UNDERSTANDING IMMUNOBIOLOGY THROUGH THE SPECIFICITY OF NF-κB

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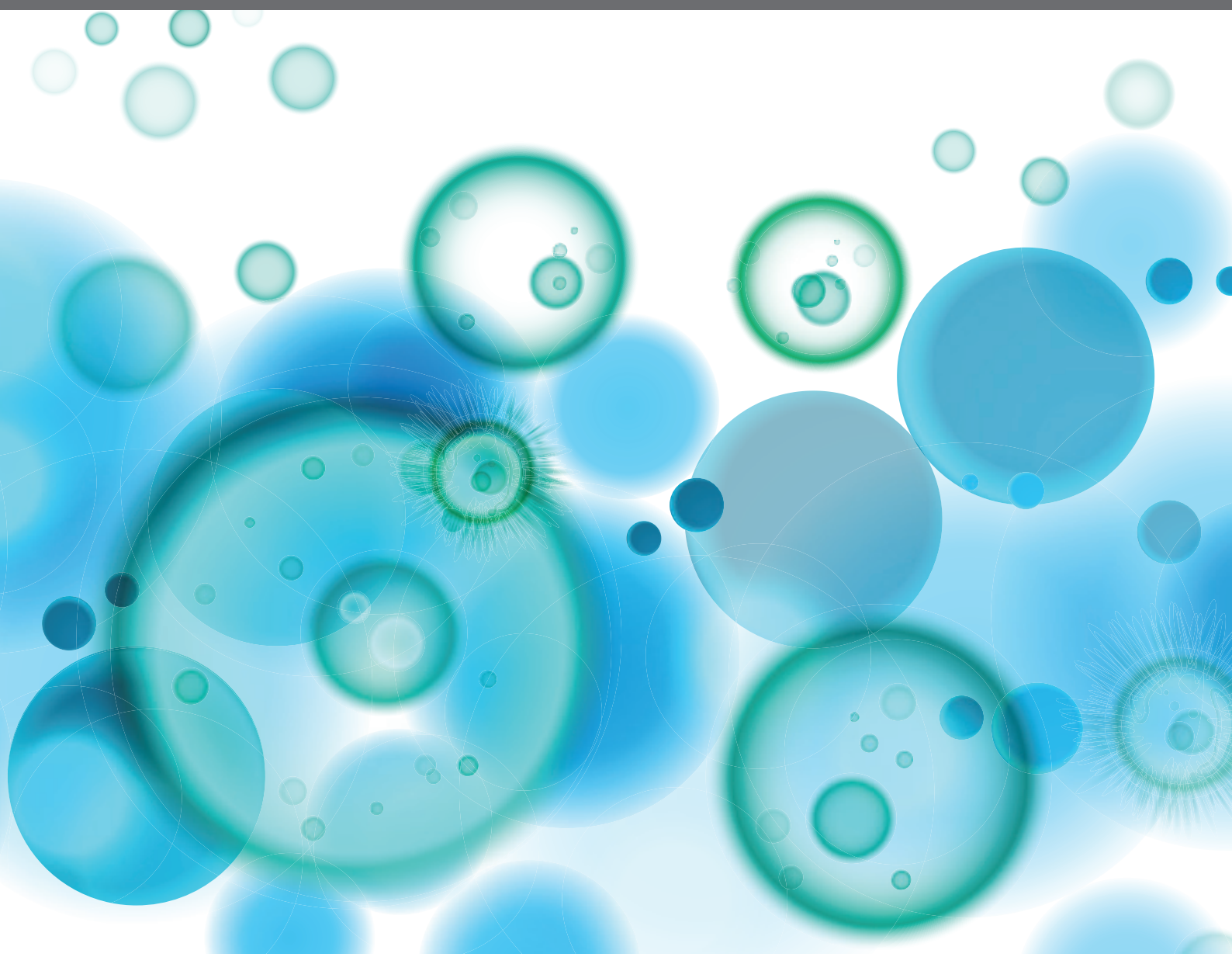

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UNDERSTANDING IMMUNOBIOLOGY THROUGH THE SPECIFICITY OF NF-κB

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EDITORIAL
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Editorial: Understanding Immunobiology Through the Specificity of NF-κB

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Keywords: NF-kappaB (NF-κB), microscopy, systems biology, quantitative methodologies, high throughput profiling, mathematical modeling

Editorial on the Research Topic

Understanding Immunobiology Through the Specificity of NF-κB

Since its discovery more than 30 years ago, the significance of NF-κB transcription factors has penetrated virtually all areas of biomedical research. While it was originally found from immune cells, these evolutionarily conserved proteins are expressed in most cell types. Dysregulation of NF-κB has been observed in many devastating diseases such as cancer, autoimmunity, and neurodegenerative diseases. NF-κB biology has been intensely studied over the years, and numerous regulatory mechanisms and target genes have been identified. Briefly, NF-κB exists as hetero/homo-dimers whose subunits share a Rel homology domain which mediates the dimerization. Even though NF-κB constitutively shuttles between the cytoplasm and the nucleus, NF-κB dimers are efficiently retained in the cytoplasm by the inhibitor of NF-κB (IκB) proteins in a resting state. Upon stimulation, the ubiquitin-proteasome-mediated degradation of IκBs results in nuclear accumulation of NF-κB, where these transcription factor proteins scan the tissue-specific epigenome and bind to κB motifs within accessible chromatin in a matter of minutes, to regulate tissue-specific gene expression programs. At individual promoters or enhancers, NF-κB collaborates with or antagonizes other transcriptional regulators which brings about distinct regulatory outcomes. The magnitude and duration of NF-κB action are governed by multiple negative and positive feedback regulators.

In light of its ubiquitous expression and the conserved core regulatory module described above, the function of NF-κB seems remarkably specific to the distinct signals from the microenvironment or intracellular stress. Despite the great advances in the field, we still lack the knowledge about detailed workings of transcriptional regulation by NF-κB and the functional relevance. For example, recent technological advances, some of which discussed in this Research Topic, have shown unexpected complexity regarding the temporal and spatial regulation of NF-κB activity. However, the role of NF-κB dynamics in fine-tuning epigenetic and transcriptional programs remains poorly understood. The remaining frontiers of investigation into NF-κB are likely to hold the key to the information that we need to control this transcriptional regulator for therapeutic gains in several pathological settings. Here, we have collected reviews and research reports from some of the investigators who have shaped our current knowledge and continue to shed light on NF-κB biology.

Brignall et al. contributed a Review which presents an insightful in-depth discussion of a range of subtopics, from NF-κB dimer specificity to genomic binding site selection. These are critical areas that will need further elucidation for a quantitative understanding of NF-κB functioning as a transcription factor.

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In a Review, Jeknić et al. summarize technical approaches that address an aspect of NF-κB regulatory mechanisms which received a relatively late attention: how the temporal patterns of NF-κB signaling dynamics are used to encode functional information and how they are decoded by individual cells. They discuss the challenges of generating informative single-cell measurements and survey currently available analysis and engineering tools, as well as recent improvements in throughput and information content of various assay platforms.

Nelson and Nelson's Mini Review highlights the importance of single cell analysis in examining host-pathogen interactions to account for the different infection status in individual cells. The article explains key findings from the pioneering studies of cellular responses to intact microbes which allow the investigation of not only a full host defense response but also how NF-κB is modulated by invading pathogens for their survival.

Tissue-specificity of NF-κB function is influenced by the repertoire of intracellular factors interacting with the regulators of NF-κB within a given cellular context. Macrophages are important effectors of innate immunity, and recent evidence suggests that findings about NF-κB signaling in non-immune cells such as fibroblasts may not apply to this cell type. Macrophage-specific NF-κB signaling mechanisms is the focus of a Review by Dorrington and Fraser. The central nervous system (CNS) has become an exciting context for NF-κB in recent years, since the brain represents an organ where NF-κB can have strikingly distinct functions depending on the cellular context such as neurons or glia. Moreover, microglia, the resident immune cells in the brain, emerge as the relevant cells for manifesting the phenotypes of SNPs associated with neurodegenerative diseases such as Alzheimer's disease. The Review by Dresselhaus and Meffert is a timely exposition of NF-κB neurobiology in various CNS cell components and their roles in several neurodegenerative diseases.

A Review by Adelaja and Hoffmann summarizes data on how NF-κB signaling is modulated by crosstalk mechanisms between pathways that are downstream of TLR ligands, IL-1, TNF-α, lymphotoxins, and interferons.

The Research Topic also includes three primary research articles from studies using diverse tools. As discussed extensively in the Review by Brignall et al., the dimer specificity of NF-κB proteins and its functional relevance are poorly understood. Martin et al. analyzed the dimerization status of RelA subunit of NF-κB using a quantitative live cell microscopy technique termed Number and Brightness. The result suggests that a higher than expected proportion of NF-κB dimers exist as RelA:RelA homodimers.

Chatterjee et al. show that surprises can still be found regarding biochemical networks of NF-κB. They describe an unexpected role of p100 in regulating the canonical NF-κB pathway downstream of TNF-α.

Mitchell et al. took a mathematical modeling approach and present a simplifying tissue-level NF-κB activity "calculator," showing that the activation status of a pathway as complex as NF-κB can be projected onto a simple measure with useful predictive features. Their calculator was able to dissect distinct macrophage mechanisms of type I and II interferons in amplifying NF-κB activity.

While the Research Topic showcases some important recent fruits and open problems in the field, we acknowledge that this collection does not encompass all the significant lines of research that deserve our attention. Exciting technological innovations are enabling us to address previously intractable questions about how the NF-κB system is used for interpreting danger- or stress-associated signals with a robust functional specificity. At the same time, this collection also reminds us that we still have a long way to go toward understanding the biology of NF-κB in the immune system and beyond.

AUTHOR CONTRIBUTIONS

M-HS and SR wrote the manuscript.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Signal Distortion: How Intracellular Pathogens Alter Host Cell Fate by Modulating NF-κB Dynamics

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By uncovering complex dynamics in the expression or localization of transcriptional regulators in single cells that were otherwise hidden at the population level, live cell imaging has transformed our understanding of how cells sense and orchestrate appropriate responses to changes in their internal state or extracellular environment. This has proved particularly true for the nuclear factor-kappaB (NF-κB) family of transcription factors, key regulators of the inflammatory response and innate immune function, which are capable of encoding information about the mode and intensity of stimuli in the dynamics of NF-κB nuclear accumulation and loss. While live cell imaging continues to serve as a useful tool in ongoing efforts to characterize the feedbacks that shape these dynamics and to connect dynamics to downstream gene expression, it is also proving invaluable for recent studies that seek to determine how intracellular pathogens subvert NF-κB signaling to survive and replicate within host cells by providing quantitative information about the pathogen and changes in NF-κB activity during different stages of an infection. Here, we provide a brief overview of NF-κB signaling in innate immune cells and review recent literature that uses live imaging to investigate the mechanisms by which bacterial and yeast pathogens modulate NF-κB in a variety of different host cell types to evade destruction or maintain the viability of an intracellular growth niche.

Keywords: NF-κB, dynamics, live cell imaging, macrophage, host:pathogen interactions, innate immunity

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INTRODUCTION

The nuclear factor-kappa B (NF-κB) pathway is considered a master regulator of inflammation and is intimately involved in the cellular response to infection (1). Similar to other mammalian transcription factor pathways, such as p53 (2–4), and NFAT (5), the NF-κB pathway can exhibit distinct dynamic responses to different stimuli (6–9). These dynamics, which include damped oscillations (7), allow cells to encode complex information about the modality, concentration, and duration of a particular stimulus in the amplitude, frequency, and persistence of oscillations (6, 10, 11). These dynamics are essentially decoded at the level of gene expression with different patterns of behavior leading to differing cell fates and phenotypes (10, 12, 13). This phenomenon, which is often referred to as dynamic multiplexing, allows cells to efficiently use a limited number of signaling pathways to deal with highly complex signaling environments (11). The dynamic behavior of the NF-κB pathway can be challenging to study using standard biochemical techniques that use population averaging because the responses of individual cells to a given stimulus can differ markedly (7, 9). This may be due to the difficult to control effects of paracrine and autocrine

signaling (12), inherent differences between cells and the signaling history of the cell (extrinsic noise), and the stochasticity of certain elements of the signaling pathway (e.g., transcription and translation; intrinsic noise) (8, 14, 15). For these reasons, live cell imaging (often in combination with mathematical modeling) has become an invaluable tool for studying NF-κB signaling (16, 17), and has been used to characterize the specific feedbacks that shape the behavior of the pathway (6, 7, 18, 19). For similar reasons, live cell imaging is being increasingly used to improve our understanding of the role NF-κB signaling plays during infection with intracellular pathogens (20–23). In tissue culture models of infection, only a fraction of the cells within the population may become infected and this will occur at different times between cells making it difficult to build an accurate picture of how NF-κB signaling is affected during each stage of the pathogenic process. Live cell imaging provides a means to deconvolve events occurring during different stages of an infection (20), distinguish between non-infected and infected cells (23), as well as keeping track of changes in intracellular microbial burden within individual cells (22).

In the following review, we will provide a brief overview of NF-κB signaling and describe how live cell microscopy has been used to investigate the capacity of the pathway to encode information about the signaling environment of the cell in the dynamics of NF-κB transcription factors. We will discuss the duality of NF-κB signaling within the context of host:pathogen interactions and how it can both aid and hinder the response to an infection. Finally, we describe how recent live cell studies have provided new insights into the ways in which different microbial pathogens incorporate NF-κB modulation as a part of intracellular survival strategies.

BASIC INSIGHTS INTO NF-κB REGULATION FROM LIVE CELL IMAGING

At the core of the NF-κB pathway are the Rel family of transcription factors: p65 (RelA), RelB, c-Rel, p100/p50, and p105/p52, each containing a central DNA binding motif, known as the Rel homology domain (24). These proteins can form homo- or heterodimers in virtually any combination with p65:p50 dimers appearing to be the most common. In the absence of stimulus, NF-κB activity is suppressed by inhibitor kappaB (IκB) proteins, which anchor NF-κB transcription factors in the cytoplasm. The canonical wing of the NF-κB pathway, defined by the activity of p65-containing dimers, can be activated by diverse stimuli. These range from the proinflammatory cytokines, tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β), to microbe-associated molecular patterns (MAMPs) like lipopolysaccharide (LPS) and flagellin, which are recognized by surface or phagosomal pattern recognition receptors (PRRs), including the toll-like receptors (TLRs) (25). In each case, activation proceeds via the IκB kinase (IKK) complex, a convergence point for the NF-κB pathway. The IKK complex phosphorylates both NF-κB and IκB proteins (26, 27), regulating the activity of the former and stimulating the degradation of the latter. In the case of IκBα, an IκB isoform associated with the regulation of canonical NF-κB signaling, the protein is phosphorylated at serine 32 and 36, creating a phospho-degron, which is recognized by the E3 ubiquitin ligase complex, $SCF^{\beta-TRCP}$, and leads to polyubiquitination and proteasomal degradation of IκBα (28). IKK-dependent phosphorylation also promotes the degradation of other IκB isoforms (i.e., IκBβ and ε) and the processing of p100 and p105 to p52, and p50, respectively [reviewed in (29)].

In addition to regulating genes involved in innate immunity and inflammation, p65 also promotes the expression of a core set of negative regulators, IκBα, IκBε, and tumor necrosis factor alpha-induced protein 3 (TNFAIP3/A20, **Figure 1**) (6, 7, 19). The inherent delay in the expression of these proteins is thought to be responsible for the oscillatory behavior of the pathway. While each of these feedbacks was first identified in genetic and biochemical studies (19, 30–33), the individual roles played by these in shaping NF-κB dynamics was clarified by subsequent studies using live imaging and mathematical modeling. As RNA polymerase II associates with the IκBα promoter prior to stimulation (6), this feedback is rapidly activated on nuclear translocation of p65 and is perhaps most closely linked to the oscillatory behavior of the pathway (7). Expression of IκBε is delayed relative to IκBα and this may play a role in increasing the heterogeneity of the response between cells in addition to helping terminate NF-κB activation after transient stimulation (6, 19). Finally, A20 provides a non-redundant feedback that operates over longer timescales (34), inhibiting IKK activity by antagonizing upstream regulators (35). Expression of the *TNFAIP3* gene, which encodes A20, is temperature sensitive and may imbue the NF-κB pathway with the ability to adjust the expression of select NF-κB-regulated genes across physiologically relevant temperatures during infection and inflammation (36).

