Control of RelB during dendritic cell activation integrates canonical and noncanonical NF-κB pathways

Vincent F-S Shih1, Jeremy Davis-Turak1, Monica Macal2, Jenny Q Huang1, Julia Ponomarenko3, Jeffrey D Kearns1,5, Tony Yu1, Riku Fagerlund1, Masataka Asagiri1,4, Elina I Zuniga2 & Alexander Hoffmann1

The NF-κB protein RelB controls dendritic cell (DC) maturation and may be targeted therapeutically to manipulate T cell responses in disease. Here we report that RelB promoted DC activation not as the expected RelB-p52 effector of the noncanonical NF-κB pathway, but as a RelB-p50 dimer regulated by canonical IkBα and IkBε. IkB control of RelB minimized spontaneous maturation but enabled rapid pathogen-responsive maturation. Computational modeling of the NF-κB signaling module identified control points of this unexpected cell type–specific regulation. Fibroblasts that we engineered accordingly showed DC-like RelB control. Canonical pathway control of RelB regulated pathogen-responsive gene expression programs. This work illustrates the potential utility of systems analyses in guiding the development of combination therapeutics for modulating DC-dependent T cell responses.

DCs are specialized sentinel immune cells essential in both innate and adaptive immunity. DC progenitors differentiate to become immature DCs that populate both nonlymphoid and lymphoid tissues and perform immune-surveillance functions. When encountering pathogens or pathogen-associated molecular patterns (PAMPs), immature DCs undergo a maturation program that determines their role in the adaptive immune response1. A hallmark of DC maturation is expression of major histocompatibility complex molecules (MHC), T cell co-stimulatory molecules (CD40, CD80 or CD86) and cytokines (for example, interleukin 23; IL-23) in addition to a gene expression program of intracellular factors that enable effective antigen uptake, processing and presentation, and T cell activation. In addition, production of inflammatory molecules such as nitric oxide and cytokines such as tumor necrosis factor (TNF) and interferon underlies DC functions in innate immune responses2-3. DCs have thus attracted attention for engineering or modulating immune-based therapies4.

The transcription factor NF-κB protein RelB is highly expressed in antigen-presenting cells5 and is critical for DC maturation, DC function as antigen-presenting cells6 and DC-mediated immunity. Specifically, small interfering RNA–mediated silencing of RelB expression radically alters the DC maturation process and results in blunted antigen–specific T cell responses in vitro and in vivo7. RelB-deficient mice have deficiencies in splenic DC subsets8,9 but other critical roles of RelB in DCs may be masked by other cell types in which RelB deficiency leads to functionally opposite phenotype: notably, T cells are hyperactive in these null mice, whereas DC-specific deletion of the RelB–controlling kinase NIK results in deficient T cell responses10. Indeed, the extent of RelB activation determines the tolerance or rejection of allogenic organ transplants by determining the balance of associated activated or regulatory T cells7. These insights have prompted investigations of cell-based therapies for autoimmune diseases using RelB-silenced DCs11.

Despite the potential clinical importance of RelB, the molecular mechanisms that control its activity in DCs have remained unclear. Mouse embryonic fibroblasts (MEFs) have served as a useful model system for many signaling studies. Detailed biochemical studies in MEFs have shown that unlike classical NF-κB (the RelA-p50 dimer), RelB is not activated from a latent cytoplasmic pool via the NEMO-dependent, ‘canonical’ signaling pathway but via the ‘noncanonical’ NF-κB pathway that involves proteolysis and processing of newly synthesized NF-κB2 p10012-14. Consistent with the critical role of RelB in DCs, noncanonical signaling pathway components such as the signaling protein NIK and Nfkbia gene have been reported to be required for proper DC functions10,15. However, RelB has also been found to be rapidly activated in DCs by canonical pathway stimuli TNF and lipopolysaccharide (LPS)16-19, and the canonical signaling pathway component TRAF6 has been shown to be essential20. These reports suggest that the mechanism by which RelB activity is controlled in DCs may be different than what has been described in MEFs. In DCs, the molecular control mechanisms must provide for constitutive RelB expression to enable rapid and decisive induction of maturation programs after exposure to pathogens or PAMPs but must limit spontaneous maturation of DCs in their absence.

