling and shaping NF- κ B oscillations. Different aspects of NF- κ B oscillations, such as the timing and amplitude of peaks and troughs, are sensitive to different parameters in the original model, as measured by sensitivity coefficients (an analog of metabolic control coefficients). Some parameters are predicted to be broadly important for nearly all aspects of oscillations, and they all relate to reactions involving I κ B α (Ihekwa *et al*, 2004; Joo *et al*, 2007). These parameters cooperate in a complex, nonlinear way to modulate oscillations (Ihekwa *et al*, 2005), and overall, their effects on the timing and amplitude of the initial peak can be rationalized based on their contribution to total I κ B levels and the speed of the feedback loop (Cheong *et al*, 2006; Mathes *et al*, 2008; Moss *et al*, 2008). Interestingly, the highly sensitive parameters correlate well with a minimal subset of reactions from the original model that sustain oscillations (Box 2). Additionally, a condensed model involving only NF- κ B, I κ B α , and I κ B α mRNA still oscillates (Krishna *et al*, 2006), and in principle, a model with only NF- κ B and I κ B α with transcriptional delay can as well (Monk, 2003). Taken together, these theoretical perspectives indicate that the I κ B α portion of the module is indeed the strongest generator of oscillations.

Interest in oscillations was further spurred by observations in which the NF- κ B activity spiked repetitively ('spiky oscillations') in cells overexpressing fluorescent protein-tagged NF- κ B or I κ B α , with the timing and frequency of spikes varying from cell to cell (Nelson *et al*, 2004). This is distinctly different than the biphasic dynamics observed in the population average (Hoffmann *et al*, 2002), and reconciling the two has become an important goal of mathematical analysis. Our statistical analysis of NF- κ B activity measured by immunocytochemistry in single wild-type cells indicates that biphasic population dynamics is easily distinguished from an ensemble of individually oscillating cells, regardless of the mechanism underlying spiky oscillations (Barken *et al*, 2005). The intuitive conclusion, also supported by computational analysis, was that overexpression of NF- κ B or I κ B α components alters the oscillatory potential of the module. Others have attempted to attribute spiky oscillations and their variations from cell to cell to fluctuations in the rates of the chemical reactions comprising the pathway. Full stochastic simulation of a module in which the only I κ B species is I κ B α indicates that intrinsic biochemical randomness results in minimal deviation from the deterministic NF- κ B response unless transcription and translation rates have been badly estimated (Hayot and Jayaprakash, 2006). Rather, fluctuations in extrinsic factors, such as the number of molecules of active IKK or NF- κ B, need to be invoked to reconcile single live cell and average responses. However, these conclusions are at odds with simulations of other I κ B α -only models (Lipniacki *et al*, 2006, 2007), in which only a few biochemical reactions need to be stochastic to generate distributions of responses similar to those obtained in live cells. Differences in parameter values or the inclusion of an A20 feedback loop in the latter models (Lipniacki *et al*, 2004) may explain these differing conclusions. In any case, at minimum, accurate measurements of I κ B transcription and translation rates are needed to test the role of stochasticity in individualized cell responses.

Emerging developments in mathematical modeling of NF- κ B signaling

As seen above, computationally oriented studies have led to numerous and varied insights into the molecular mechanisms that regulate NF- κ B dynamics and inflammatory gene expression, and will surely continue to do so in the future. In this section, we highlight other aspects of NF- κ B biology for which mathematical modeling is likely to play an important role.

Information encoding and decoding

Secretion of NF- κ B-activating cytokines like TNF α is one way in which one cell can communicate to another and alter its behavior. One general question is what information is conveyed by secreted signals, how this information is encoded by the signaling cell, and how it is interpreted by the receiving cell. The unique temporal dynamics of NF- κ B responses to TNF α provides a model system to address the principles underlying cell-cell communication.