The core negative feedbacks are supplemented by additional cell type and stimulus-specific feedbacks. The best example of this is the feedback dominance switching observed in macrophages exposed to LPS (18), which enables cells to discriminate between high and low LPS concentrations. In response to high concentrations of LPS, p65 is able to transactivate expression of the *Rela* gene, increasing the expression of p65 and overcoming negative feedbacks that would otherwise curtail NF-κB activity. This mechanism is likely specific to macrophages or at least lymphoid cells as it requires expression of Ikaros, a transcription factor involved in lymphoid development (37). The NF-κB-regulated expression of TNFα could also be considered a second positive feedback, acting as an autocrine or paracrine signal to prolonging the NF-κB response to LPS in mouse embryonic fibroblasts as well as increasing the heterogeneity of the response in murine macrophages (12, 38).

The challenging task of assigning meaning to NF-κB dynamics has been addressed by recent studies that supplement live cell imaging with microfluidics and transcriptional profiling to either shape and synchronize NF-κB dynamics across a cell population through periodic forcing (10) or link the dynamics in individual cells to single cell RNAseq transcriptional profiles (12). These studies, together with earlier work (6), collectively show that different dynamic responses can produce distinct patterns of gene expression and changes in cellular

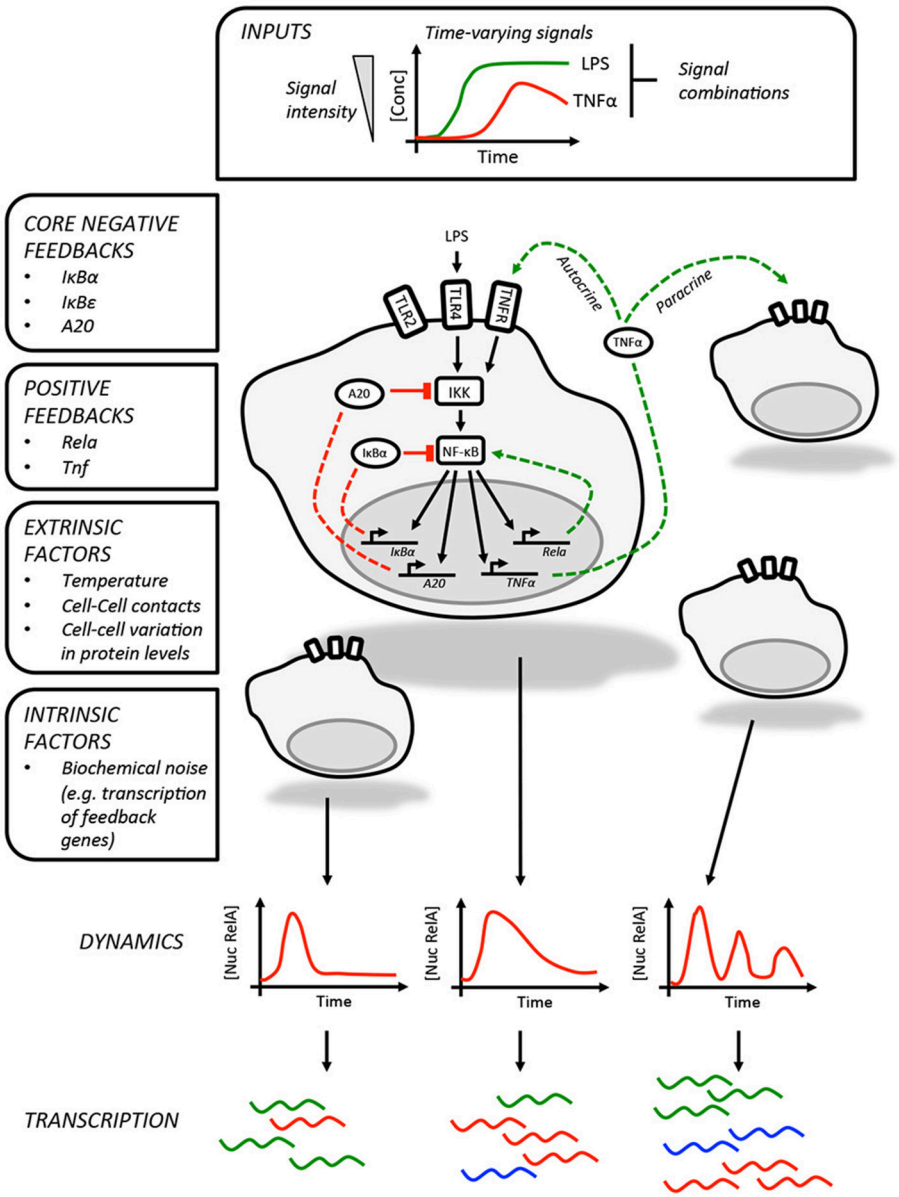

FIGURE 1 | Information processing by the NF-κB pathway. The NF-κB pathway is able to encode information about time-varying stimuli. In this illustration, which depicts LPS-induced NF-κB activity in macrophages, we list the factors that influence the dynamics of the response in individual cells. These include the core negative feedbacks (red dashed lines) and positive feedbacks (green dashed lines). The variability in single cell NF-κB dynamics are contributed to by a variety of factors, including paracrine signaling, and result in different patterns of gene expression between cells. The intrinsic biochemical noise of gene expression will also create variability within the responses of individual cells.

function. This appears to be because the transcripts of NF-κB target genes with related functions are expressed with similar kinetics or have similar stabilities. In this way, the expression of cytokines and cytokine receptors closely track NF-κB dynamics and will even oscillate, whereas the transcripts for target genes associated with other processes, including remodeling the extracellular matrix, accumulate more slowly and require repeated cycles of NF-κB nuclear accumulation in order to be expressed at biologically meaningful concentrations (10). Therefore, it seems logical that exogenous factors that influence NF-κB dynamics could effectively alter their meaning, impacting gene expression, and potentially compromising the response.

A large number of microbial pathogens are known to utilize effectors that directly target components of the NF-κB system and those that replicate or survive within host cells may also indirectly affect NF-κB as a consequence of other pathogen-encoded activities (39, 40). In most studies, these

effects are characterized as simply inhibiting or activating NF-κB signaling in host cells. Given our current understanding of the relationship between NF-κB dynamics and gene expression, we assert that a more nuanced view of these effects is called for if we are to fully understand the role NF-κB signaling plays in innate immunity and host: pathogen interactions.

LIVE IMAGING AS A TOOL TO STUDY NF-κB MODULATION BY INTRACELLULAR PATHOGENS

Overall, the use of live cell imaging to investigate NF-κB responses in cells exposed to live pathogens is surprisingly uncommon and is dwarfed by a wealth of similar studies using purified microbial ligands. Perhaps for this reason, the earliest publications in this area compared the kinetics of the TLR4-NF-κB response in cells co-cultured with intact, extracellular *E. coli* or LPS isolated from the same organism, showing similar effects (41). Other early publications used live cell imaging to correlate the attachment of bacteria to the surface of host cells with the timing of an NF-κB nuclear accumulation and disentangle the asynchronous responses between cells. This was used in two separate studies by the Meyer group to show that *H. pylori* with an intact type IV secretion system could induce p65 oscillations in human gastric epithelial cells (42), and that the force of type IV pilus retraction could stimulate waves of p65 nuclear translocation as *Neisseria gonorrhoeae* microcolonys form and fuse on the surface of infected cells (21).

While these studies using extracellular pathogens have been informative, they are mainly descriptive and do not provide deeper insights into how NF-κB signaling alters during the course of an infection or how it impacts outcome. During intracellular infections, NF-κB activity is very much a double-edged sword that can benefit both host and pathogen. It can strengthen the innate immune response of the host through expression of pro-inflammatory cytokines and directly enhance the microbicidal activity of macrophages by promoting expression of *Nos2* and other markers of M1 polarization (43–45). However, by positively regulating the expression of anti-apoptotic proteins, prolonged NF-κB activation can extend the survival of infected cells, providing a niche for the intracellular persistence and replication of the pathogen. Perhaps for these reasons, a wide variety of bacterial and eukaryotic pathogens, including *Salmonella* (23, 46, 47), *Legionella pneumophilia* (20, 48), and *Toxoplasma gondii* (49, 50) target NF-κB during infection. It is also common for individual pathogens to express multiple effectors, regulating different components of the NF-κB system to contrasting effect, deploying them individually or in combinations at different stages of an infection (39).

Delineating the various events that impact NF-κB activity during intracellular infection can be especially challenging. Intracellular pathogenesis is a multistage process, involving the microbe-active or -passive entry into host cells, intracellular survival of the pathogen, which may be accompanied by replication, and eventual exit (51). Changes in NF-κB activity may be associated with any phase of the process, driven by recognition of microbial antigens by host cell PRRs, either pre- or post-entry, or through the delivery of microbial effectors into the host cell. Even in cell culture models of infection, these events will happen asynchronously and, indeed, intracellular microbial burden will vary between cells. Furthermore, non-infected cells may exhibit so-called bystander effects, either through interaction with shed MAMPs, paracrine signaling, or a combination of both, complicating analysis (23). However, as many intracellular pathogens can be genetically modified to express fluorescent markers or are large enough to be identified in brightfield images, live cell microscopy can be used to track the progress of infection in individual cells while simultaneously monitoring changes in the localization of NF-κB proteins (**Figures 2A,B**) (22, 23). Quantitative time-resolved measurements of this type, and the ability to separate the responses of bystanders from those occurring in infected cells would be impractical (if not impossible) to achieve using bulk cell analysis techniques.

This approach was used in a recent study by Ramos-Marquès et al. to characterize the effect of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) on NF-κB signaling in fibroblasts (23). *S.* Typhimurium is a cause of inflammatory enteric disorders in mammals and is able to colonize fibroblasts after penetrating gut epithelium (52). While it was known that exposure to *S.* Typhimurium was capable of triggering NF-κB activity in these cells through recognition of shed MAMPs, LPS and flagellin by TLR4 and TLR5, respectively, it was previously unclear whether intracellular persistence of the bacterium affected the response. In order to explore this, the investigators used live imaging together with microfluidics in order to transiently expose fibroblasts to live bacteria for 10 min. This approach both limited the exposure of non-infected cells to shed extracellular MAMPs while also minimizing the effects of paracrine signaling. Although infected cells exhibited a heightened initial NF-κB response to *S.* Typhimurium exposure, presumably due to engagement of both surface and intracellular TLRs, subsequent exposure to bacteria or TNFα elicited a much-diminished response. The decreased nuclear translocation of p65 in these cells was accompanied by decreased *IL1B* and increased *SOCS3* expression, a cytokine signaling suppressor. These effects required a functional type III secretion system expressed from the *Salmonella* pathogenicity island 1 (T1) but not pathogenicity island 2 (T2). While the specific *S.* Typhimurium effectors responsible were not identified, it is known that a variety of T1 and T2 effectors directly target NF-κB pathway components and are capable of both increasing and decreasing NF-κB activity in different cellular contexts (47, 53, 54). These include AvrA, which inhibits p65 nuclear translocation by deubiquitinating IκBα (47). The ability to selectively employ combinations of these effectors in different host cell types may provide *S.* Typhimurium with the capability to tune host NF-κB responses to contrasting effect, either leading to the apoptosis of the host cell or extending its viability for use as a growth niche.

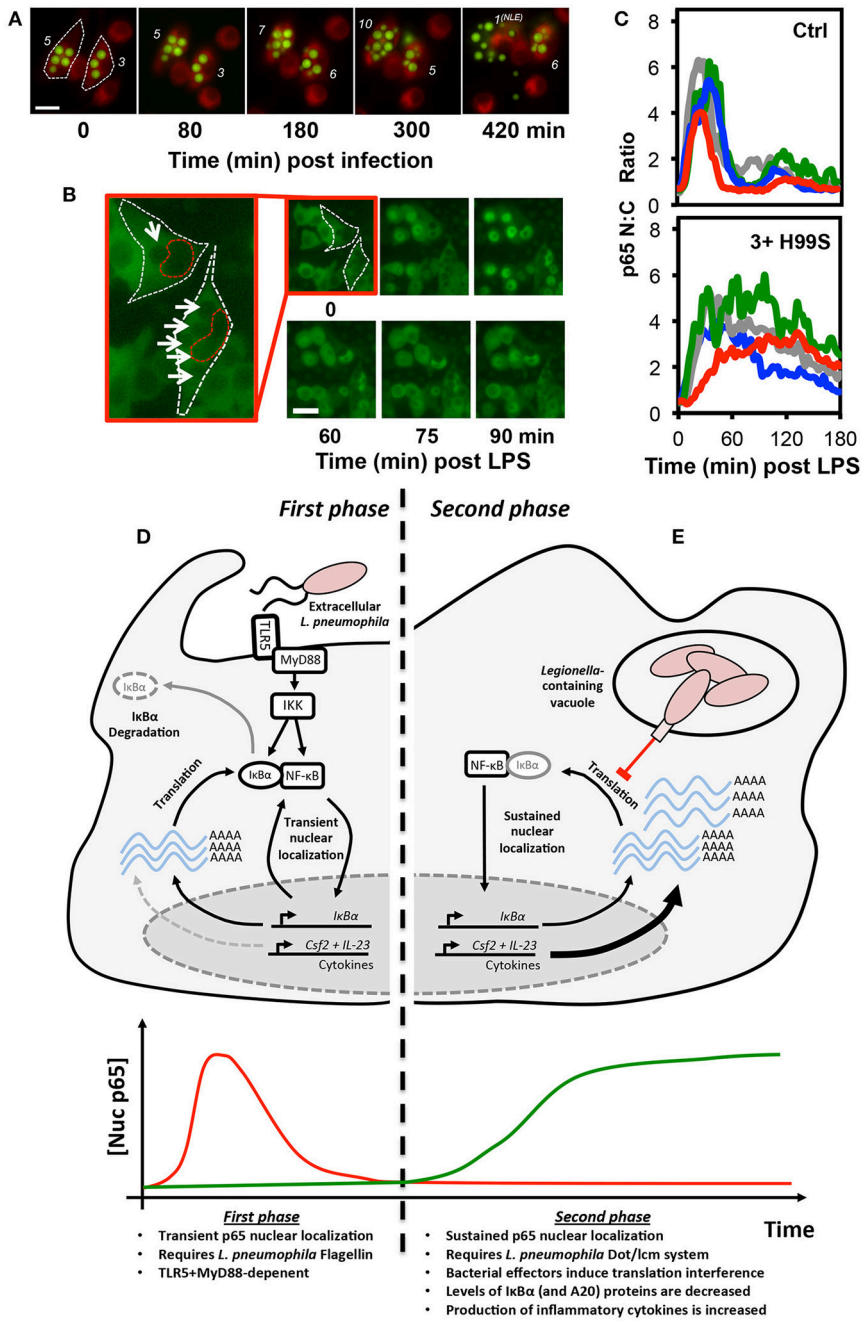

FIGURE 2 | Translational interference by intracellular pathogens alters NF-κB signaling dynamics. Both the fungal pathogen, *C. neoformans* **(A–C)**, and bacterial pathogen *L. pneumophila* **(D–E)**, alter NF-κB signaling by inducing translational interference in host cells. In *C. neoformans* infected cells, these effects are influenced by microbial burden. **(A)** Changes in burden can be tracked in live host macrophages. RAW 264.7 murine macrophages were stained with the membrane dye CellTracker™ Red CMTPX dye (Red) and infected with GFP-expressing *C. neoformans* (Green) then imaged by live cell fluorescence microscopy. The number of intracellular *C. neoformans* in each cell is marked in white. Burden can increase or decrease due to *C. neoformans* replication and non-lytic extrusion (NLE), respectively. **(B)** RAW264.7 cells expressing p65-EGFP were infected with *C. neoformans* and imaged by live cell fluorescence microscopy in the presence of LPS. For the two infected cells, white and red dashed lines indicate cell and nuclear boundary, respectively. Intracellular *C. neoformans* are marked with arrows. **(C)** Quantification of p65-EGFP nuc:cyto ratio in 4 representative non-infected and infected cells (containing ≥3 yeast per cell). Scale bars represents 20 μm. **(D,E)** Epithelial cells exhibit a biphasic NF-κB response to *L. pneumophila*. **(D)** During the first phase, flagellin from extracellular *L. pneumophila* stimulates transient TLR5:MyD88-dependent nuclear localization of p65. **(E)** In contrast, the second phase is flagellin, TLR5, and MyD88-independent and requires the *L. pneumophila* Dot/Icm secretion system. Delivery of effectors into host cells induces translational interference, the partial inhibition of new protein synthesis. This results in a net decrease in the levels of IκBα (and A20) proteins, labile negative regulators of NF-κB signaling. The resulting stable accumulation of p65 proteins in the nucleus promotes increased expression of a subset of pro-inflammatory cytokines, including GM-CSF and IL-23, encoded by the *Csf2* and *Il23a* genes, respectively. The images and data depicted in **(B,C)** were originally published in Hayes et al. (22), reproduced with permission. © The American Society for Biochemistry and Molecular Biology.