In this study, we elucidated the molecular mechanisms responsible for regulating RelB in DCs. We used a systems biology approach of iterative computational modeling and quantitative experimental
analyses of the NF-κB signaling network in DCs to reveal that RelB activity was limited by classical iκBs, IκBα and IκBβ, and regulated via the canonical pathway. Modeling studies identified two DC-specific control points that render RelB subject to regulation by the canonical pathway. Finally, gene expression profiling revealed that RelB-dependent gene expression programs regulated by the canonical pathway activity control DC-orchestrated immune responses.

RESULTS
Developing a DC-specific model for NFκB signaling
The established view of NF-κB signaling comprises two separate pathways (Fig. 1a)12. The canonical pathway, involving the NEMO-dependent kinase IKK, triggers degradation of NF-κB inhibitors, the classical iκBs: IκBα, IκBβ and IκBε. Resulting activation of latent RelA-containing and c-Rel-containing NF-κB dimers controls inflammatory and proliferative gene expression programs. The noncanonical pathway, involving the kinases NIK and IKK1, triggers processing of p100 to p52 and generation of the RelB-p52 transcription factor, which is implicated in cell survival and maturation. To examine NF-κB RelB signaling in DCs in a quantitative manner, we developed a mathematical model that describes the formation and regulation of RelA and RelB dimers in terms of mass-action kinetics (Supplementary Note). The first version of the model involves 41 molecular species, 132 reactions and 53 unique kinetic parameters based on published and newly made measurements that constrain the model to a single parameter set ensemble; it recapitulates well-documented NF-κB control in MEFs20–22, such as prompt LPS-induced RelA activation and delayed lymphotoxin β-dependent RelB activation (Fig. 1b).

To adapt the model to DCs, we first measured the expression of key NF-κB proteins in bone marrow–derived DCs (BMDCs) in comparison to that in MEFs and bone marrow–derived macrophages (BMDMs). Relative to the expression of the housekeeping gene β-actin, expression of Rela mRNA was similar in BMDMs, BMDMs and MEFs, and the relative amount of RelA protein in these cell types correlated (Fig. 1c). In contrast, we observed threefold to sixfold more Relb mRNA and protein expression in BMDCs than MEFs and BMDMs (Fig. 1c and Supplementary Fig. 1a). p100, encoded by the Nfkb2 gene, is known to inhibit RelB. We therefore tested whether p100 expression correlated with enhanced RelB expression in BMDCs. We observed 3.5-fold more Nfkb2 mRNA in BMDCs, but quantitative immunoblotting showed little difference in the p100 protein abundance among the cell types analyzed (Fig. 1c and Supplementary Fig. 1b). Lack of correlation between the relative p100 protein and RNA abundance suggested that p100 degradation may be elevated in BMDCs. We noted a 2.5-fold increase in the amount of p52 in BMDCs, which suggests that both complete p100 degradation and p100 processing to p52 may occur in BMDCs (Fig. 1c and Supplementary Fig. 1b). Consistent with this hypothesis, protein expression of IKK1, the kinase determining the activity of noncanonical NF-κB pathway, gradually increased during DC differentiation with concomitant p100 processing to p52 (Fig. 1d), potentially via the control of microRNAs23. Our data indicate that DC differentiation involves not only increased expression of RelB but also elevated constitutive activity of the noncanonical NF-κB signaling pathway.

Based on the measurements, we made specific modifications to the computational model to recapitulate RelB control in DCs (Supplementary Note). First, we increased Relb and Nfkb2 expression threefold, which increased the abundance of RelB but not its
nuclear localization. Then we destabilized p100 by the IKK1-dependent pathway to achieve comparable p100 expression as in MEFs (Fig. 1c). This change resulted in a substantial increase of nuclear RelB activity (Fig. 1e). To test experimentally whether RelB in DCs primarily localizes into the nucleus, we separated BMDCs into cytoplasmic and nuclear extracts but found that more than 75% of nuclear RelB activity (Fig. 1f). Band intensities of the antibody-ablation analysis (Fig. 1g). The fact that sequester RelB in the cytoplasm.

**IκBα restrains RelB-p50 and spontaneous DC maturation**

To search for inhibitors of RelB in DCs, we immunoprecipitated RelB from BMDC whole-cell lysates and analyzed the associated proteins (Fig. 2a). As expected, p100, the known RelB inhibitor and noncanonical regulator, was associated with RelB. Unexpectedly, IκBα and IκBε, the classical IκBα inhibitors regulating the canonical NF-κB pathway, were also immunoprecipitated with RelB, but IκBβ and p105 were not. Substantial amounts of p50, known as the binding partner of RelA in the canonical pathway, were found in RelB immunoprecipitates, and this complex was primarily cytoplasmic (Supplementary Fig. 2a,b). Reciprocal immunoprecipitation of various NF-κB inhibitors confirmed that RelB not only directly interacted with p100 but also associated with IκBα and IκBε in BMDCs (Fig. 2b and Supplementary Fig. 2c), and RelA associated with IκBα, as expected (Supplementary Fig. 2d).