For TNF α , it is possible to use changes in its concentration over time to transmit information about the distance between the signaling and receiving cells. Specifically, in a local infection, a macrophage will secrete a brief pulse of TNF α in a self-limited manner. Because of the effect of diffusion, nearby cells experience temporal patterns of changes in TNF α concentration that depend on the separation distance: the concentration experienced by a cell drops exponentially and while the duration of exposure to the cytokine increases modestly with distance. Experimentally, we observed that NF- κ B is able to respond to amounts of TNF α that vary over several orders of magnitude, including very small ones. A model incorporating these observations, therefore, predicted that cells in a wide region around a local infection would mount an inflammatory defense. Moreover, because the amplitude of NF- κ B activity scales according to the logarithm of TNF α concentration, the model also predicts that NF- κ B responses drop roughly linearly with distance. Thus, cells near the infection would mount a vigorous inflammatory defense, whereas cells further away would have a tempered response, suggesting that the TNF α -NF- κ B pathway is optimized so that cells respond in a way commensurate with their distance from danger (Cheong *et al*, 2006).

We anticipate that mathematical and computational models tightly coupled to experimental analysis will be indispensable in further understanding the information processing characteristics of NF- κ B pathways. Because modeling to date has been very successful in demonstrating how dynamic IKK signals are transformed into dynamic NF- κ B signals by the I κ B-NF- κ B module (Werner *et al*, 2005; Cheong *et al*, 2006), we especially look forward to progress in understanding events upstream of IKK or downstream of NF- κ B. For example, multiple cytokine signaling pathways converge on IKK, but how each transmits information through IKK is poorly understood, as is how multiple cytokines convey information simultaneously through the same module. On the downstream end, different NF- κ B-responsive genes are expressed after different durations of NF- κ B activity (Hoffmann *et al*, 2002; Barken *et al*, 2005), but the basis of these differential responses is unknown. Combining pathway models with mathematical

analysis of promoters and enhancers is likely to shed light on this issue (Krishna *et al*, 2006).

Rational drug targeting

NF- κ B is involved in numerous physiologic responses, such as inflammation and apoptosis, and is implicated in myriad diseases like arthritis, autoimmune and inflammatory disorders, and cancer (Kumar *et al*, 2004). As such, numerous anti-inflammatory compounds are under development to target NF- κ B (Karin *et al*, 2004), and mathematical models are beginning to be used to understand how these potential drugs affect NF- κ B signaling.

One initial study in this direction examined the effect of three drug classes—inhibitors of IKK, the proteasome, and nuclear import machinery—on NF- κ B oscillations in response to TNF α (Sung and Simon, 2004). The effect of each class was simulated by altering the appropriate kinetic rate parameters in a simplified version of the original model containing only one I κ B-like species. In this way, NF- κ B oscillations were predicted to be disrupted with high doses of IKK or proteasome inhibitors, or low doses of nuclear import inhibitors. Similarly, another study predicted that an IKK inhibitor dampens the NF- κ B response to interleukin-1 (Ihekweba *et al*, 2007). These types of simulations could potentially be used to further understand drug specificity or the effect of multiple drugs applied simultaneously.

In addition, drug-targeting studies may benefit from extending this idea further, that is, by performing a ‘computational drug screen.’ Each kinetic rate parameter in the I κ B–NF- κ B module represents a potential target for modulation by a drug, so we are studying how sensitive the biphasic NF- κ B response to TNF α is to alterations in each parameter. For example, we find that the initial transient phase but not the late sustained phase of NF- κ B activity is robust to variations in the values of the parameters that control the half-life of I κ B α (D Barken *et al*, in preparation). This suggests that even drugs that target reactions within the central NF- κ B signaling module may in fact have selective effects, for example, by inhibiting prolonged inflammation without completely abrogating acute responses. This surprising possibility would be difficult to foresee by qualitative reasoning alone, but quantitative predictions provided by modeling are crucial in rationally identifying rate-limiting reactions for specific phases of the NF- κ B temporal profile. We anticipate that similar methods will prove useful in rational selection of drug targets to mediate highly specific therapeutic effects.

Other trends in applications of NF- κ B models

Paralleling the many physiological roles of NF- κ B, models of NF- κ B signaling are beginning to be applied in a variety of contexts. For example, TNF α -induced NF- κ B dynamics were measured in liver cells infected with or without hepatitis B virus. A model of TNF α signaling to NF- κ B suggests that an unknown IKK upregulating factor can reconcile subtle changes in NF- κ B dynamics due to infection (Park *et al*, 2006). Another model examined the role of NF- κ B in neural stem cells and predicts that the level of NF- κ B activity correlates with the rate of cell proliferation (Piotrowska *et al*, 2006). Tests of

predictions from these and related models are likely to be useful in elucidating the role of NF- κ B dynamics in physiological and pathophysiological contexts.