TRANSLATIONAL INTERFERENCE: A RECEPTOR-INDEPENDENT MECHANISM OF ALTERING NF-κB SIGNALING IN HOST CELLS

Facultative intracellular pathogens, by definition, do not require a mammalian host for replication. It is thought, therefore, that many of the strategies employed by these pathogens to evade host macrophages evolved in order to survive interactions with environmental protozoa, such as amoeba (55). These strategies may involve the expression of virulence factors that enable pathogens to either avoid ingestion by phagocytes or by targeting highly conserved, essential eukaryotic processes within the host cell in order to survive ingestion. It is notable then that a variety of bacterial and eukaryotic intracellular pathogens are able to induce translational interference, the partial suppression of nascent protein synthesis in host cells [reviewed in (56)]. While the primary purpose of this might be simply to increase the availability of free amino acids within the intracellular environment for microbial growth and attenuate innate immune function, its effects on cellular signaling should not necessarily be dismissed as "collateral" or a secondary effect. As feedback in the NF-κB system requires protein synthesis, translational interference will alter NF-κB dynamics and downstream gene expression. This has been illustrated by experiments where partial inhibition of ribosome function in the absence of external stimulus or microbial pathogens have driven a rapid reduction in IκBα (and slower loss of IκBβ and IκBε) and nuclear accumulation of p65 in murine fibroblasts (57). Within the context of an intracellular infection, this could hypothetically aid the pathogen by disrupting the normal operation of the pathway but it may also provide a receptor-independent mechanism by which intracellular microbial activity could be detected and responded to by host cells. These possibilities have been explored in a number of recent studies utilizing live cell imaging to measure NF-κB activity during infection with the facultative intracellular pathogens, *L. pneumophilia* and *Cryptococcus neoformans* (20, 22).

The encapsulated fungal pathogen, *C. neoformans*, is ubiquitous in urban environments and infects most individuals during childhood. It rarely causes disease in immune-competent hosts (58). Instead, it can enter a chronic, dormant state in host macrophages, often for many years, before later emerging should the immune system become compromised, leading to pneumonia and meningitis. As such, it is generally characterized as an AIDS-associated infection and is thought to be responsible for approximately 181,000 deaths per year worldwide, with most occurring in sub-Saharan Africa where HIV is endemic (59).

The *C. neoformans* polysaccharide capsule is essential for virulence and is largely made up of glucuronoxylomannan (GXM). GXM is synthesized and deployed as capsule rapidly after the inhalation of *C. neoformans* spores, increasing the effective radius of the yeast particle, impeding ingestion by host phagocytes and masking cell wall antigens that could be detected by PRRs. GXM is continually shed during growth as polysaccharide-filled vesicles both pre- and post-phagocytosis and appears to have immunomodulatory activities in this form (60). While there is disagreement in the literature about the precise effects of free GXM (61), possibly due to differences in the cell models used and GXM purification methods, several groups have shown that it is capable of suppressing TLR4 and MyD88-dependent NF-κB activation in a FcγRIIb and SHIP-dependent manner both *in vitro* and in a murine model of endotoxic shock (22, 62, 63).

Interestingly, the effects of GXM and capsular polysaccharides on NF-κB signaling may differ when secreted by phagosomal *C. neoformans*. This was explored in a recent study by Hayes et al. (22), which utilized the RAW264.7 NF-κB reporter cell line first described by Sung et al. (18), in order to simultaneously monitor p65 localization, the expression of an mCherry reporter of *TNF* promoter transactivation, and intracellular microbial burden. During these experiments, microbial burden was highly variable, as *C. neoformans* is able to both replicate within the acidified environment of the phagolysosome and also exit host cells without inducing cell death by non-lytic extrusion (**Figure 2A**) (64). While phagocytosis of encapsulated *C. neoformans* alone did not have an immediate effect on NF-κB signaling in host macrophages, it was capable of influencing the response of infected cells to pro-inflammatory stimulus. Specifically, when infected cells were challenged with LPS, the amplitude and duration of the response was increased and this was found to be dose-dependent, escalating with intracellular microbial burden (**Figures 2B,C**). This effect was lost when macrophages were infected with the capsule-deficient, GXM-negative *C. neoformans* mutant strain, CAP59, indicating that this effect was GXM-dependent. Interestingly, only live GXM-positive *C. neoformans* strains but not CAP59 or heat killed yeast induced a measurable decrease in nascent protein production in host cells, as measured by ribopuromycylation, suggesting that the altered NF-κB response was a product of GXM-induced translational interference. These data were consistent with the findings of an earlier independent study showing a reduction in protein translation rate in *C. neoformans*-infected J774.1 murine macrophage-like cells (65). Even though the overall change in NF-κB dynamics in the live cell imaging study were slight and would be difficult to detect in biochemical assays, it seems likely that it would be sufficient to influence the pattern of NF-κB regulated gene expression given the strong association between NF-κB dynamics and transcriptional output, which has been clearly demonstrated in macrophages (12).

The strategies employed by intracellular pathogens to subvert signaling may differ by cell type and can also alter as an infection progresses. For example, *L. pneumophila*, the causative agent of Legionnaires' disease can directly activate IκBα degradation and NF-κB in host macrophages through secretion of LegK1 effector proteins, an IKK mimic (48), promoting host cell survival. However, in epithelial cells, *L. pneumophila* induces biphasic NF-κB activation, which was resolved in a live cell imaging study by the Meyer group (20). The first phase of activation involves the recognition of flagellin, a component of *L. pneumophila* flagella, by TLR5, triggering transient MyD88-dependent nuclear translocation of p65 in infected cells (**Figure 2D**). This was associated with NF-κB-dependent expression of IL-8, likely

benefiting the host (66, 67). The second phase was TLR5 and MyD88-independent and instead required a functional Dot/lcm type IV secretion system, used by the bacterium to deliver effector proteins into host cells from the *Legionella*-containing vacuole (**Figure 2E**). This stimulated long-lasting, non-oscillatory p65 nuclear localization and was associated with a reduction in IκBα levels and expression of the anti-apoptotic proteins cIAP1, cFLIP, and XIAP, which the authors hypothesized would aid the pathogenesis of *L. pneumophila* through preservation of the intracellular growth niche. It is notable that earlier studies interpreted the TLR5-dependent and Dot/lcm-dependent responses as separate effects achieved at different multiplicities of infection rather than sequential events occurring during infection (68, 69). In this regard, the use of live cell imaging was instrumental in correcting this misconception.

Subsequent studies by an independent group indicate that the second phase of NF-κB activation in *L. pneumophila* infected cells is a product of translation interference (40), requiring the *L. pneumophila* Dot/Icm type IV secretion system to deliver a cocktail of five bacterial effectors into host cells to globally decrease—but not completely inhibit—mRNA translation. As IκBα proteins are particularly labile and turn over quickly, under these conditions, the rate of IκBα degradation exceed the rate of production, resulting in a rapid decrease in IκBα protein levels accompanied by stable nuclear accumulation of NF-κB in host cells. This results in the selective "superinduction" of specific transcripts that are not normally responsive to transient PRR-mediated NF-κB activity. While the precise mechanism remains unclear it seems likely that the shear number of these transcripts and possibly the stability of the protein products overcome the translational bottleneck in *L. pneumophila* infected cells. Proteins upregulated in these cells included the proinflammatory cytokines, interleukin-23 and GM-CSF, suggesting that this stable nuclear localization of p65 may not be entirely beneficial to the pathogen and may represent a receptor-independent mechanism of NF-κB activation, providing a means to initiate an innate immune response.

CONCLUDING REMARKS

Live cell imaging has transformed our understanding of how the NF-κB system coordinates the cellular response to stimuli, especially in innate immune cells. The ability of this technique to disentangle differing and asynchronous responses of individual cells has also made it ideal for investigating how intracellular pathogens manipulate NF-κB signaling in host cells, particularly in instances where the effects on this pathway are influenced by intracellular microbial burden or the changing repertoire of microbial ligands and effectors presented or deployed during the course of an infection (22). Despite the various advantages of the technique, to the author's knowledge, it has seldom been used for this purpose and this mini-review represents a relatively complete overview of the current literature in this area.

Prior genetic and biochemical studies have shown that modulation of host cell NF-κB signaling is relatively common among gastrointestinal pathogens, including *Helicobacter pylori* (70), *Shigella* (71), and *Yesinia* (72), and has been demonstrated in other invasive bacteria, such as *Mycobacterium tuberculosis* (73). Overall, it appears that NF-κB modulation is utilized by pathogens to either "buy-time" for intracellular replication, as employed by *Mycobacterium tuberculosis* and *Shigella* (71, 73) by stimulating the expression of pro-survival NF-κB-responsive genes, or to do quite the reverse, by using effectors that inhibit host cell NF-κB-activity to blunt an inflammatory response or promote apoptosis in order to evade destruction by innate immune cells or aid escape and dissemination (72). While these previous studies have successfully identified the molecular players required for subversion of NF-κB signaling in host cells, a reexamination of these effects using live cell imaging is merited. As demonstrated by the research highlighted in this review, this method could help to resolve otherwise hidden bi- or multiphasic responses to intracellular pathogens (20), and perhaps most interestingly, link the different NF-κB responses of individual cells to specific transcriptional responses using fluorescent reporters (18, 22) or downstream single cell transcriptomics (12) and different infection outcomes (e.g., intracellular replication, non-lytic exocytosis, host cell death, killing of the pathogen etc.).

Despite the potentially very useful insights that can be obtained through the application of this technique, our enthusiasm should be tempered by an awareness of its inherent limitations, which stem from the absolute requirement to modify the system being studied through the use of fluorescent tags and the over-expression of exogenous proteins, both of which have the potential to affect the behavior of the pathway. The former is perhaps least concerning as careful characterization of p65 fluorescent fusions has suggested that GFP-tags neither interfere with the ability of the protein to transactivate gene transcription or correctly associate with regulators, including the IκB proteins (74), although it may have as yet unrecognized consequences. The effects of protein overexpression on the behavior of the NF-κB are less clear-cut. Experimental evidence has suggested that p65 overexpression has little effect on pathway behavior (75), although separate studies have indicated IκBα levels recover more rapidly after stimulation in cells expressing p65-GFP in addition to the endogenous protein (42). More recent studies have attempted to minimize the effects of overexpression by using BACS or stable transduction of viral constructs to express p65 fusions under the control of the endogenous promoter (18, 36). In these ways, expression levels of tagged p65 can be regulated appropriately by the cell and kept more closely to endogenous levels than might be achieved through transient transfection of plasmid constructs. It also seems likely that future studies will utilize CRISPR/Cas9-based gene editing to introduce fluorescent proteins into the endogenous locus of NF-κB genes to avoid protein overexpression.

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REVIEW
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Considering Abundance, Affinity, and Binding Site Availability in the NF-κB Target Selection Puzzle

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The NF-κB transcription regulation system governs a diverse set of responses to various cytokine stimuli. With tools from *in vitro* biochemical characterizations, to omics-based whole genome investigations, great strides have been made in understanding how NF-κB transcription factors control the expression of specific sets of genes. Nonetheless, these efforts have also revealed a very large number of potential binding sites for NF-κB in the human genome, and a puzzle emerges when trying to explain how NF-κB selects from these many binding sites to direct cell-type- and stimulus-specific gene expression patterns. In this review, we surmise that target gene transcription can broadly be thought of as a function of the nuclear *abundance* of the various NF-κB dimers, the *affinity* of NF-κB dimers for the regulatory sequence and the *availability* of this regulatory site. We use this framework to place quantitative information that has been gathered about the NF-κB transcription regulation system into context and thus consider questions it answers, and questions it raises. We end with a brief discussion of some of the future prospects that new approaches could bring to our understanding of how NF-κB transcription factors orchestrate diverse responses in different biological contexts.

Keywords: NF-κB, transcription regulation, specificity, accessibility, competition

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INTRODUCTION

The nuclear factor-κB (NF-κB) family of transcription factors regulate the expression of genes that are crucial to a wide variety of biological processes, ranging from immune, stress, and inflammatory responses, to cell apoptosis. The NF-κB family is made up of five proteins, p105/p50 (encoded by *NFKB1*), p100/p52 (encoded by *NFKB2*), RelA (also known as p65), RelB, and c-Rel, which can form a range of homo- and hetero-dimeric complexes [**Figures 1A,B**; (6)]. When partnered with inhibitory IκB proteins, NF-κB dimers are preferentially shuttled to the cytoplasm where they are held inactive. In response to stimuli, IκB is phosphorylated and subsequently degraded, thus releasing NF-κB and allowing it to accumulate in the nucleus (**Figure 1C**). Once in the nucleus an NF-κB dimer can bind to κB sites to activate or repress the transcription of its target genes. The best-studied κB sites fit the consensus κB site pattern, 5′-GGGRNWYYCC-3′ (where R, W, Y, and N, respectively denote purine, adenine or thymine, pyrimidine, and any nucleotide) (7–9). In the human genome encompassing 3×10^9 base pairs, there are undoubtedly myriads of sequences matching the consensus κB site. Indeed, early on, ChIP-chip (chromatin immunoprecipitation to

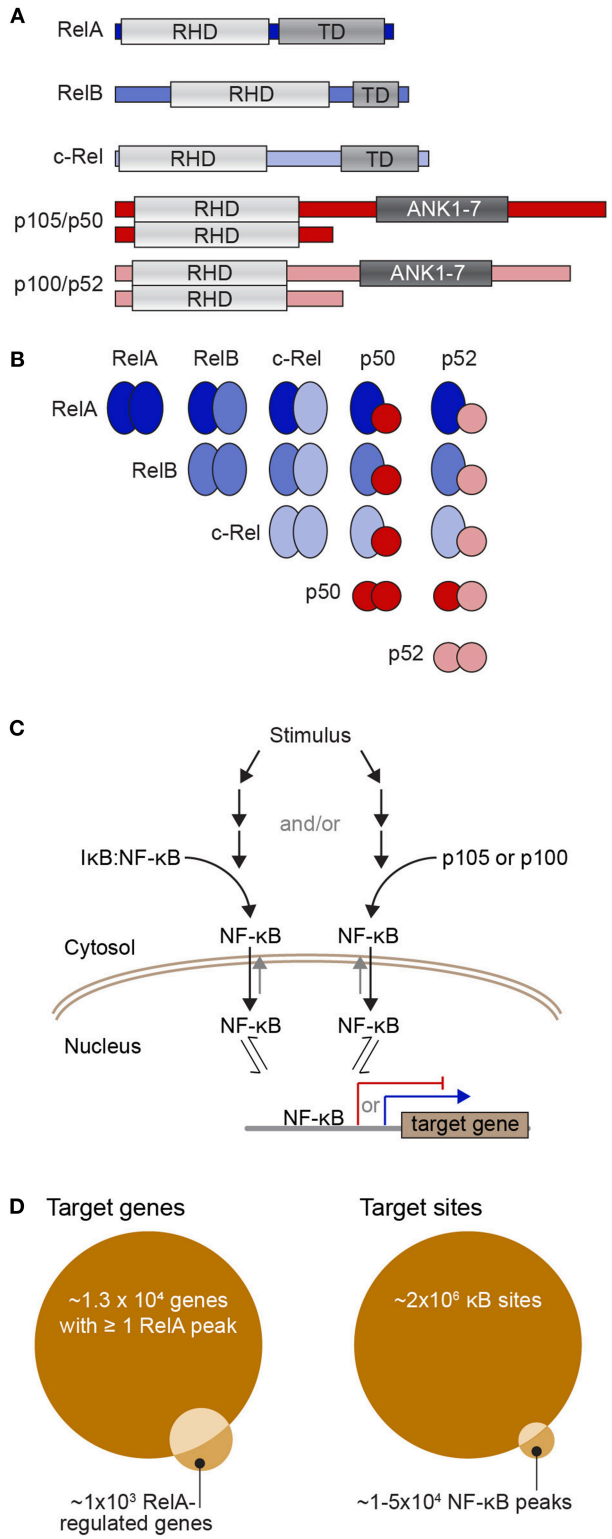

FIGURE 1 | The NF-κB transcription factor family and its dimerization and DNA interactions. **(A)** Diagrams of the five NF-κB subunits showing their Rel homology domain (RHD), which encompasses both their DNA-binding

(Continued)

FIGURE 1 | domain and dimerization region, the transactivation domains (TD) of RelA, RelB, and c-Rel, as well as the ankyrin-rich region of p105 and p100 (repeats 1-7; ANK1-7), which is cleaved to yield p50 and p52. **(B)** Diagram of the ways in which the NF-κB subunits can partner to form dimers that contain zero (all red), one (blue/red), or two (all blue) transactivation domains. **(C)** Simple schematic of the process of activation of NF-κB dimers. Upon stimulation, a series of events leads to phosphorylation and proteasome-mediated degradation of IκB to release NF-κB dimers (left) and/or cleavage of p105 or p100 to remove their IκB-like ankyrin-rich domain and again release NF-κB dimers (right). Free NF-κB dimers are preferentially shuttled into the nucleus where they have access to the regulatory sequences of NF-κB target genes. TD-containing NF-κB dimers can activate transcription of target genes (blue arrow), while TD-lacking NF-κB dimers can act as transcriptional repressors (red). **(D)** Venn diagrams representing potential target sites (right) and potential target genes (left). There may be up to 2×10^6 consensus κB sites or half-sites in the human genome (1) although various ChIP-seq studies have reported that there may be between 1 and 5×10^4 NF-κB-bound peaks in a mammalian genome across a population of stimulated cells [e.g., (2–4)], of which 30–50% contain a consensus κB site. Because many gene regulatory sequences have multiple NF-κB-bound peaks, one estimate is that around 1.3×10^4 genes have at least one RelA peak in their regulatory region (5). However, the same study found only ∼1,000 genes were detectably regulated by RelA-containing NF-κB with ∼60% of these having a RelA ChIP-seq peak in gene-proximal regulatory regions (5).

microarray) experiments interrogating the sequence of human chromosome 22 suggested that there are more than 1.4×10^4 of these consensus sites contacted by NF-κB dimers during a response to stimulus (2, 10). More recent ChIP-seq experiments have identified 20,000–50,000 RelA-bound peaks, although it is unclear whether RelA directly contacts the DNA at all of these sites (3, 4). Just considering the RelA subunit, 1×10^5-1.5×10^5 molecules enter the nucleus following stimulation (11), a portion of these molecules bind to DNA and this regulates the expression of just ∼600 genes [a curated list of known NF-κB target genes can be found at the Boston University NF-κB Transcription Factors website; (12)]. The large number of RelA molecules in comparison to the relatively small number of regulated transcripts suggests a complex relationship between the amount of NF-κB in the nucleus and the subsequent expression of target genes.