The observations that IκBα immunoprecipitates did not contain other IκB isoforms confirmed the specificity of the antibodies used and that only one IκB isoform associated with each RelB molecule. Analyses of the amounts of RelB captured and remaining in the flow-through after immunoprecipitation with various IκB antibodies provides a quantitative understanding of RelB protein distribution in BMDCs (Fig. 2c). This analysis revealed that 37–45% of RelB was associated with p100 and 12–17% with IκBε. A substantial proportion of RelB (19–34%) associated with IκBα, which prompted us to investigate the function of this interaction.
To test whether IκBα may inhibit RelB activity in BMDCs, we took advantage of IκBα-deficient mice and developed two strategies to focus our experimental analysis on RelB activity. First, we bred the mice onto a c-Rel−/− background (Rel−/−); then we modified the standard electrophoretic mobility shift assay (EMSA) with IκB site-containing probes (xκB EMSA) to include shift-ablating antibodies for RelA, resulting in a specific RelB EMSA. Using these tools, we found that RelB activity was more than twofold elevated in IκBα-deficient BMDCs (Fig. 2d). Supershift analysis with antibodies that were shown to be specific for p50 and p52 (Supplementary Fig. 2e) revealed that whereas control BMDCs contained primarily constitutive RelB-p52 activity, ablation of IκBα resulted in a substantial increase in active RelB-p50 dimer, rendering RelB-p50 the predominant NF-κB RelB DNA binding activities in BMDCs depleted for indicated proteins by knockout (KO), monitored by EMSA (left). (f) Computational simulations of NF-κB RelB DNA binding activities in BMDCs depleted for indicated proteins by knockout (KO), monitored by EMSA (left). Signals were quantified and graphed relative to respective resting cells (right). Data shown in b,d,e,g are representative of at least three independent experiments. Data shown in c are representative of two independent experiments (error bars, s.d.; n = 3).

TLRs activate RelB-p50 via the canonical NFκB pathway

To explore the regulatory consequences of RelB-p50 interactions with IκBα and IκBβ proteins during DC maturation, we incorporated them into the mathematical model as kinetic rate equations and used the quantitative immunoprecipitation results as constraints in a multidimensional parameter optimization protocol (Supplementary Note). We simulated NF-κB regulation during Toll-like receptor (TLR)-induced DC maturation using experimentally measured time-course data of the NEMO-dependent IKK kinase activity as an input. Such simulations indicated rapid and substantial activation not only of RelA but also of RelB (Fig. 3a). To test this prediction experimentally, we stimulated BMDCs and MEFs with the TLR9 ligand CpG, the TLR2 ligand Pam3CSK4 and the TLR4 ligand LPS during TLR-mediated DC maturation. We observed rapid RelB activation in mathematical models, based on RelB activity in IκBα−/− BMDCs (Fig. 3a), correlating with previous studies of RelB-deficient DCs6, and suggesting a specific function for RelB in regulating the antigen uptake and processing program of antigen-presenting cells. Together, these data demonstrate that the classical NF-κB inhibitor, IκBα, not only restrains the expression of RelB by controlling RelA or c-Rel24, but in immature DCs it also has a critical functional role in restraining RelB activity to prevent inappropriate spontaneous maturation.
observed rapid activation of RelB in splenic DCs stimulated with CpG or Pam3CSK4 (Supplementary Fig. 3d). Computational simulations suggested that this induced RelB activity consists of RelB-p50 rather than RelB-p52 dimer (Fig. 3c). Experimentally, supershift analyses of nuclear extracts revealed that both RelB-p50 and RelB-p52 activities were present under unstimulated conditions but that CpG stimulation primarily increased RelB-p50 activity (Fig. 3c and Supplementary Fig. 3e), unlike LTbR stimulation of MEFs, which induces RelB-p52. These data suggest that during DC maturation RelB activation is regulated by the canonical pathway.