Another trend that we anticipate continuing is merging of the NF- κ B signaling models with models of other pathways. Initial steps have been in the areas of modeling LPS-induced TNF α signaling (Covert *et al*, 2005; Werner *et al*, 2005), TNF–EGF–insulin crosstalk and autocrine signaling (Janes *et al*, 2005, 2006), and crosstalk between TNF-induced caspases and NF- κ B-induced antiapoptotic factors (Rangamani and Sirovich, 2007). The interest in linking NF- κ B models to other pathway models is likely to grow, and since TNF α induces JNK activity, and because interleukins, T-cell receptors, B-cell receptors, and other stimuli activate NF- κ B (Hayden and Ghosh, 2004), we can expect expansion into these areas. Elements of some existing models may prove useful in this regard (Schoeberl *et al*, 2002).

As a corollary to this trend, we expect existing NF- κ B models to merge with each other. Most of the published NF- κ B models described above are ‘backwards compatible,’ in the sense that they recapitulate the essential dynamic properties of their predecessors while demonstrating some new dynamic properties. However, the descendant models are not necessarily compatible with each other. To address this issue, we have, for example, developed a ‘consensus model’ that recapitulates a multitude of combined experiments (Hoffmann *et al*, 2002; Werner *et al*, 2005; Cheong *et al*, 2006; Kearns *et al*, 2006; O’Dea *et al*, 2007) and is thus increasingly predictive (R Cheong and A Levchenko, in preparation). Advanced parameter fitting techniques are likely to emerge as important tools in developing highly comprehensive consensus models (Fujarewicz *et al*, 2007).

Finally, an increasing use of the core I κ B–NF- κ B model is in illustrating new modeling environments and techniques. For example, the NF- κ B pathway has been used to illustrate a new technique to graphically represent models in a way that is easy to interpret and yet is mathematically precise (Cho *et al*, 2003). Another application used NF- κ B to exhibit an agent-based stochastic modeling method (Pogson *et al*, 2006). NF- κ B models have also been used to illustrate efficient ways to investigate parameter sensitivity (Fujarewicz *et al*, 2007; Joo *et al*, 2007). Models of the I κ B–NF- κ B signaling module are attractive in these settings because they are usually moderate in size (a few dozen parameters and equations) yet display complex behavior, much of which can be rationalized through careful analysis of these models. As modeling becomes more accessible to non-specialists, we anticipate model analysis and applications will grow rapidly. In fact, one can already interactively explore the NF- κ B model online through the Sigmoid project (<http://www.sigmoid.org/>) (Cheng *et al*, 2005). The growth and dissemination of such new tools can only contribute to NF- κ B modeling efforts.

Conclusion

Signal transduction pathways are dedicated sets of chemical reactions responsible for detection, processing, and delivery of the information about changes in the cell environment to the ‘decision centers’ of a cell. Unlike wires and antennas used in

human-built devices designed for information transfer, cells are limited to using chemistry as the basis for the sophisticated and robust passing of signals within complex and convoluted intracellular spaces. The underlying complexity may thus be foreign to our anthropomorphic attempts to confer the ideas of wires, transistors, and resistors to sophisticated liquidity of biological processes. Nevertheless, as much as the behavior of electrical circuits can be captured by mathematical equations, so too can the intricacies of signal transduction be understood through computational techniques.

A myriad of soluble signaling molecules, coupled to each other through feedback loops and pathway crosstalk, impinge upon NF- κ B. The regulation and dynamics of the resulting signaling network are rich and complex and their underlying mechanisms are not immediately transparent. Mathematical modeling has cut through the haze by helping to summarize experimental observations and develop a deep and coherent understanding of the NF- κ B signaling. Such computational approaches are essential for continued advancements in the field of signal transduction, as exemplified by the profound qualitative and quantitative insights obtained thus far for NF- κ B signaling.

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