Numerous ChIP-seq and whole genome sequencing experiments have shown that the recruitment of many transcription factors, including NF-κB, to chromatinized DNA is dependent on the cellular context and therefore must be highly regulated [reviewed in (10)]. However, despite the wealth of genomic data now available, the mechanisms by which NF-κB-DNA interactions generate specific gene expression profiles following stimulation remain largely unknown. For example, by allocating ChIP-Seq peaks to their nearest gene, RelA-containing NF-κB was found to bind ∼13,600 genes in TNF-stimulated HeLa cells, yet only ∼1,000 genes were up or down-regulated in response to RelA perturbation, and only ∼600 of these were directly bound by NF-κB [**Figure 1D**, left; (5)]. Thus, a vast majority of the genes that are bound by NF-κB in response to stimulus are not regulated. This raises the questions: how do NF-κB dimers select their binding sites and why are only

some of the bound genes transcriptionally regulated? Seeking to answer these questions, we will focus herein on three key sets of factors that regulate NF-κB recruitment to DNA: *abundance* of NF-κB dimers and κB binding sites, binding *affinity*, and the *availability* of the κB sites at any given time.

ABUNDANCE

κB Binding Sites

If, as Martone et al. (2) estimated, there are $\sim 10^4$ consensus κB sites in the genome that are bound by RelA and $\sim 1 \times 10^5$ RelA-containing dimers enter the nucleus upon cell stimulation [estimated by Hottiger et al. (11)], a simple view of the system would predict rapid saturation of these consensus κB sites (see **Box 1**). However, experiments demonstrate that many consensus κB sites are not bound and, in fact, this lack of saturation of the system is necessary to generate stimulus- and cell-type-specific gene expression profiles (16–18). One explanation for this apparent dichotomy is that, in addition to consensus κB sites, NF-κB can bind to degenerate κB sites. Structural, biochemical, and *in vivo* assays have demonstrated that NF-κB dimers can bind to κB half sites, sites whose sequences deviate from the consensus sequence, and even unrelated sites (3, 19–24). With these additional non-consensus binding sites, the total number of potential NF-κB sites in the human genome could easily climb to 2×10^6 (1). This flips the NF-κB protein vs. NF-κB binding site calculus (**Figure 1D**, right), and our first question becomes: how do the relatively *sparse* NF-κB dimers decide which of the *numerous* potential κB binding sites to interact with?

In recent years, innovative live-cell imaging techniques based on fluorescence recovery after photobleaching (FRAP), along with kinetic modeling of the collected data, have started to shed light on the dynamic nature of the transcription factor-DNA interaction process. Broadly speaking, this work indicates that most transcription factors may rapidly diffuse through the nucleus (with diffusion coefficients of $\sim$0.5–5 $\mu m^2 s^{-1}$ depending on transcription factor size) while "scanning" the genome for high-specificity sites (25, 26). Of note, the use of the term "scanning" should not necessarily evoke the image of a transcription factor gliding along chromatin, although such one-dimensional sliding models have been posited following single-molecule imaging studies of the p53 transcription factor (27, 28). Instead, many transcription factors, including NF-κB dimers, may "scan" by visiting multiple sites in a trial-and-error series of short-duration binding events (29). Therefore, transcription factors undergo thousands of these transient encounters with chromatin that ultimately will have no direct consequence on gene expression.

Interestingly, it is now thought that most *functional* NF-κB interactions with chromatin—interactions that lead to a change in transcription—are fleeting. Early, *in vitro,* bulk biochemical measurements of NF-κB interactions with κB sites indicated the formation of very stable complexes with a half-life of up to 45 min (30); using bulk, ChIP-based assays, similarly long interaction half-lives have been measured for other transcription factors (31) and shown to be regulated by ubiquitylation [reviewed in (32, 33)]. However, a subsequent study using FRAP in live cells expressing RelA-GFP showed that most RelA-DNA interactions are actually quite dynamic, with half-lives of a few seconds (16). Using stopped-flow kinetics and surface plasmon resonance, which can both directly measure association and dissociation kinetics, *in vitro* measurements made in physiological salt and pH conditions recapitulated these faster kinetics [yielding half-lives of 1.5 and 40 s, respectively for RelA:p50-DNA (34) and a range of a few seconds to a few minutes for a variety of NF-κB dimers and binding sites (22)]. Strikingly, IκBα can further accelerate the RelA:p50-DNA dissociation by up to $\sim$40-fold, "stripping" dimers from DNA in a process that has now been extensively characterized (34–39). Indeed, a recent study used single-molecule tracking of individual Halo-tagged RelA molecules in live cells to show that the majority ($\sim$96%) of RelA undergoes short-lived interactions lasting on average $\sim$0.5 s, while just $\sim$4% of RelA molecules form more stable complexes with a lifetime of $\sim$4 s (40). Because the ability of the RelA fusion proteins to induce transcription of target genes was verified in both the FRAP and single-molecule *in vivo* studies, these results suggest that long-lasting NF-κB binding may not be required for preinitiation complex assembly or for the activation of transcription. However, the mechanisms that distinguish NF-κB-DNA binding events that change transcription of a target gene from those that do not remain unclear.

Recent studies have found that while individual interactions are very brief, the integrated target site occupancy of Sox2 and Oct4 transcription factors can be highly sensitive to the nuclear concentration of the transcription factor (41). This implies that even when transcription factor occupancy at target sites is short-lived, high nuclear concentrations facilitate rapid turnover and, overall, increase the frequency of these short interactions. In this way, high rates of transcription factor sampling at binding sites may generate enough cumulative site occupancy to affect transcription (29). Having many binding sites across the human genome, NF-κB may also use this mechanism to tune the spatiotemporal patterns of gene expression it generates in response to stimulation by, for example, having a larger effect on sites that have the highest cumulative occupancy.

Intriguingly, high frequencies of transcription factor sampling have also been observed at non-consensus sites, yet these interactions were shown to have no direct effect on transcription (42). This observation has revived ideas first proposed years ago, whereby a key part of the target search process is transcription factors making non-specific contacts with DNA and then proceeding to slide or hop around the local chromatin environment until either a specific contact is formed, or contact and access to DNA is lost (43, 44). This model could partially explain the large number of sites detected by static, end-point biochemical binding assays including ChIP-Seq studies, which appear to be non-functional. Although these sites are "visited" in the search process, their cumulative occupancy may not be sufficiently long, or their interaction qualitatively suitable, to affect transcription.

If transcription factors rapidly sample many sites, would a cluster of non-functional binding sites near a target gene promoter or enhancer increases, or decreases, the local concentration of transcription factor? An increase in local

Box 1 | Computing fraction of binding sites occupied by transcription factor.

Computational models provide a powerful means to examine, interrogate, and ultimately better understand the relationships between inputs and outputs of complicated biological processes. Here, we use a simple mass-action kinetics model to illustrate how (i) binding affinity, (ii) abundance of transcription factors and their binding sites, and (iii) the availability of these binding sites due to the presence or absence of a binding competitor species affect the fraction of sites bound by the transcription factor. Although in reality, binding by a transcription factor is only a rough correlate of gene transcription in response to stimuli, this toy model shows us how the interplay between quantitative aspects of protein-DNA interactions potentially affects transcription regulation. Previous studies have used similar kinetics models to calculate fraction of binding sites (13).

In the simple scenario that we depict (**Figure Box 1A**), we model the binding of transcription factors to their cognate sites on the genome as a simple adsorption process—where molecules bind to sites, unchanged. This model therefore gives us a theoretical limit on the fraction of bound sites when the process is activation energy-limited (i.e., within-nucleus transport is much faster than DNA binding) and the process of a transcription factor finding a binding site is random. We also make additional simplifying assumptions: (1) the contents of the nucleus are well mixed and both genomic and non-genomic compartments are homogeneous; (2) all binding sites are equivalent with identical affinities for the transcription factor and competitor species; (3) the total nuclear concentrations of transcription factor and competitor species are fixed, under the assumption that any change occurs on a time scale slower than that of the binding process (and therefore, in this very simplistic model, we assume that the steady state is reached faster than changes in nuclear abundance and post-translational modifications of transcription factors). Given the stated assumptions, we will let X be free nuclear transcription factor, X^{comp} be free nuclear competitor species, and Y represent the transcription factor binding site. Y can be bound by X or X^{comp} creating the complexes $Y:X$ and $Y:X^{comp}$, respectively. Binding of transcription factor and competitor species to DNA can then be modeled by two reaction equations:

$$X + Y \leftrightarrow Y:X$$
$$X^{comp} + Y \leftrightarrow Y:X^{comp}$$

As we assumed identical affinities to DNA binding sites for the transcription factor and its competitor, we will also assume the same association rate parameter α and dissociation rate parameter γ. Using mass action kinetics and mass balance equations, our reaction system can be fully described using two ordinary differential equations (ODEs):

$$\frac{d[Y:X]}{dt} = \alpha \cdot (X_T - [Y:X]) \cdot (Y_T - [Y:X] - [Y:X^{comp}]) - \gamma \cdot [Y:X]$$
$$\frac{d[Y:X^{comp}]}{dt} = \alpha \cdot \left(X_T^{comp} - [Y:X^{comp}]\right) \cdot (Y_T - [Y:X] - [Y:X^{comp}]) - \gamma \cdot [Y:X^{comp}]$$

Here, X_T, X_T^{comp} and Y_T are the total number of molecules or sites for a given nucleus and, under our assumption of time scale separation (#3), they are assumed constant while solving the ODEs. We also define $K_D = \frac{\gamma}{\alpha}$, the dissociation constant (which is, as usual, the inverse of the binding affinity constant). Solving the system of ODEs gives the concentration of each species over time and at steady state. Solving the ODEs for different sets of parameter values and initial concentrations for X_T, X_T^{comp} and Y_T allows us to illustrate the relationships between these parameters and initial concentrations and the steady state (ss) fraction of sites bound by the transcription factor, calculated as $\frac{[Y:X]_{ss}}{Y_T}$.

To survey a biologically relevant range of concentration values, we considered a typical HeLa cell, with a total cell volume of 2,700 μm^3, a cytoplasmic to nuclear volume ratio of 3.3 (14), and ratios of transcription factors and available binding sites from 10^3:10^6 to 10^6:10^3 molecules/site. In the absence of the competitor (**Figure Box 1B**; continuous lines) and for K_D >10 nM, we need a large amount of transcription factor ($\log_{10}\left[\frac{X_T}{Y_T}\right] > 1$) to effectively saturate most of the binding sites at steady state. If we consider the RelA:p50 heterodimer, which has been reported to bind to the consensus κB site with a K_D of 12.8 ± 2.2 nM (15), and ~1.5 × 10^5 heterodimers in a nucleus (11) with ~1.4 × 10^4 binding sites (2), we obtain $\log_{10}\left[\frac{X_T}{Y_T}\right] > 1$, and the simple model finds that >90% of the DNA binding sites would be occupied. In the presence of the competitor species (in abundance equal to that of the dimers) the achievable occupancy is reduced to half (**Figure Box 1B**, dashed lines). Even a simplistic illustrative model such as the one we used here clearly shows how the interplay between abundance and affinity changes the fraction of bound sites in a nonlinear fashion, and how one mechanism for regulating availability of the sites—competition—can substantially reduce the number of sites occupied by a transcription factor. As we discuss in this review, there are many other nuances to abundance, affinity, and availability which will require more complex models to fully capture.

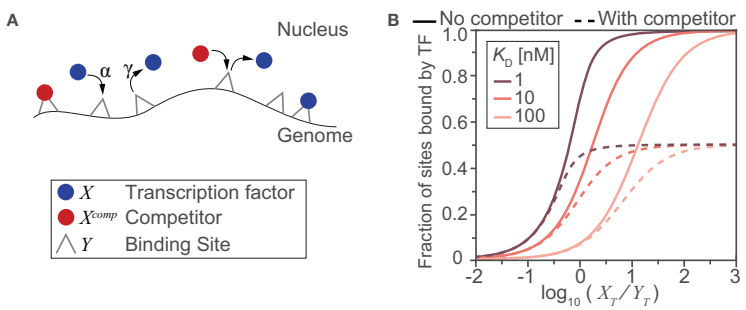

Figure Box 1 | A toy model illustrates the impact of abundance, affinity and availability on the fraction of potential sites bound. **(A)** Schematic diagram of the reactions and molecular species included in the model. DNA binding sites are present on the genome and can be bound by a transcription factor (blue) or by a competitor protein (red) with an on-rate of α and off-rate of γ. **(B)** Model-derived input-output relationships between the ratio of total transcription factor to potential binding sites ($\frac{X_T}{Y_T}$) and the steady state fraction of sites that are bound by a transcription factor. The relationship was derived for three different transcription factor binding affinities for the DNA sites (expressed using the dissociation constant, $K_D = \frac{\gamma}{\alpha}$), in the presence (dashed lines) or absence (solid lines) of a competitor species (where, $X_T = X_T^{comp}$).

concentration could occur if the brief interactions with clustered sites keep more transcription factor molecules nearby, increasing the probability that one binds to the functional target site [e.g., via an avidity effect as theoretically considered by (45)]. Alternatively, if the non-functional sites sequester transcription factors away from the functional target sites, acting as "natural decoys" (46), they would effectively decrease the local concentration of transcription factors. An early study showed that transfection of double-stranded oligonucleotides with κB sites inhibits the NF-κB-induced production of inflammatory cytokines in a rat model of myocardial infarction (47) showing a decoy-site effect. However, in that scenario, the transfected oligonucleotides likely reduce the global, not local, concentration of available NF-κB dimers and thus globally suppress transcription NF-κB-driven transcription. A more direct test of the effects of clusters of sites would be to manipulate the sequences near an NF-κB target gene promoter. In budding yeast, synthetic promoters were used to show that adding clustered sites for a transcriptional activator reduce the transcriptional output, as expected for decoys (48). In addition, the clustered sites could qualitatively change a transcriptional response from a graded response, correlated to transcription factor abundance, to a threshold-based, non-linear response (48). A combination of mathematical models and synthetic *LacI*-based constructs in *E. coli* showed that varying the number and chromosomal context of repressor binding sites can also quantitatively and qualitatively change the response (49). With so many possible nuances driving up or down the probability of transcription factor-DNA interactions, it may well be that the effect of additional sites on the transcription of an NF-κB target gene, whether enhancing or dampening, is highly context-dependent. The relative affinity, number, and two- or three-dimensional clustering of the sites could all modulate their effects and diversify the response of target genes to one NF-κB signal.