A hallmark of canonical signaling is the release of a pre-existing NF-κB dimer, whereas noncanonical signaling involves the stimulus-responsive de novo generation of the dimer. In CpG-responding DCs we did not detect increases in protein expression of RelB or p50, or Relb mRNA, whereas Nfkbia mRNA, encoding IκBα, was induced more than fourfold (Supplementary Fig. 3f). Furthermore, inhibition of protein synthesis by cycloheximide did not block CpG-induced RelB activation, whereas resynthesis of IκBα protein was blocked (Supplementary Fig. 3g), suggesting that de novo RelB protein synthesis is not required for CpG-inducible RelB activation. In contrast, immunoblotting confirmed that in DCs nuclear accumulation of RelB was accompanied by disappearance of cytoplasmic RelB after CpG stimulation, indicative of stimulus-responsive nuclear translocation of a pre-existing pool of RelB (Supplementary Fig. 3h). Inhibition of IKK2 activity, a hallmark of the canonical pathway, by the inhibitor PS-1145 (ref. 26) resulted not only in reduced RelA activity and IκBα protein degradation but also in reduced RelB activation (Supplementary Fig. 3g,i), suggesting that IKK2 signaling is required for RelB activation. We monitored the abundance of known NF-κB inhibitor proteins during the CpG time course: the abundance of the potential RelB inhibitors p100 and p105 remained unaltered; however, IκBα and IκBε were rapidly degraded, correlating with the activation kinetics of RelB activation (Fig. 3d). Notably, in coinmunoprecipitation assays, the amount of IκBα associated with RelB decreased in response to CpG (Fig. 3e). Together, these data suggest that degradation of IκBα allows for the release of RelB from pre-existing IκBα-RelB complexes.

To investigate the role of IκBα in TLR-induced RelB activation, we used the mathematical model to computationally simulate the effect of IκBα deletions on RelB activation. We found that in silico deletion of individual inhibitors had little effect, except in the case of IκBα (Supplementary Fig. 3j). Even compound deficiency of IκBβ, IκBε and IκBδ (which elevated basal RelB activity; Supplementary Fig. 3k) showed robust RelB activation in response to canonical pathway activation, as opposed to greatly diminished activation in an IκBα-deficient model (Fig. 3f). To test these computational modeling predictions, we used IκBα-deficient mice and generated Nfkbib−/−Nfkbic−/−Nfkbk2−/− mice. We confirmed the lack of protein products by immunoblotting (Supplementary Fig. 3l). Indeed, RelB activation was robust in Nfkbib−/−Nfkbic−/−Nfkbk2−/− BMDCs, whereas IκBα-deficient BMDCs showed a diminished increase and delayed kinetics (Fig. 3g and Supplementary Fig. 3m). Together, these data provide genetic and mechanistic evidence that IκBα is required for CpG-induced RelB activation in DCs.

Engineered MEFs show DC-like RelB control

We previously showed that hallmarks of the NF-κB signaling system in mature but unstimulated DCS are abundant in basal RelB expression
and basal noncanonical pathway activity. To investigate whether these mechanisms are sufficient and what their relative contributions may be, we performed computational simulations of RelB activation for a range of parameter values governing basal RelB mRNA synthesis and NIK protein half-life. These in silico analyses showed that activation of RelB mildly increased when either parameter was increased, but substantial enhancement occurred only when both parameters were concomitantly elevated (Fig. 4a). Our simulations suggest that the DC-specific, rapid RelB activation upon canonical pathway stimulation can be explained by DC-specific, constitutively elevated Relb mRNA synthesis and noncanonical IKK activity.

To test this model-derived hypothesis experimentally, we asked whether genetically engineering these two mechanisms into MEFs may be sufficient to allow for DC-like canonical regulation of RelB. We took advantage of MEFs deficient in TRAF3, an E3-ligase controlling NIK degradation, to increase constitutive noncanonical signaling. As suggested by the model simulations, we then transduced a retroviral Relb transgene to increase RelB expression about threefold relative to that in untransduced MEFs (Fig. 4b and Supplementary Fig. 4a). The engineered MEFs exhibited substantial RelB activation in response to LPS (Fig. 4c) or TNF (Supplementary Fig. 4b), whereas the parental control MEFs did not, and RelA activation by these stimuli remained unchanged. Neither single genetic alteration produced substantial RelB activation, indicating that enhanced RelB expression and noncanonical pathway activity function synergistically, as predicted by the model, to push RelB into the canonical pathway and render it responsive to TLR agonists. Antibody supershift and depletion analysis (Fig. 4d and Supplementary Fig. 4c) confirmed that canonical signaling primarily activated the RelB-p50 dimer (sevenfold) rather than the RelB-p52 dimer (twofold) as observed in DCs and predicted by the computational model (Fig. 3c).

Overexpression of a RelB-GFP fusion protein retrovirally transduced into single cells also revealed nuclear translocation upon TNF stimulation in the Traf3−/− context but not in control cells (Fig. 4e).

These iterative computational-experimental studies support a model in which the NF-κB protein RelB may function in either noncanonical or canonical pathways (Fig. 4d). In a dimer with p100 or p52, RelB is subject to control by the noncanonical pathway; in a dimer with p50, RelB may be bound by IkBz and IkBe and is regulated by NEMO-dependent canonical signals. Our analysis indicates that low constitutive RelB expression and noncanonical pathway activity characterizes one steady state (found in MEFs) and allows for RelB-p52 activation by stimuli such as LTβ that engage the noncanonical pathway (Supplementary Fig. 4d). High constitutive RelB expression and noncanonical pathway activity characterize another steady state (found in DCs) and allow for RelB-p50 activation by stimuli such as CpG that engage the canonical pathway (Supplementary Fig. 4e).
RelB and c-Rel cooperate in TLR-induced DC maturation

Given that RelB-p50 is induced by PAMPs during DC maturation, we wondered whether it controls the expression of inflammatory regulators or DC activation markers. After stimulation with the TLR9 ligand CpG or the TLR2 ligand Pam3CSK4 for 24 h, we indeed observed reduced surface expression of DC activation markers MHCII, CD86, CD80 and CD40 in Relb−/− DCs (Fig. 5a and Supplementary Fig. 5a,b). Expression of proinflammatory genes, Tnf and Il23a, correlated with the kinetics of CpG or Pam3CSK4-induced RelB activation and were reduced in Relb−/− DCs (Fig. 5b). In EMSAs, activated RelB-p50 bound to DNA probes containing the κB sites found in the Tnf and Il23a promoters (Fig. 5c), indicating that RelB-p50 can directly interact with these regulatory regions. In vivo, we observed recruitment of RelB to the promoter regions of Tnf and Il23a genes after DC maturation with CpG using the chromatin immunoprecipitation assay (Fig. 5d).

We noted that RelB bound to consensus κB site sequences28 associated with the known canonical NF-κB pathway effectors, RelA and c-Rel, rather than the unconventional sequences previously ascribed to RelB in splenic stromal cells29 or MEFs30. Because single knockouts did not show overt defects in CD11c+ cell generation in bone marrow cultures (Supplementary Fig. 5d), we tested whether c-Rel and RelB have overlapping functions in regulating the DC maturation program by examining gene expression in c-Rel and RelB doubly-deficient DCs. Genome-wide expression profiling activated by TLR ligands CpG and Pam3CSK4 revealed a group of 157 genes that were substantially downregulated in Relb−/− Relb−/− BMDCs (Fig. 5e and Supplementary Table 1). To delineate the contribution of RelB in activating these genes, we examined the expression phenotype of the 50 most severely c-Rel–RelB–dependent genes in Relb−/− BMDCs stimulated with TLR ligands. Expression phenotypes in fold induction were calculated between wild-type and null DCs, and the order of genes was sorted in increasing degree of RelB dependency (Fig. 5f and Supplementary Table 2). This analysis revealed a continuous spectrum of RelB dependency rather than two distinct classes (of RelB-dependent and RelB-independent genes), suggesting an overlap in DNA interaction specificities between c-Rel and RelB dimers. Tnf and Il23a were identified in this analysis as regulated by both RelB and c-Rel. Quantitative RT-PCR validated the requirements of RelB and c-Rel in activating Cxcl2, Ccl40 and Il1b gene expression (Supplementary Fig. 5c).

Given overlapping functions of c-Rel and RelB in regulating DC gene expression programs, we investigated their relationship within the signaling system. Whereas RelA and c-Rel protein expression were similarly abundant in wild-type BMDCs and those lacking RelB, Relb−/− BMDCs exhibited decreased RelB protein expression (Fig. 6a). Relb transcripts were reduced by ~40% in Relb−/− relative to wild-type BMDC (Fig. 6b). This reduction resulted in severely diminished activation of RelB DNA-binding activity in Relb−/− BMDCs in response to LPS (Fig. 6c). These data indicate that one of the key determinants of RelB control by the canonical pathway, namely RelB expression, is in fact controlled by c-Rel. The feed-forward circuit architecture suggests that expression of RelB in differentiated but immature DCs may reflect the exposure of differentiating cells to c-Rel–inducing stimuli. We therefore tested whether c-Rel–deficient DCs may also be defective in RelB-responsive gene expression by comparing the expression of RelB target genes in Rel−/− DCs and Rel−/−Relb−/− DCs. Indeed, Relb−/− BMDCs showed reductions of surface marker and inflammatory cytokine expression (Supplementary Fig. 6a,b). These data support a model in which RelB acts as a downstream mediator of c-Rel in DC activation programs.