In one more layer of complexity, many NF-κB target genes have multiple κB sites *within* their regulatory regions [a common feature of many transcription factor binding motifs; (50)]. In fact, 95% of up-regulated and 91% of down-regulated NF-κB target genes have been shown to contain more than three κB sites in their regulatory regions [e.g., (2, 5)]. For many years, the predominant model for transcriptional regulation was that having multiple sites within gene promoters would drive cooperativity in DNA binding by the cognate transcription factors (51). This cooperative binding was then thought to lead to rapid, binary switching between fully unoccupied inactive promoters and fully occupied active promoters, yielding a largely all-or-none transcriptional activation (or repression) response. However, more recent studies have shown that NF-κB (via RelA) does not generally show cooperative binding to DNA, and instead NF-κB-dependent transcriptional activity scales gradually with NF-κB nuclear concentration (52). Therefore, Giorgetti and colleagues propose that the presence of multiple κB sites in one regulatory region increases the dynamic range of transcriptional outputs, with promoters with more consensus κB sites driving higher transcription at the same nuclear concentration of NF-κB, thus providing yet another means to quantitatively modulate NF-κB-dependent gene expression.

In summary, these observations indicate that the distribution of κB sites in the genome is non-uniform and clustering of the consensus and non-consensus sites in combination with highly frequent interactions of NF-κB with these sites can influence the transcriptional logic as well as shape the dynamic range of transcription. As, in fact, most human transcription factors are generally observed to bind to only a fraction of their consensus sites in any given cell type (53); this site selection process may be a generalized mechanism to achieve specific transcriptional responses.

Nucleus-Localized NF-κB Dimers

One challenge for anyone surveying the NF-κB literature with a quantitative mindset is that the terms "NF-κB" and "RelA" are often used interchangeably, and most studies reporting on the abundance of "NF-κB" in the nucleus focus exclusively on the RelA subunit. By ignoring other dimer species, these numbers muddle the relationship between signal, nuclear "NF-κB," and DNA binding or transcription output. Different stimuli can lead to the nuclear accumulation of specific NF-κB dimers, indicating the importance of considering more than just the RelA subunit [e.g., (54)]. As other reviews have considered stimulus-specific activation of particular NF-κB dimers (17, 55); here, we specifically consider how the nuclear abundance of different dimer species can modulate NF-κB-driven transcriptional responses (**Figure 2**).

The five NF-κB subunits can dimerize in almost every combination, each with unique but overlapping DNA and protein binding affinities [**Figure 1B**; (3, 22, 23, 56)]. RelA, RelB, and c-Rel each contain a transactivation domain (TD), capable of recruiting the transcription machinery, and thus NF-κB dimers including at least one of these subunits can activate transcription. In contrast, p50 and p52 do not have a TD and homodimers or heterodimers made up of only p50 and p52 are not capable of inducing transcription without recruiting an additional TD-containing transcription factor. Bound to the same κB site, a TD-containing NF-κB dimer will likely act as a transcriptional activator while a TD-lacking NF-κB dimer can act as a transcriptional repressor.

Although RelA:p50 is frequently cited as the most abundant NF-κB dimer, this may be dependent on cellular context. Other dimer species can also be highly expressed, and some are more likely to be found in the nucleus prior to stimulation. For example, p50 homodimers localize to the nucleus in resting mouse bone marrow derived macrophages (BMDMs) at a concentration of ∼200 nM, which is similar to the maximum nuclear RelA:p50 concentration following stimulation in these cells (57). Thus, a quantitative framework that seeks to explain or predict NF-κB-DNA interactions and NF-κB-driven transcription at target genes but considers only RelA:p50 dimers is greatly oversimplifying the system. The 1.5×10^5 molecule per cell figure that we have considered overlooks contributions from other dimer species, the nuclear concentration of which is not necessarily correlated with that of RelA:p50. Therefore, the simple assumption that nuclear RelA:p50 is the major contributor

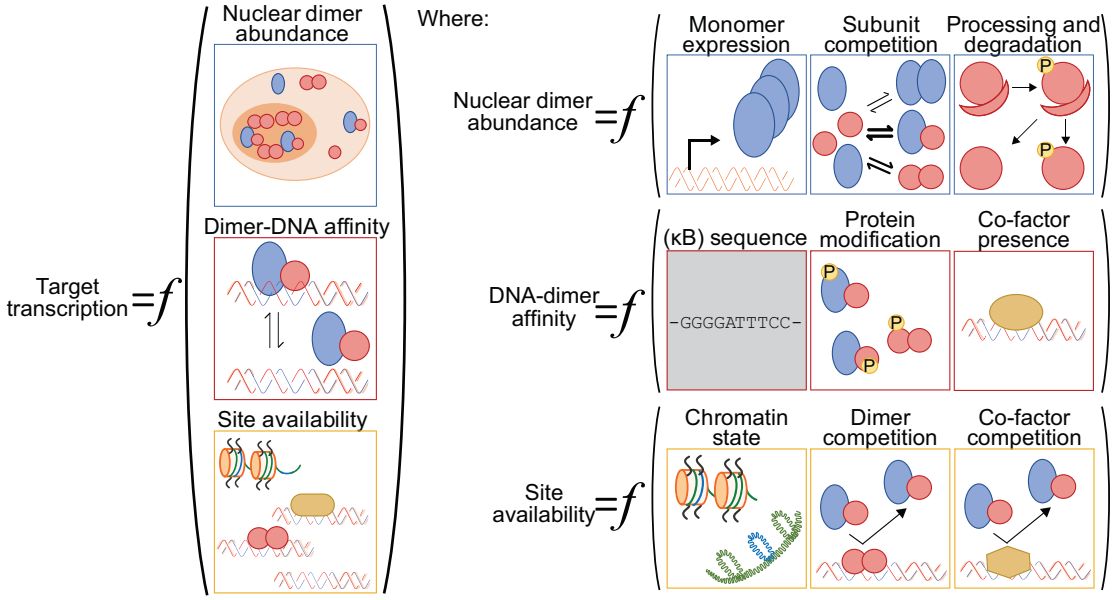

FIGURE 2 | A generalized function for NF-κB-driven gene regulation. Schematic diagram of quantitative and qualitative factors that can differentially modulate NF-κB-driven gene regulation gene-by-gene and across various cellular contexts. Broadly speaking, target gene transcription is set by the nuclear abundance of NF-κB dimers, the NF-κB-DNA binding affinity and the availability of the DNA binding sites (left). The nuclear abundance of NF-κB dimers is itself a function of NF-κB subunit monomer expression, of NF-κB subunit competition in the various dimerization reactions, and of processing and degradation of inhibitory domains (ankyrin-rich domains of p105 and p100) and inhibitory proteins (IκBs) (top right panel). The NF-κB-DNA binding affinity is influenced by the DNA sequence (for both consensus κB sites and non-consensus sites), by NF-κB dimer identity and their post-translation modifications, and by the presence of regulatory co-factors that may help recruit NF-κB dimers to DNA or stabilize the interactions (center right panel). We note here that the DNA sequence is, arguably, the only factor that is not cell-type specific (gray shading). Finally, the availability of DNA binding sites for interaction with an NF-κB dimer is a function of the chromatin state, including the presence of histones and histone modifications, of competition from other NF-κB dimers and their relative affinities for the same site, and of competition with other regulatory factors that may bind to and occlude the potential binding site (bottom right panel).

to NF-κB-driven transcription not only underestimates total nuclear NF-κB abundance, but may also obscure the true relationship between "NF-κB" abundance and DNA-binding and transcription activation (see also **Box 1**).

Finally, the abundance of the different NF-κB dimers is not a static quantity. Some stimuli induce the production of specific dimer species, for example via processing of the p100 subunit to p52, which leads to an increase in p52-containing dimers [**Figures 1C**, **2**; (55)]. There is also competition between the various NF-κB subunits for dimerization, due to the similar affinity of multiple subunits for a given subunit dimerization partner [**Figure 2**; (58)]. Therefore, if, for example, p52 abundance increases, not only could this induce an increase in repressive p52:p52 dimers, competition for NF-κB dimerization will reduce the abundance of lower dimerization affinity subunit pairs, which could lead to splitting of dimers containing two TD domains to generate p52-containing heterodimers, and effectively increase the abundance of transcription activating NF-κB dimers. Overall, although many studies consider only one protein, the RelA subunit, the total nuclear abundance of NF-κB factors could be substantially higher and the relative abundances of various dimers dynamically modulated. In the section 'Competition between NF-κB dimers' below, we come back to this and discuss how different dimer abundances can impinge on κB binding site availability.

As we add resolution to quantitative understanding and models of NF-κB-driven transcription in various cellular contexts, we will need to reevaluate simplifying assumptions about the abundance of NF-κB dimers and consider the contributions of the combinatorial possibilities of the "NF-κB dimer network" (17). Because of dimer-specific activities, transcription is certainly impacted by subunit abundance and competition for partnering with TD-containing subunits.

AFFINITY OF NF-κB DIMERS FOR κB BINDING SEQUENCES

Biochemical DNA binding studies of a wide variety of 9–12 base-pair sequences have revealed that different NF-κB dimers bind far more sequences than previously thought, with different dimer species exhibiting specific but overlapping affinities for consensus and non-consensus κB site sequences (3, 22, 23). Although specific NF-κB dimer-DNA affinity values are hard to pin down because they are strongly condition-dependent (15), a constant is that for a given sequence and assay, the affinities of different dimers are consistent with more than one dimer being able to bind this sequence in cells [e.g.,(15, 22, 34)]. Many sequences that contain only a single consensus half-site also show substantial dimer binding (22). Furthermore, structural studies

showed that in certain conformations, only one subunit of NF-κB dimers is involved in sequence-specific DNA interactions (24). Taken together, these studies indicate that κB half sites are sufficient for functional NF-κB dimer binding and that the state of the dimer may direct its binding toward certain sequences. Importantly, just as dimers exhibit preferences for different DNA sequences (**Figure 2**), the corollary must be true, that different DNA sequences may recruit one specific dimer combination over another.

Interestingly, once bound to DNA, each NF-κB dimer has been shown to induce different amounts of transcriptional activity from target genes [reviewed in (56)]. The clearest example, as mentioned above, is that because neither p50 or p52 possesses a TD, dimers containing just these subunits are unable to activate transcription alone. More subtle differences have also been reported, for example, the decreased recruitment of RNA polymerase II (RNAPII) as the *IL12B* promoter switches from binding RelA-containing dimers to RelB-containing dimers (59). The combination of dimer specificity with dimer switching during a response can thus provide a mechanism to generate temporally diverse NF-κB-dependent transcription responses. On the one hand, a response could be abbreviated when TD-containing dimers driving transcription are replaced with TD-lacking repressing dimers, to switch off gene transcription. In a specific example, the stabilization of p50 homodimers during the response of macrophages to LPS stimulation leads to curtailing of the pro-inflammatory transcription of *TNFA* (60), likely via a switch from transcriptionally active dimers to inactive p50 homodimers at the promoter region. By contrast, a switch to the p52/RelB heterodimer, which is insensitive to inhibitory IκB proteins, was found to facilitate the sustained activation of target genes such as *NFKBIA* and *NFKB2* [encoding IκBα and p100/p52, respectively; (59)]. Therefore, the intricacies of sequence-specific affinities of NF-κB dimers and dimer-specific RNAPII-recruiting activities can enable not only tuning of the strength but also the duration and temporal patterns of transcriptional responses at target gene promoters.

How might different consensus κB sites modulate the activity of the NF-κB dimers? Structure-function studies have shown that binding to different consensus κB sites can alter the conformation of the bound NF-κB dimers, thus dictating dimer function [(61, 62), reviewed in (10, 63)]. When an NF-κB dimer interacts with a DNA sequence, side chains of the amino acids located in the DNA-binding domains of dimers contact the bases exposed in the groove of the DNA. For different consensus κB site sequences different bases are exposed in this groove, and NF-κB seems to alter its conformation to maximize interactions with the DNA and maintain high binding affinity (61). Changes in conformation may in turn impact NF-κB binding to co-regulators of transcription, whether these are activating or inhibitory, to specify the strength and dynamics of the transcriptional response (64). These findings again highlight how the huge array of κB binding site sequences must play a key role in modulating the transcription of target genes.

Finally, as an additional layer of dimer and sequence-specific regulation, each of the subunits can be phosphorylated at multiple sites with, depending on the site, effects on nearly every step of NF-κB activation [reviewed in (55)]. While the function of each phosphorylation site is still emerging, there are clear examples of phosphorylation events that have κB-sequence-specific effects on DNA binding and transcription (**Figure 2**). One of these is the phosphorylation of serine 329 (Ser329) of p50. This phosphorylation attenuates the affinity of p50 for consensus κB sites with a cytosine (C) vs. adenosine (A) at position −1, leading to differential transcriptional activation at A- vs. C-containing sites (65). In addition to effects on NF-κB dimer affinity for DNA, we note that phosphorylation at other sites on the NF-κB subunits has also been shown to affect dimer abundances, via effects on dimerization, monomer and dimer stability, affinity of IκBs, and nuclear translocation rates [reviewed in (55)].

Overall, when considering the various ways in which NF-κB dimer abundances and their affinity for DNA can be modulated, it becomes clear that with these multiple cascading effects, small differences in consensus κB site sequences and small *a priori* differences in interaction affinities can ultimately have a large impact on the transcriptional response to NF-κB pathway activation.

AVAILABILITY OF HIGH AFFINITY κB BINDING SEQUENCES

Chromatin State

So far, in our discussion of the large numbers of κB sites on DNA and the high nuclear abundance of NF-κB dimers upon stimulation, we made a strong implicit assumption that all the consensus κB sites and half sites are available for binding. Given their high abundance, nuclear NF-κB dimers should be able to locate and bind to many consensus κB sites and half sites within minutes of an initial cell stimulation. However, ChIP-PCR studies in the murine monocytic cell line Raw 264.7, have shown that while recruitment of NF-κB occurs rapidly after LPS addition for a subset of genes (e.g., *NFKBIA* and *CXCL2*), other gene promoters containing high affinity κB sites remain unbound by NF-κB dimers for over an hour (e.g., *CCL5* and *IL6*) despite the continued presence of nuclear NF-κB dimers (66). This kinetic complexity of the recruitment of NF-κB dimers to DNA during a stimulus-induced response has been largely attributed to variable, chromatin-state-dependent accessibility following stimulation.

The promoter regions of early response genes have abundant histone acetylation or trimethylation prior to stimulation [e.g., H3K27ac, (67) and H4K20me3, (66)], a chromatin state "poised" for immediate activation. This chromatin state may lead to a more open chromatin structure, constitutively accessible to transcription factor binding (66, 67). In contrast, promoters of late genes often have hypo-acetylated histones, requiring conformational changes to the chromatin to become accessible. They are therefore unable to recruit NF-κB for up to several hours after stimulation (68), due to the slow process of chromatin remodeling. Of note, we recently observed that recruitment of RelA-containing dimers displayed similar rapid binding kinetics at highly and poorly acetylated H3 HIV LTR insertions, but recruitment of RNAPII was different, with highly acetylated H3

correlating with more efficient transcription (69). Others have also reported early appearance of nascent transcripts of late genes, again hinting that, at least in some contexts, recruitment of NF-κB dimers may take place early, but that differential stability or processing of the transcript leads them to accumulating only later (70–72). Nevertheless, taken together, these different studies of chromatin state and NF-κB dimer binding suggest that despite the large repertoire of potential binding sites, only a fraction of these sites are available for binding, or for active recruitment of RNAPII, at any given time. This accessible repertoire can change upon stimulation and is dictated by the epigenetic state of the cell.

Indeed, another aspect of the NF-κB DNA-binding response that has been revealed by ChIP-seq experiments is its cell-type- and stimulus-specific nature, with different NF-κB subunits binding to diverse sites under different experimental conditions. For example, Xing et al. (5) compared the genes that were bound and regulated by RelA in TNF-treated HeLa human cervical carcinoma cells, to the direct, transcriptionally regulated target genes identified in LPS-treated U937 and THP-1 human monocytic cells. They found a strikingly small overlap between the sets of genes directly regulated by NF-κB in all three of these scenarios. Although deeper and less stringent analyses of these data may reveal a greater overlap, it is clear that cell type and stimulus combine to regulate chromatin accessibility and focus NF-κB dimer-DNA interactions at a subset of all consensus κB binding sites. Therefore, one role of the very large number of potential κB binding sites may be to allow context-specific and diverse use of the NF-κB pathway in response to a variety of stimuli and across different cell types and states.