DISCUSSION

RelA and c-Rel had been previously considered as effectors of the canonical NF-κB signaling pathway, and RelB as the effector of the noncanonical pathway, based on its role as a RelB-p52 transcription factor in secondary lymphoid organogenesis. However, we showed here that RelB is also an effector of the canonical pathway in DCs. During DC differentiation RelB expression increased, and elevated steady-state noncanonical pathway activity resulted not only in the expected Relb-p52 dimer but in formation of the Relb-p50 dimer. Unlike Relb-p52, which is mostly nuclear in immature DCs, Relb-p50 is inhibited by the IkB proteins, IkBα and IkBε, which allows for rapid activation of Relb-p50 activity via the canonical pathway upon exposure to maturation stimuli. Conversely, with the recent discovery of 1KβB21,22, chronic inflammatory conditions rendered RelA an effector of the noncanonical signaling pathway. Thus, both RelA and RelB are potential effectors of the canonical and noncanonical signaling pathways; whether they are functionally relevant effectors is determined by the physiological steady state of the NF-κB signaling system.

Our observations imply that Relb-p50 and Relb-p52 present different molecular surfaces to IkB proteins, providing physiological relevance to previous studies of protein–interaction specificities26,27. Similarly, the DNA interaction characteristics of Relb-p50 and Relb-p52 may be distinct22,33. Relb residue Arg125 in the Relb-p52 dimer makes an additional base contact with DNA that allows Relb-p52 to recognize a broader range of κB sites. This may account for the Relb-p52–specific function in regulating chemokines involved in secondary lymphoid organogenesis, such as secondary lymphoid tissue chemokine (SLC), EBI1 ligand chemokine (ELC), B lymphoblastoid cell chemokine (BLC) and stromal cell–derived factor 1α (SDF-1α)20,29. In contrast, Relb-p50 interacts with DNA sequences similarly to RelA-p50, and a role for Relb in TNF production, GM-CSF and Bc1-εl expression has been reported34,35. Together, these studies suggest that the dimerization partner of RelB determines not only the signaling pathway that RelB is responsive to but also the RelB target gene program.

Why then, would DCs use Relb as an effector of the canonical NF-κB signaling pathway along with RelA and c-Rel? One possibility...
is that RelB-p50 target genes are distinct from those controlled by c-Rel or RelA. Our transcriptomic profiling suggests overlap between c-Rel–dependent and RelB–dependent gene programs, but c-Rel turned out to control RelB expression; thus, other tools are required to address the question of RelB-p50 versus cRel-p50 specificity. A second possibility is that the stimulus-responsive dynamic control of RelB is distinct from that of RelA or c-Rel. Although RelB-p50 is inhibited by IκBα in resting cells, it may make for a poorer substrate for IκBα feedback control than RelA, which is efficiently stripped off the DNA by IκBα36. We speculate that the involvement of RelB-p50 in DC biology ensures irreversible execution of a terminal maturation and activation program in response to transient PAMP exposure.

Mathematical modeling, which we used here to describe biochemical reactions in terms of kinetic rate equations, lends itself as a tool for studying the regulation of signaling networks. Iteratively refined mathematical models of the NF-κB–IκB system have addressed the dynamic and homeostatic control of the NF-κB RelA-p50 dimer by IκBα proteins in fibroblasts21,22,37-41. In this study, we developed to our knowledge the first kinetic model that accounts for the generation and regulation of multiple NF-κB dimers, namely RelA- and RelB-containing dimers. We contrasted the steady-state and dynamic control mechanisms in two cell types, MEFs and DCs, and found that the key biochemical differences are two kinetic rate constants (Relb mRNA synthesis and NIK half-life); a threefold increase was sufficient to shift the in silico model from MEF-like to DC-like regulation of the NF-κB signaling system. We confirmed this prediction experimentally by genetically engineering MEFs to produce DC-like RelB control. There was no need to invoke cell type–specific protein experimentally by genetically engineering MEFs to produce DC-like RelB- containing dimers. We contrasted the steady-state and dynamic expression and regulation of multiple NF-κB dimers, specifically expression in dendritic antigen-presenting cells. Development 118, 1221–1231 (1993).