Beyond the binding events monitored by ChIP-seq and other protein-DNA interaction assays, the "function" of a binding event is generally assessed by determining the transcriptional outcome of the *nearest* gene. However, this simple view may need to be revisited. Indeed, until recently, it was assumed that the regulatory elements of a gene must be located within several kilobases of its locus, and situated on the same chromosome (73). Contrary to this, there is mounting evidence of functional long-range interactions occurring between genomic regions that are situated megabases apart, and even located on different chromosomes (74, 75). Moreover, chromosome organization studies have implicated RelA-containing NF-κB dimers in the initiation or maintenance of higher-order intra- and inter-chromosomal complexes (76, 77). In particular, Apostolou and Thanos (77) found that RelA-containing NF-κB binding to specialized *Alu* repeats plays an important role in initiating interchromosomal interactions, and in the initiation of the *IFNB1* enhanceosome assembly during the early stages of Sendai virus infection (77, 78). *Alu* repeats are ubiquitous repetitive DNA transposable elements that had been shown to contain putative κB-binding sites; they were later shown to represent 11% of p52-, RelB-, and RelA-bound sites in HeLa cells (1). What becomes clear is that NF-κB dimers, at least RelA-containing dimers, can use long-range intra- and inter-chromosomal interactions to regulate gene expression, meaning that the "nearest gene" method of assessing impact of consensus κB sites likely misestimates the number of functional sites.

Taken together, chromatin state and chromatin organization strongly influence the selection of DNA binding sites by NF-κB dimers and, most likely, the selection of the target genes that are regulated by these protein-DNA interaction events. Analyses that consider binding events in the context of three-dimensional nuclear organization and chromatin composition will be required to generate a more accurate view of the ways in which NF-κB-DNA binding affects gene transcription.

Competition Between NF-κB Dimers

In addition to cell-state specific chromatin modifications and chromatin conformation, NF-κB-driven transcriptional responses can also be modulated by competition between different dimer species for response element binding (**Figure 2**). Indeed, while global NF-κB dimer abundance may set the global number of sites that are occupied, which dimers are present pre- and post-stimulus will modulate which sites are transcriptionally activated or repressed, based on relative abundances and affinities. In particular, competition between dimers is consequential when dimers lacking a TD occupy consensus κB sites and limit site availability for newly translocated TD-containing NF-κB dimer binding. As mentioned above, this mechanism of transcription repression has been studied most extensively for the p50 homodimer, which has been shown to play a critical role dampening the inflammatory response [reviewed in (79, 80)]. Specifically, *NFKB1* (p50-encoding) knockout mice have been shown to be more susceptible to several types of infection or infection models [e.g., (81–83)], and some of these responses have been linked to disruption of the transcriptional regulation of inflammatory signals (83, 84). By contrast, perturbations that increase nuclear p50:p50 lead to increased promoter binding by p50:p50 and reduced transcription in response to stimulation of many inflammatory genes (60, 85). Those two examples represent relative extremes of dimer concentrations modulation. Yet, given that, as we discussed above, the nuclear abundances of TD-containing NF-κB dimers appear far from saturating conditions, even moderate changes in nuclear concentration of TD-lacking dimers should affect consensus κB site availability to TD-containing, transcription activating dimer binding (see also **Box 1**). Taken together, differences in dimer abundances, along with competition for κB sites, help explain why different cell types or states exhibit varied responses to NF-κB-activating stimuli.

Co-regulators of Transcription

Because DNA binding by NF-κB may not necessarily require high affinity and high specificity [e.g., (3, 22)] and non-NF-κB transcription factors can also bind consensus κB sites due to degeneracies in recognized sequences (86) or as they search for their targets (43, 44), it follows that other transcription factors could act as co-regulators of transcription by competing with or helping recruit NF-κB dimers (**Figure 2**). In addition, TD-containing NF-κB subunits are also known to interact via their TD with a variety of transcriptional co-factors that modify the chromatin landscape to facilitate NF-κB recruitment and initiate transcription [e.g., (87, 88), and reviewed in (89, 90)]. Here, there are two potential scenarios: (1) the partner transcription

regulators pre-exist at the κB sites and activation is rapid, or (2) the partner transcriptional regulators must be activated by the same stimulus that activates NF-κB. In the latter scenario, the time scale of NF-κB's ability to affect transcription will be dependent on the time scale of activation of its co-regulator. If this partner is required for releasing a binding site from a competitor or other barrier to site accessibility, then delayed partner activation is another factor that could explain the delayed NF-κB occupancy at high affinity sites. With these different possibilities, co-regulators could effectively modulate the abundance and identity of available κB sites over time.

How do NF-κB dimers interact with the chromatin modifying machinery? One way is via post-translationally modified NF-κB dimers that are known to recruit the histone acetyltransferases (HATs) p300 and CBP, which promote chromatin accessibility and transcription factor binding. For example, RelA interacts with the CBP/p300 complex once RelA Ser276 has been phosphorylated, following the degradation of IκBα (87, 91). When Ser276 is mutated to alanine to prevent phosphorylation, RelA cannot recruit CBP/p300 and fails to induce transcription at a subset of NF-κB-dependent genes (92). Intriguingly, which genes are affected does not seem directly related to whether they normally show delayed expression or not; some genes whose response normally peaks early show reduced transcription when RelA cannot recruit HATs (92), so there may be additional factors that come into play to determine site accessibility and its timing.

In contrast to RelA, the p50 and p52 NF-κB subunits lack a TD and as such bind to histone deacetylases (HDACs) instead of HATs [e.g., (93)]. HDACs are associated with formation of closed chromatin and transcriptional repression (94). In the context of κB sites found in the HIV genome, the recruitment of HDACs by the p50:p50 homodimer acts to maintain transcriptional repression and latency (93). Upon stimulation with cytokines that activate the NF-κB pathway, RelA-containing NF-κB dimers can displace p50:p50 homodimers and HDACs, leading to restoration of an acetylated histone state and transcriptional reactivation of the HIV genome (93). A similar mechanism has been suggested for the transcriptional regulation of pro-inflammatory cytokine genes in hepatic cells (84, 95). Taken together, these studies show that the interactions of NF-κB dimers with different modifiers of chromatin can result in the dynamic regulation of the chromatin state and of the availability of consensus κB sites for transcriptionally repressive or activating interactions with these dimers.

NF-κB has also been reported to bind cooperatively to many general transcription factors *in vitro*. For example, the *in vitro* assembly of the interferon-β (*IFNB1*) enhanceosome was shown to be dependent upon interactions with the AP-1 family member c-Jun, interferon regulatory factors (IRFs) and activating transcription factor 2 (ATF2) [reviewed in (10) and in (96)]. However, *in vivo* these factors are recruited to the enhanceosome in a stepwise manner, without any dependence upon cooperative interactions (77, 78). As such, there is currently little *in vivo* evidence that NF-κB dimers binding to consensus κB site is enhanced by association with partner transcription factors. Nevertheless, ChIP-seq experiments have detected many instances where NF-κB dimers may be brought to enhancer or promoter sequences lacking κB sites via interactions with another transcription regulator such as PU.1 or ZNF143 (3). Overall, with promoter and enhancer sequences replete with transcription factor binding sites, NF-κB dimers likely partner with other transcription factors to modulate target genes transcription, but how these interactions are coordinated and how they impact transcription is still unclear for most of these targets.

Although here we have only discussed a few specific examples, there are several other points of cross-talk between NF-κB dimers and other families of transcription factors [e.g., nuclear hormone receptors (97) and (33, 98), as well as STATs and IRFs, recently reviewed in (99)]. Overall, it is clear that the differing abilities of NF-κB dimers to recruit other transcriptional regulators via protein-protein interactions and the specific sets of DNA-protein interactions that can take place at each gene's regulatory region could potentially be combined to produce a vast diversity of context-specific, temporally distinct NF-κB-dependent gene expression patterns.

THE FUTURE OF NF-κB RESEARCH

Understanding the mechanisms that regulate NF-κB-DNA interactions is critical to elucidating the intricacies of NF-κB-specific gene expression profiles. In this review we have discussed the relationships between NF-κB and κB binding sites, and some of the many known complexities of these relationships that affect the regulation of target genes (**Table 1**). However, despite the wealth of information that has already been uncovered by studies of the NF-κB transcription factors, a comprehensive understanding of the mechanisms underpinning NF-κB-DNA interactions that explain stimulus- and cell type-specific responses remains elusive as numerous questions are yet unanswered.

In recent years, our ability to probe chromatin and visualize transcription has considerably advanced, and these advances will be key to developing a better understanding of the complex regulatory processes in the NF-κB system. Chromosome conformation capture (3C) and its subsequent iterations, most recently Hi-C (100), have facilitated the observation of chromatin folding and identification of long-range interactions on a genome-wide scale [reviewed in (101)]. Of particular interest for the study of the interactions of promoters of NF-κB target genes with other regions of the genome, the Promoter Capture Hi-C assay takes promoter-containing fragments from Hi-C libraries and performs paired-end sequencing to identify long-range promoter interactions with distal regulatory elements (102). However, it is important to keep in mind that such methods inform us on the enrichment of particular interactions in bulk populations of cells, an average readout of chromosomal interactions and conformation. Other approaches will be required to understand how different instances of the system vary and how this variability translates into different NF-κB-driven gene expression programs.

TABLE 1 | Summary of layers of regulations influencing NF-κB-driven gene transcription.

Quantity	Influential factors
ABUNDANCE	
κB binding sites	Number of sites Consensus vs. non-consensus sites Duration and frequency of interactions with DNA Cumulative occupancy of binding sites Avidity vs. decoy site effects of clusters Cooperative vs. independent binding at promoters
Nucleus-localized NF-κB dimers	Homodimerization and heterodimerization Transcription activators vs. repressors Stimulus- and time-dependent changes Competition for subunits in dimerization
AFFINITY	
NF-κB dimers for κB sequences	Diversity of bound sequences Dimer-specificity of binding sites Dimer switching and temporal patterns Sequence-specific conformational changes Post-translational modifications of NF-κB subunits
AVAILABILITY	
Chromatin state	Histone acetylation and poised chromatin state Cell-type specificity and stimulus-dependence Nearest gene: accessed in 2- vs. 3-dimensions
Competition between NF-κB dimers	Relative abundances Activating vs. repressive dimers Pre- and post-stimulus changes
Co-regulators of transcription	Pre-existing vs. recruited co-regulators HDACs and HATs recruitment Cell-type specificity and stimulus-dependence

Factors that influence NF-κB-driven gene transcription, its "layers of regulation," organized by the quantity, abundance, affinity, or availability, with which they were associated in the organization of this review.

Simultaneous developments in the fields of biomolecular labeling and imaging technology have facilitated the visualization of transcription factor dynamics in living cells [reviewed in (29)]. These approaches offer unparalleled insights into the interactions occurring between transcription factors and DNA at the single-cell, single-molecule level. Early studies of transcription factor diffusion and DNA-binding dynamics often used fluorescent proteins and fluorescence recovery after photobleaching (FRAP) assays (29, 103). In FRAP assays, the rate of fluorescence recovery after bleaching then provides information regarding the diffusion and binding kinetics (k_{on} and k_{off}) of a large population of fluorescently labeled molecules (104, 105). However, FRAP measurements fail to accurately capture the heterogeneity in binding dynamics (26, 106).

Single-molecule tracking approaches promise a more complete picture of the different types of dynamic interactions, slow and fast, between NF-κB dimers and DNA. However, two difficult challenges from the use of fluorescent protein tags in these approaches are that the low photostability of fluorescent proteins can severely limit the duration of tracking and the generally high number of expressed fusion proteins yields densely packed, difficult to resolve, transcription factors. The advent of high-brightness, photostable, self-labeling dye tags, relying on fusion with the enzymatic HaloTag and SNAPTag (107, 108) is facilitating long-term imaging of single-molecules at high signal-to-noise ratios. In addition, the development of genome-editing techniques has enabled the tagging of endogenous proteins instead of relying on high-expression exogenous promoters for fusion proteins, thus generating more sparsely labeled populations of molecules to track. Combining these approaches with super-resolution imaging modalities such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) should open the door to the development of a clearer picture of the NF-κB-DNA interactions and subsequent gene expression.

Another quantitative aspect of NF-κB biology that merits a revisit in the future is how the landscape of dimer abundances changes across different cellular contexts and across time. Our current understanding of this landscapes relies mostly on bulk, population-based, endpoint biochemical assays, as well as inferences made from lots of accumulated knowledge from *in vitro* affinity measurements and disruptions of cellular contents with knockout of specific NF-κB subunits [reviewed in (56)]. From many single-cell studies of the dynamics of RelA translocation to the nucleus in different cell types and under different stimuli, we have learned that these dynamics are quite variable [e.g., (109–113)] and, importantly, that the observed variability is absolutely consequential for target gene expression and cellular outcomes [e.g., (14, 69, 72, 111, 114–121)]. However, in all these studies, we are left to assume which RelA-containing NF-κB dimers are actually present in each cell, and each nucleus, and we still lack a similar body of knowledge on NF-κB dimers that do not contain RelA. Capturing live-cell dynamics of the nuclear abundance of other NF-κB subunits and how these dynamics affect transcriptional output of target genes should help us figure out whether what we learned for RelA also applies to these other subunits. Finally, the application of fluorescence correlation spectroscopy (FCS) and fluorescence-lifetime imaging microscopy (FLIM) has begun to reveal aspects of protein dimerization and multimerization in other systems [e.g., p53 tetramerization in irradiated human cells (122) and cell type-determining transcription factors in the *Arabidopsis* root (123)]. In the future, similar approaches should help us broaden our understanding of how NF-κB transcription factors interact with each other, interact with other transcription regulators and interact with DNA in a complex, tunable system that regulates gene expression in many cellular decision processes.

CONCLUSIONS

Over the years, studies have dissected the NF-κB pathway, uncovering many factors and nuances that influence the outcome of DNA binding in this complex system. With hundreds of thousands of DNA binding proteins, and millions of potential DNA binding sites, the recruitment of NF-κB to DNA is regulated in complex ways. This regulation generates gene-, stimulus- and cell type-specific

NF-κB responses, allowing NF-κB to respond to numerous different inputs, with a diverse array of outputs. However, a complete, mechanistic understanding of these processes remains unresolved. As we collect better measurements from single-molecule to genome-wide scales, systems biology models may now help us reassemble this dissected system into a framework that can predict ensembles of transcriptional responses.

AUTHOR CONTRIBUTIONS

RB, AM, SM, and SG all contributed to the conceptualization, writing and editing of this review. SM performed the simulations presented in the **Box 1**.

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REVIEW
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NF-κB Signaling in Macrophages: Dynamics, Crosstalk, and Signal Integration

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The nuclear factor-κB (NF-κB) signaling pathway is one of the best understood immune-related pathways thanks to almost four decades of intense research. NF-κB signaling is activated by numerous discrete stimuli and is a master regulator of the inflammatory response to pathogens and cancerous cells, as well as a key regulator of autoimmune diseases. In this regard, the role of NF-κB signaling in immunity is not unlike that of the macrophage. The dynamics by which NF-κB proteins shuttle between the cytoplasm and the nucleus to initiate transcription have been studied rigorously in fibroblasts and other non-hematopoietic cells, but many questions remain as to how current models of NF-κB signaling and dynamics can be translated to innate immune cells such as macrophages. In this review, we will present recent research on the dynamics of NF-κB signaling and focus especially on how these dynamics vary in different cell types, while discussing why these characteristics may be important. We will end by looking ahead to how new techniques and technologies should allow us to analyze these signaling processes with greater clarity, bringing us closer to a more complete understanding of inflammatory transcription factor dynamics and how different cellular contexts might allow for appropriate control of innate immune responses.

Keywords: NF-κB, macrophages, innate immunity, cell signaling, technologies

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INTRODUCTION

Transcriptional regulation of gene expression provides the basis for the responsiveness of cells to external stimuli such as changing microenvironment, infectious interlopers, or chemokine gradients. The bridge between stimulation and transcription is formed by a complex network of signaling pathways that work to activate transcription factors, which translocate to the nucleus and initiate discrete transcriptional programs. In the thirty-plus years since its discovery by Sen and Baltimore (1), few (if any) inducible signaling pathways have been studied in greater detail than that of nuclear factor-κB (NF-κB). First discovered in human B cells, it was quickly discovered that NF-κB is expressed in nearly all cells across the animal kingdom, dating back to invertebrates (2) and jawless fish (3). First shown to regulate the expression of the κ light-chain of antibodies in B cells, NF-κB was soon found to regulate an enormous range of genes in varying cell types and contexts, opening up an exciting new era in the study of signaling pathways driving gene transcription (4).