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ONLINE METHODS

Reagents. GM-CSF and IL-4 were from Peprotech. We used 0.1 μM CpG (Invivogen), 500 ng/ml Pam3CSK4 (Invivogen), 100 ng/ml LPS (Sigma, B5:055) and 0.5 μg/ml LTβR agonist (Biogen) to stimulate cells. Cycloheximide and IKK2 inhibitor (PS-1145) were from Sigma. Antibodies to RelA (sc-372), RelB (sc-226), c-Rel (sc-70), IkBα (sc-945), IkBε (sc-7155), IKK1 (sc-7606), TRAF3 (sc-6933), USP2 (sc-861), α-tubulin (sc-5258), β-actin (sc-1615) and CD16/CD32 (sc-18867) were from Santa Cruz Biotechnology. p50/p50, p100/p52 and antibody to p100 C terminus were from US National Cancer Institute, Biological Resources Branch, Frederick, Maryland, USA. NIK antibody (4994) was from Cell Signaling. Immunoprecipitation beads and HRP-conjugated anti-rabbit secondary antibody (18-8816) were from eBioscience.

Antibody staining and flow cytometry. Single-cell suspensions were collected and blocked with anti-mouse CD16/CD32 (553157, BD Biosciences) from spleens of C57BL/6 mice for 10 min at 4 °C. Staining was performed with PerCP-labeled anti-CD4 (G043-10), APC-labeled anti-CD8 (53-6.7), or FITC-labeled anti-CD25 (MPC-11) and PE-labeled anti-CD69 (SH101). Stained cells were acquired in a FACSCalibur (BD Biosciences) or an Accuri C6 and data analysis was performed with FlowJo software.

Animal rearing. Wild-type and gene-deficient C57BL/6 mice were maintained in specific pathogen–free condition at the University of California, San Diego. All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Nfkbia−/−Tnf−/−Rel−/− and Nfkbia−/−Tnf−/−Rel−/−Relb−/− mice were generated by cross-breeding B6.Cg-Tg (TcraTcρb μOV A 323-339) mice with wild-type (WT), Relb−/− and Rel−/−Relb−/− BMDCs were made from bone-marrow suspensions prepared from mouse femurs. We seeded 2 × 10^6 bone marrow cells on 10-cm plate and cultured them for one week with L929-conditioned DMEM to derive BMDCs or cultured them for 6–11 d with 20 ng/ml GM-CSF and 10 ng/ml IL-4 to derive BMDCs. BMDC medium was replaced on days 3, 6 and 8, and floating cells were collected and subjected to experimental analyses as previously described. Typically, day 6–7 BMDCs were used to investigate TLR-induced DC maturation, and day 9–11 BMDCs for spontaneous DC maturation studies.

Spleenic DC purification. Spleens were cut into small fragments and digested with collagenase D (2 mg/ml, Roche) for 30 min at 37 °C followed by incubation with 10 mM EDTA pH 8.0 for 5 min. Single-cell suspension of splenocytes were enriched for CD11c+ cells by immunomagnetic cell sorting using MACS CD11c microbeads (Miltenyi Biotec) according to manufacturer’s protocol.

Spleen cell preparation. Single-cell suspensions were collected and blocked with anti-mouse CD16/CD25 in PBS containing 5% FCS for 10 min. Cells were stained with 7-aminocanthinocyncin D (7-AAD) to exclude dead cells and indicated antibodies for DC maturation analyses. All antibodies were purchased from BD Pharmingen: anti-CD11 (HL3, anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL-1) and anti-Iaα (AF6-120.1). Stained cells were acquired in either a FACS Calibur (BD Biosciences) or an Accuri C6 and data analysis was performed with FlowJo software.

Antigen presentation in DC–T cell cocultures. GM-CSF–derived bone marrow DCs were pulsed with 200 μg whole ovalbumin (Sigma, 5 μg OVA 323-339 (OT-II) peptide (Anaspec) or medium alone for 2 h at 37 °C. Naive CD4+ T cells (5 × 10^4 cells/well) were obtained by negative enrichment (>90% purity; Stem Cell Technologies) from spleens of B6.Cg-Tg (TcraTcρb)425 Cbn/J mice transgenic for ovalbumin 323-339-specific αβTCR (Jackson Laboratory) and labeled with carboxyfluorescein succinimidyl ester (CFSE) (Dye). DCs were washed and cultured with CFSE-labeled CD4+ T cells (5 × 10^4 T cells/well) at the indicated DC:T cell ratios as described. T cells were restimulated 72 h later with 5 μM OT-II peptide for 5 h in the presence of brefeldin A and examined for CFSE dilution and production of TNF and IL-2 by flow cytometry (BD LSRII). Data were analyzed using FlowJo software (Treestar).