NF-κB signaling is crucial for a multitude of important immunological transcriptional programs, including inflammatory responses to microbes and viruses by innate immune cells (2, 5, 6), development and activation of adaptive immune cells (7, 8), as well as the development of secondary lymphoid organs (9). In this review, we will focus on the innate immune aspects of

NF-κB signaling, especially in the mononuclear myeloid cell compartment, where NF-κB regulates thousands of primary and secondary response genes including cytokines, chemokines, transcription factors, antimicrobial peptides, and interferon (IFN)-stimulated genes (ISGs) (10–14). While NF-κB gene knock-out (KO) studies, next-generation sequencing, and advances in computational biology have provided us with a wealth of information regarding the transcriptional *outcomes* of NF-κB signaling, there is still much to be learned about the signaling process itself, which is complicated by cell type-, tissue-, and stimulus-specific variability in signaling components and their spatio-temporal dynamics.

With this review, we aim to outline recent work on the dynamics of NF-κB signaling in macrophages and other innate immune cells, with an emphasis on pattern recognition receptor (PRR) stimulation. First, we will describe the key findings of the many studies on NF-κB signaling in cell-free conditions and in non-immune cells such as fibroblasts and epithelial cells so as to contrast these with macrophage-based studies. We will also touch on how crosstalk between NF-κB and other signaling pathways, thresholding of pathway activation, and feedback loops can modulate the inflammatory response in macrophages by altering NF-κB activation. We will then cover the much smaller body of research on NF-κB signaling in other innate immune cells before discussing new tools that are being used to gain better spatio-temporal resolution of the NF-κB pathway, including novel reporter-based assays and their use in the ever-expanding field of computational modeling.

NF-κB SIGNALING DYNAMICS

The NF-κB signaling module consists of five NF-κB monomers (RelA/p65, RelB, cRel, NF-κB1 p50, and NF-κB2 p52) which can dimerize to form up to 15 unique transcription factors and interact with the κB consensus motif found in many gene promoters, as well as five inhibitory proteins (IκBα, β, ε, γ, and δ) that make up the IκB protein family. Although the specific DNA sequence that constitutes a κB site is quite broadly defined, sites associated with individual genes have been shown to be highly evolutionarily conserved (15). Furthermore, unique NF-κB dimers can induce disparate transcriptional responses based on differences in these κB sequences that are as small as one nucleotide (16). This should be considered when evaluating the comparative functions of different NF-κB dimers activated concurrently in the same cell, as certain sites may preferentially bind specific dimers. Unlike the other NF-κB Rel proteins, NF-κB1 p50 and NF-κB2 p 52 are translated as precursor proteins (p105 and p100) that are autoinhibited by their c-terminal domains (also known as IκBγ and IκBδ, respectively), which are homologous to the "professional" IκB proteins (17). In their processed forms, p50 and p52 can form homodimers which lack the transcriptional transactivation domain present in the Rel proteins and can thus function in an inhibitory capacity. A subset of the NF-κB and IkB proteins are constitutively expressed in all mammalian cell types, including erythrocytes (18), with the activity of the NF-κB dimers inhibited at low levels of pathway activation through binding to one of the IκB proteins. The IκB proteins inhibit NF-κB transcription by occluding DNA-binding sites on the Rel proteins and preventing the translocation of the bulk of NF-κB dimers into the nucleus, resulting in only small amounts of inactive NF-κB trafficking between the nucleus and cytoplasm periodically (19). The particular dimer combinations present in a given cell are dependent on multiple factors, including cell type and tissue environment, which likely contribute to differential outcomes of NF-κB signaling depending on context.

Along with diversity in dimer repertoire, NF-κB signaling is also governed by two separate activation strategies, known as "canonical" and "non-canonical" signaling. Non-canonical NF-κB signaling occurs upon stimulation of a subset of the tumor necrosis factor superfamily receptors (TNFRs), and is slower and longer-lasting than canonical signaling. In this case, stimulation leads to the proteolytic processing of the p100 precursor protein into its active form p52, releasing it from auto-inhibition and leading to transcriptional activation by p52:RelB dimers. As such, these two NF-κB proteins are often termed "non-canonical NF-κBs" (7). While non-canonical signaling is important (especially in lymphoid organ development), for the bulk of this review we will focus on canonical signaling, as this is the primary pathway initiated after the ligation of either inflammatory cytokine receptors or PRRs. The canonical NF-κB response is also much faster than non-canonical signaling (20), making this pathway especially important during innate immune responses in first-responder cells such as macrophages.

Canonical NF-κB Signaling

Studies in non-hematopoietic cells have provided a strong foundation of information for our understanding of canonical NF-κB signaling (**Figure 1**). Cytokine or PRR stimulation and signal transduction result in the phosphorylation of the IKK2 complex, which is made up of IKKβ and NF-κB essential modulator (NEMO, also known as IKKγ). The activated IKKβ then phosphorylates IκB with NEMO acting as a scaffold (21), leading to tagging of IκB for degradation via K48-linked ubiquitin chains by the F-box-containing E3 ligase β-TrCP [comprehensively reviewed in Kanarek and Ben-Neriah (22)]. IκB is then degraded by the proteasome, leaving the NF-κB dimer free to translocate to the nucleus and initiate the transcription of primary response genes such as *TNF* and *IL1B*.

While seemingly simple, this classic NF-κB activation cycle can lead to countless variations in gene expression depending on a host of factors. The first level of complexity in NF-κB signaling arises from the multitude of ligands and receptors that stimulate this pathway. For example, NF-κB activation can arise through the stimulation of many cytokine receptors like TNFRs and IL1Rs (23), PRRs such as TLRs (24), MAVS (25), STING (26), and NOD-like receptors (27), as well as T and B cell receptors (28, 29), among many others. While macrophages do not express all of these receptors (e.g., T and B cell receptors), there remain numerous ways in which NF-κB can be stimulated in these cells. For the purpose of brevity, we will focus mostly on PRRs such as TLRs when discussing NF-κB signaling in macrophages. It

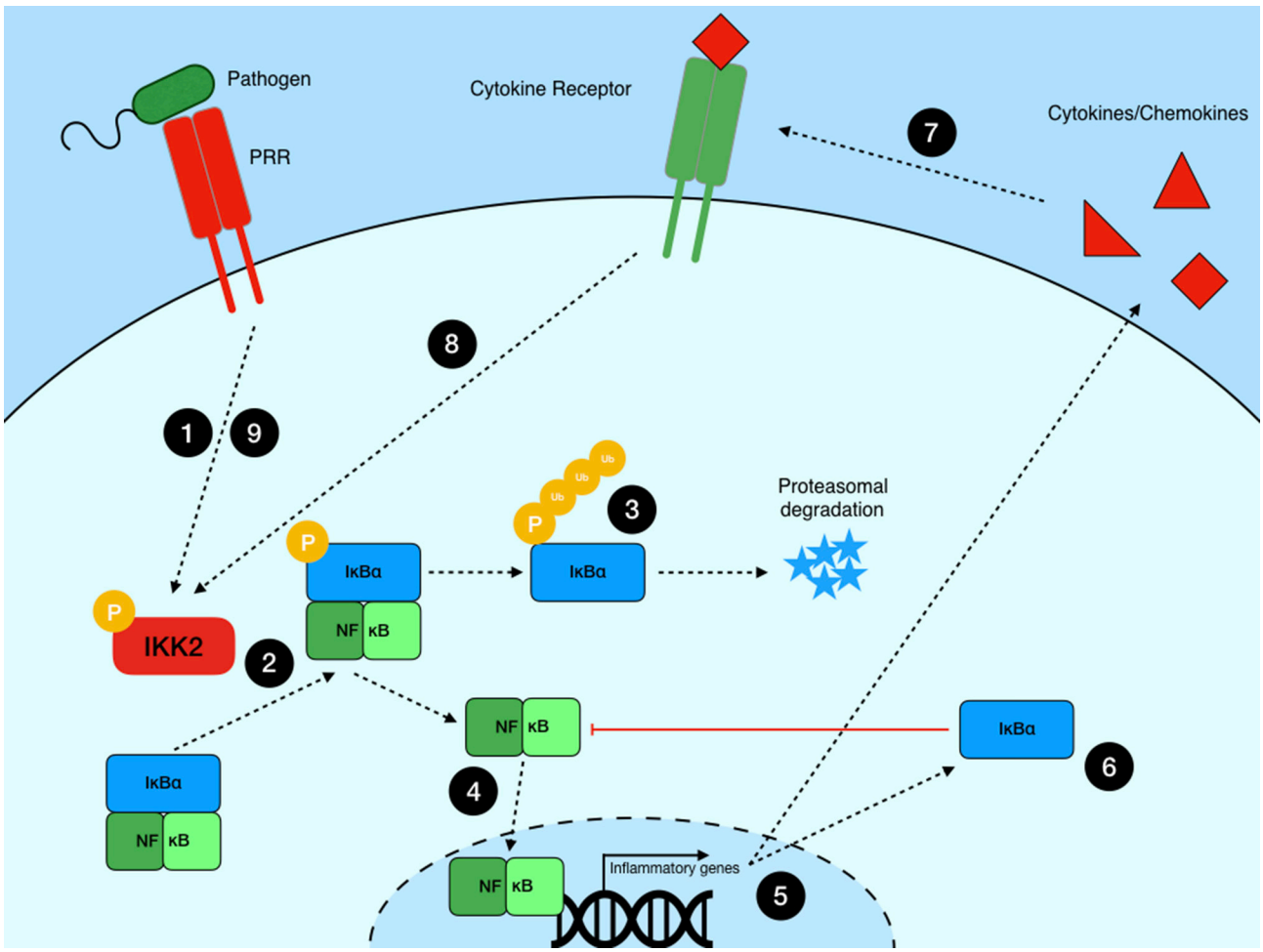

FIGURE 1 | Canonical NF-κB signaling pathway. NF-κB signaling is initiated when a PRR or cytokine receptor recognizes its ligand, starting a signaling cascade (1) that converges on the phosphorylation of the IKK2 complex. IKK2 then phosphorylates IκBα (2), leading to its polyubiquitination and (3) subsequent degradation by the proteasome. This releases NF-κB dimers from negative regulation and (4) allows them to translocate to the nucleus to (5) initiate inflammatory gene transcription. (6) *De novo* synthesis of IκBα acts as a negative regulator of NF-κB-dependent transcription, limiting inflammation in the absence of further signaling events. (7) Primary response genes include those encoding cytokines such as TNF. (8) Release of these proteins leads to autocrine signaling through cytokine receptors. This, or (9) continued PRR ligation, create a positive feedback loop wherein NF-κB is periodically activated until these signals are eliminated.

should be noted however that the question of how variable NF-κB dynamics in response to a wide range of input pathways (often activated at different times during pathogen infection with a multi-PAMP microbe) contribute to transcriptional outcomes in the same cell type, remains an important topic for future study.

While the ligation of each of the receptors mentioned above can directly activate NF-κB signaling, they also feed into other signaling pathways that can interact, directly or indirectly, with NF-κB pathway components. One of the best studied examples of this regards the stimulation of TLR4 with lipopolysaccharide (LPS). TLR4 signaling through the adaptor molecule MyD88 leads to the activation of NF-κB, mitogen-activated protein kinase (MAPK), and IRF5 pathways (30), while subsequent endosomal TLR4 signaling through TIR-domain-containing adaptor protein inducing interferon-β (TRIF) leads to the activation of IRF3, and production of type I interferons (IFNs) and other antiviral genes (31). Therefore, *at least* four major signaling pathways are being activated by one stimulus. However, this does not take autocrine signaling through cytokine receptors into account. For example, triggering the TRIF-IRF3-IFN pathway leads to the release of type I IFNs, which will then bind to the type I IFN receptor (IFNAR) on the surface of the same cell (32), modulating signaling events that are still happening due to the original response to LPS or other cytokines (33). Therefore, there is the potential for considerable signal crosstalk after a cell is stimulated with one molecule. Needless to say, these interactions need to be considered when attempting to dissect seemingly "simple" pathways like canonical NF-κB signaling.

A recent study in human nasopharyngeal epithelial cells showed that the DNA binding sites of an NF-κB dimer are highly stimulus-specific (34). This work demonstrated that

stimulation of epithelial cells with four common stimuli [LPS, TNF, Pam2CSK4, and Poly(I:C)] led to four significantly different patterns of RelA binding. Interestingly, the genes where RelA binding was enriched after stimulation with poly(I:C), a double-stranded RNA analog, were associated with the antiviral program. This shows that a stimulus from a specific class of pathogen can lead to a response tailored to that pathogen, even though RelA activation itself may appear to be stimulus-neutral in terms of IκB degradation and RelA nuclear entry. It will be interesting to see if future studies can determine the mechanism by which NF-κB binding sites are altered in a stimulus-specific manner. One possibility is that other stimulus-specific transcription factors activated alongside NF-κB act to augment or inhibit NF-κB chromatin binding and transcription at particular loci.

Stimulus identity is not the only complicating factor in the outcome of NF-κB signaling. Negative regulation of NF-κB signaling through synthesis of new IκB proteins and subsequent re-activation of the pathway can lead to a periodic oscillation of active NF-κB translocation to the nucleus, modulating subsequent gene expression dynamics. Because IκB genes are primary response gene targets of NF-κB transcriptional activity, they represent a powerful negative feedback loop (**Figure 1**). Newly synthesized IκB proteins bind to active NF-κB dimers, removing them from DNA binding and shuttling them back to the cytoplasm, where the complex can be reactivated and IκB can again be ubiquitinated and degraded via the proteasome. Some of the earliest studies on NF-κB activation dynamics demonstrated that this activation and negative feedback cycle can lead to periodic oscillations if two criteria are met (35). Firstly, the strength of negative feedback must be sufficient to exceed a threshold to favor cytosolic NF-κB sequestration, and secondly, after the consequent reduction in IκB gene synthesis, the input signal to the IKK complex must be sufficient to initiate another round of IκB degradation. The period during which the stimulus is sensed by the cells can therefore have profound effects on the resultant transcriptional response (35–37). Moreover, even in the presence of continued stimulation, negative regulation via deubiquitinases like A20 can arrest signaling by deubiquitinating both components of the IKK complex (38) and important signaling proteins such as TRAF6 (39). Therefore, the balance of positive and negative feedback signals has a profound impact on the transcriptional outcome of NF-κB activation.

The period and amplitude of NF-κB nuclear-cytoplasmic oscillations has been linked to differential gene expression in fibroblasts and epithelial cell lines using single-cell imaging and transcriptome analyses (40–42). Cell lines lacking individual or multiple NF-κB and IκB genes have reinforced these conclusions and have formed the basis for computational models of feedback dynamics and their effects on gene expression (43). However, as discussed below, recent studies performed in immune cells challenged with traditional immune stimuli have shown that these dynamics can vary substantially in different contexts and even change at different times in the cell cycle (44), and models need to be adapted depending on the cell type and status, as well as stimulus identity.

NF-κB SIGNALING IN MACROPHAGES

NF-κB signaling in macrophages follows many of the principles elucidated using fibroblasts and other non-hematopoietic cells, with some exceptions. Some of these differences are simple, such as the increased importance of c-Rel in NF-κB dimers in macrophages, whereas RelA/p50 dimers predominate in fibroblasts. For example, c-Rel is especially important for the transcription of *IL12B* (IL-12 p40) in macrophages (12), as well as the resolution of inflammation via the transcription of an enzyme important for melatonin synthesis (45). Additionally, mice lacking both c-Rel and p50 NF-κB proteins have impaired innate immune responses to bacterial sepsis, with macrophages being deficient in phagocytosis, bacterial killing, and antimicrobial peptide production (10). c-Rel is also a vital part of an NF-κB-ATF3-CEBPδ transcriptional circuit that allows macrophages to scale the inflammatory response based on transient vs. persistent TLR4 stimulation (46). This circuit prevents hyperinflammatory responses to relatively miniscule LPS challenges. It should also be noted, however, that not all macrophages are the same, even when it comes to the effects of particular NF-κB proteins on gene expression. For example, c-Rel activity has differential effects on gene expression depending on whether the macrophage cells are tissue-resident or elicited from the blood (11). Once again, the complete context within which the signaling is occurring must be considered when predicting the transcriptional outcomes of NF-κB signaling.

NF-κB Dynamics

Much of the work described above provides a framework in which NF-κB signaling specificity can be encoded by the period and amplitude of NF-κB nuclear/cytosol oscillations upon TNF stimulation of fibroblasts. However, work in our lab and others has shown that NFκB oscillation is a relatively rare occurrence in macrophages stimulated with TLR ligands (41, 47–50). While oscillation is observed in a small proportion of cells (41), most LPS activated macrophages show a single, longer-lasting NF-κB nuclear translocation event. This is, in part, supported by a positive feedback loop wherein RelA drives its own transcription and favors sustained nuclear occupancy of NF-κB in an LPS dose-dependent manner (50). This positive feedback and sustained NF-κB nuclear occupancy may also be supported by cRel, which is also dose-dependently induced by LPS (46). The sustained nuclear occupancy of LPS-activated NF-κB can be correlated with target gene transcription, as demonstrated by analysis of single macrophage cells expressing both GFP-RelA and a TNF promoter-driven mCherry reporter (50). The critical role of the TRIF pathway in supporting LPS-driven TNF responses (14, 47–49, 51–54), is also reflected in the NF-κB nuclear dwell time as sustained nuclear NF-κB is diminished in TRIF-deficient cells (47–49).