Biochemical analyses. Whole cell extracts were prepared in RIPA buffer and normalized for total protein or cell numbers before immunoblot analysis. Cytoplasmic and nuclear extracts from BMDCs and BMDMs were prepared by high salt extraction buffer (Buffer A: 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA and 0.1 mM EDTA; Buffer C: 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA and 25% glycerol). Immunoprecipitation–immunoblotting analysis, EMSA, chromatin immunoprecipitation were performed as previously described. In EMSAs focusing on RelB–DNA binding activity, nuclear extracts were ablated of RelA and c-Rel–containing DNA binding activities by preincubating them with RelA and c-Rel antibodies (Fig. 3b and Supplementary Fig. 3b). Similarly, nuclear extracts were preincubated with RelB and c-Rel antibodies when RelA DNA binding activity was the focus (Fig. 3b). Antibody-shift ablation analysis (for RelB, p50 and p52) was performed as previously described and the specificities of antibodies were confirmed (Supplementary Fig. 2e).

Retrovirus-mediated gene transduction. RelB−/− or RelB-GFP expressing pBabe-puro constructs were generated by standard methods and transfected together with pCL.Eco into 293T cells with Lipofectamine 2000 transfection reagent (Invitrogen) for 48 h. Supernatant was filtered and used to infect MEFs. Transduced cells were selected with puromycin hydrochloride (Sigma). Images were acquired with a Zeiss Axio Z1 microscope.

Gene expression analysis. RNA extraction was performed with RNAeasy Mini Kit (Qiagen). RNA was collected from one set of time-course experiments (1 h, 8 h and 24 h) using wild-type (WT), Relb−/− and Rel−/− Relb−/− BMDCs stimulated with 0.1 μM CpG (Invivogen) or 500 ng/ml Pam3CSK4. Labeling and hybridization to the Illumina v.2 gene expression chip was performed by UCSD Biogem core facility. The raw data were preprocessed and normalized by mioloss method. Genes differentially regulated between WT and Rel−/− Relb−/− BMDCs were determined by a two-class paired SAM method implemented in the MeV program (multiple expression viewer). Class pairing was defined by corresponding time points between WT and Rel−/− Relb−/− BMDCs. Differentially expressed genes identified at the false discovery rate below 5% were deemed significant. Genes with at least twofold induction during TLR-elicted DC maturation are listed in Supplementary Table 1. In heat maps, expression values of each gene were normalized to its maximum fold induction and clustered by hierarchical clustering with Euclidian distance. For phenotypic analyses (Fig. 5f and Supplementary Table 2), the average fold induction (FI) in log2 scale across either timecourse (CpG and Pam3CSK4) was calculated for different genotypes, for example, FIWT, FIrel−/−, and FRelb−/−/Relb−/−. The RelB phenotype was defined as FIWT–FIrel−/−, the c-Rel–RelB phenotype was defined as FIWT–FIrel−/−/Rel−/−. Quantitative qRT-PCR was performed after first-strand cDNA synthesis with oligo(dT) and SuperScript RT II (Invitrogen), using SYBR Green PCR Master Mix reagent (Strategene), Eppendorf Mastercycler realpex system and gene-specific primers (Supplementary Table 3). Data analysis used the Δ(ΔCt) method with β-actin as normalization control to relate signals to those in MEFs or derive fold induction over basal levels. qRT-PCR and chromatin immunoprecipitation data shown are representative of three independent experiments (mean ± s.d.). Quantification of mRNA and protein abundance are representative of four independent experiments.

Computational modeling. The RelA–RelB mathematical model (version 5.0) involving mass action kinetic equations was developed based on a previously published model (version 3.1)22 and experimental data20 that allowed for constraints-based parameterization. Refinement of the model (version 5.1) and MEF- and DC-specific parameterization were based on experimental data presented in this paper. Computational simulations were performed in Matlab using the ode1s solver. Detailed descriptions are included in the Supplementary Note.

Statistics. Statistical significance was calculated by two-tailed Student’s t-test with Prism software (GraphPad). Error bars were shown as either s.d. or s.e.m. as indicated.

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