The duration of NF-κB nuclear occupancy in activated phagocytes is also regulated by additional transcription factors. Unbiased genome-scale gene perturbation screens have identified a critical role for the transcription factor Ikaros in supporting both RelA positive feedback and TNF production (50, 55, 56). Sustained NF-κB chromatin binding was shown to be

severely diminished in Ikaros-deficient cells (55), which brings up the possibility that not only do multiple transcription factor pathways converge to support sustained NF-κB nuclear activity after LPS challenge, but also that long-lasting NF-κB chromatin binding is necessary to integrate signals from a complex array of inputs onto a broad landscape of activated enhancers and promoters.

The complex relationship between NF-κB dynamics and transcriptional control in macrophages was recently investigated further by coupling the dynamics of fluorescently labeled RelA with single cell RNA-seq (41). In this study, cells were categorized based on either transcriptome analysis or NF-κB dynamics and it was found that a strong, long-lasting nuclear RelA signal correlated with increased expression of inflammatory cytokine genes, while oscillatory behavior was rare. This supports the concept that robust cytokine expression in macrophages requires sustained NF-κB nuclear occupancy. By analyzing single cells, the authors were also able to show that the behavior of individual cells did not necessarily reflect the population as a whole and that different modes of activation existed in the population. This echoes prior experiments showing that many of these distinct dynamics (even to the same stimulus), and their resulting effects on gene expression, are lost in population-based analyses (42).

While the amplitude of NF-κB translocation to the nucleus can be correlated with inflammatory gene induction and sustained expression, multiple recent studies hint at the importance of the integration of multiple signaling processes (47–49). Attempts at modeling NF-κB dynamics in macrophage cell lines stimulated with LPS show that both the MyD88 and TRIF signaling pathways are necessary for robust TNF production. Two of these studies argue that MyD88 is necessary for the initiation of *Tnf* transcription by activating NF-κB through the canonical pathway (47, 48). TRIF activation downstream of TLR4 signaling from the endosome then contributes to sustaining this response via the activation of the MAPKs p38 and Erk. These kinases subsequently act to stabilize TNF mRNA via the phosphorylation of MK2, as well as supporting the translation and secretion of TNF protein (47). Further modeling of MyD88- and TRIF-associated NF-κB activity showed again that MyD88 signaling is indispensable for initiating NF-κB shuttling to the nucleus while cell-to-cell variation in nuclear occupancy after the initial translocation depends primarily on TRIF activity (48). Whether IRF3 activation and nuclear occupancy downstream of TRIF signaling, or a separate arm of the TRIF pathway, support NF-κB dynamics remains elusive. However, a more recent study using dual TNF promoter and NF-κB reporters showed that, while initial NF-κB activation is independent of TRIF activation, *Tnf* promoter activity depends greatly on TRIF's involvement downstream of LPS stimulation (49). This suggests that TNF expression may require co-operation between NF-κB activation and that of AP-1, which is induced downstream of TRIF signaling through the MAPKs (57). One possibility is that the relative timing of MyD88- and TRIF-mediated signaling plays a critical role and that significant TNF production requires a delayed, but longer-lasting, TRIF-mediated signaling event leading to synergism between NF-κB- and AP-1-driven transcription of *Tnf* (**Figure 2**). All told, these studies suggest that while the dynamics of NF-κB translocation to the nucleus are important in determining the quality and quantity of the inflammatory response in pathogen-challenged macrophages, the overall outcome of NF-κB activation is substantially affected by crosstalk with other signaling pathways and transcription factors.

There has been a great deal of work performed in an attempt to accurately model NF-κB translocation dynamics in stimulated cells. Many of these models are based on the idea of recurrent NF-κB oscillations between the cytoplasm and nucleus, with the period and amplitude of the oscillations being linked to gene expression (37, 41, 58–60). The results of the above studies in macrophages, however, imply that these oscillations are rarer in innate immune cells than in non-hematopoietic cell models (41, 50). Perhaps a more accurate model for how NF-κB dynamics affect gene expression in macrophages would replace amplitude and period of oscillation with "height" and "width" of the single translocation peak observed in these cells. A further simplification of these would be to integrate the area under the curve when looking at RelA nuclear occupation over time (**Figure 3**). A possible benefit of this simplified model of NF-κB translocation could be that it is easier to integrate into more complex models of signaling involving the engagement of multiple pathways and transcription factors, as crosstalk among multiple pathways has a significant effect on gene expression.

Long-lasting nuclear occupancy of RelA increases the chances that significant numbers of NF-κB dimers are present in the nucleus when κB sites become available for binding, while also allowing time for other signaling pathways and transcription factors to modulate chromatin accessibility, tailoring the response to a particular stimulus or group of stimuli. For example, NF-κB must be present in the nucleus in order for the recruitment of the positive transcription elongation factor, p-TEFb, to primary response genes such as *TNF* (61). These genes are poised for transcriptional elongation before stimulation, but cannot be expressed as coding RNAs until they are bound sequentially by NF-κB, Brd4, and finally p-TEFb, releasing RNA polymerase II from its paused state (62). NF-κB and Brd4 also work together to establish super enhancers after TNF stimulation, profoundly altering the transcriptional landscape of the cell. In fact, chromatin binding of Brd4 at enhancer sites is nearly abolished when NF-κB activation is inhibited (63). It is possible that NF-κB translocation is extended in macrophages in part to facilitate these processes during acute immune events, though this has not been studied directly.

As we have mentioned, NF-κB dynamics vary greatly from cell-to-cell within a population, making single-cell analyses very important for analyzing how different signal kinetics affect transcriptional outcomes (42). Single-cell-based analyses in fibroblasts have shown that TNF stimulation leads to a quasi-digital response at the cellular level. Put simply, a cell either responds to the cytokine or not, with increasing dose leading to more cells being activated, accompanied by single-cell level increases in activation strength at higher concentrations of TNF (37, 42). However, studies in macrophages stimulated with LPS suggest a more analog phenotype, with almost all cells responding across a dose range and individual cell responses strengthening at

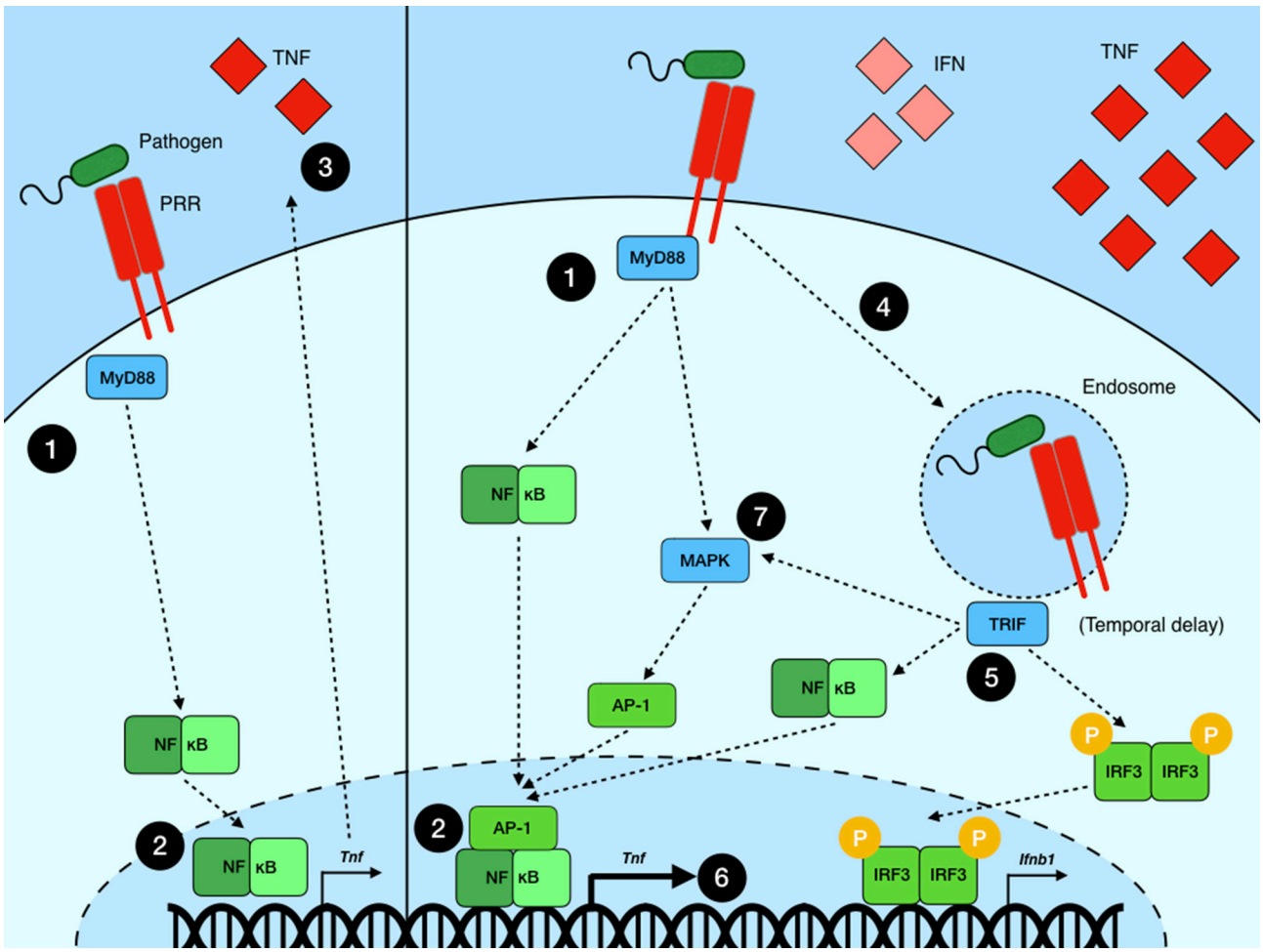

FIGURE 2 | Signal crosstalk in NF-κB transcriptional regulation. **(Left)** An example of a simplified NF-κB signaling model wherein a pathogen is recognized by a PRR (e.g., TLR4), which (1) signals through MyD88 to (2) initiate NF-κB-mediated transcription and (3) subsequent cytokine production. **(Right)** A more accurate model of NF-κB signaling wherein a pathogen is recognized by a PRR (e.g., TLR4) on the surface of the cell, leading to (2) NF-κB activation via MyD88. (4) Subsequently, the pathogen is brought into a sub-cellular compartment where it is sensed by an alternate PRR (or, in the case of TLR4, the same PRR but in a new sub-cellular context) which signals through a second adaptor TRIF (5), leading to the activation of more NF-κB dimers as well as other transcription factors, such as IRF3, leading to type I IFN production, and AP-1, which is activated via the MAPK pathway. Upon translocation to the nucleus, these multiple transcription factors act to synergize or antagonize each other, more precisely tailoring the inflammatory response to the pathogen. (6) In this example, NF-κB and AP-1, each activated by multiple inputs, together produce significantly greater amounts of TNF than singly activated NF-κB on its own. (7) It should be noted that, while both MyD88 and TRIF are known to activate MAPKs, it remains unclear what their relative contributions are in regard to AP-1 activation and TNF production in the context of LPS stimulation.

higher ligand doses (41, 50, 64). This discrepancy has important implications in the macrophage response to infection wherein stimulus concentration is highly heterogeneous throughout a tissue. Analog responses that increase at the cellular level with ligand concentration may allow for increased tuning of an individual cell's response to its immediate environment, limiting bystander damage during inflammatory processes. This alteration of response thresholds will be discussed in further detail in the next section, but it is important to note that these thresholds will change based on NF-κB dynamics.

Signaling Crosstalk

As mentioned above, one of the biggest complicating factors in NF-κB signaling outcomes is the fact that this pathway interacts, directly and indirectly, with a multitude of other signaling pathways during an immune response. As the predominant initiators of the inflammatory response to pathogens, macrophages must collect and integrate information, not only from multiple PRR pathways, but also from released host-derived mediators such as cytokines and interferons, leading to significant crosstalk among signaling pathways (**Figure 2**). In order to better simulate infectious stimuli, the outcome of stimulating macrophages with combined TLR ligands has been compared to single-ligand stimulations (65–68). These studies show that crosstalk between multiple TLRs simultaneously synergizes and antagonizes different gene subsets when compared to simply adding up the responses to individual TLR ligands alone. For example, stimulating macrophages with

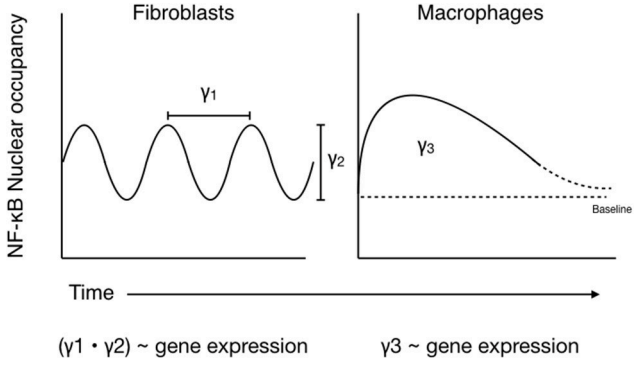

FIGURE 3 | Comparing models of NF-κB translocation dynamics in macrophages vs. fibroblasts. ***(Left)*** NF-κB activation in fibroblasts is associated with periodic oscillations of NF-κB dimers between the nucleus, where they initiate transcription, and the cytoplasm, where they exist in complex with IκBα. Gene expression dynamics scale with the period (γ_1) and amplitude (γ_2) of these oscillations, which are influenced by variables such as signal strength, duration, and receptor identity. ***(Right)*** Conversely, NF-κB shuttling in macrophages is better represented by a single, strong nuclear translocation event which persists for as long as the stimulus remains and tends to remain above baseline for an extended period of time. As such, activation signals from additional pathways are likely integrated over this time, and gene expression dynamics correlate with area under the resultant nuclear NF-κB occupancy curve (γ_3).

the TLR3 ligand poly(I:C) and the TLR7 ligand R848 together led to significantly greater production of IL-12p40 and IL-6 than that seen at saturating doses of either ligand alone (65, 69). This synergy only occurred when combining TLR ligands in such a way that both MyD88 and TRIF were utilized (i.e., triggering one TLR that signals through each adaptor), again showing that connecting these two pathways leads to enhanced inflammatory gene transcription likely by activating multiple transcription factor classes. Interestingly, work performed in fibroblasts stimulated with both TLR2 and TLR4 ligands showed that these cells make a signaling decision between the two pathways, depending on the relative dose of each of the ligands (53). This study did not record gene expression outputs, but rather looked at NF-κB shuttling dynamics, so it is not clear if dual-ligand stimulation leads to synergistic cytokine production in these cells. The studies mentioned here focus on combinatorial TLR stimulation but, as mentioned above, NF-κB stimulation is governed by much more than just TLRs, so further studies will be necessary to address how NF-κB signaling, and its outcomes, are altered by the co-stimulation of different classes of NF-κB-inducing receptors.

So far, we have only discussed how host signaling pathways interact to tailor the NF-κB-mediated immune response to a particular pathogen. However, this ignores the other side of the host-pathogen relationship. All pathogens have evolved ways in which to alter the immune response to allow for their survival and replication, and the NF-κB pathway is no exception. In fact, targeting immune signaling molecules via virulence factors is a very common strategy for pathogens as it allows them to alter receptor signaling without the need to target each receptor individually (70). For example, MyD88 and TRIF are common targets of pathogen-encoded proteases, preventing TLR signaling in infected cells (71–73). Other pathogens, such as pathogenic *E. coli* and *Clostridia spp.* target the NF-κB proteins themselves (74–77). Alterations in NF-κB signaling by pathogens have profound effects on the ability to mount an effective immune response, and the actions of pathogens on infected cells need to be considered when studying NF-κB signaling in these contexts. For more in-depth reviews of immune subversion by pathogens, please refer to Roy and Mocarski (78) and Hodgson and Wan (70).

Much like signal thresholding, explained below, the ability for macrophages to synthesize information from multiple stimuli in order to tailor the inflammatory response to a particular level of danger (in this case, a complex pathogen vs. a single stimulus) likely creates a balance between mounting an effective immune reaction and protecting the tissues from hyperinflammation.

Thresholding

Placing thresholds on signaling inputs is an important determinant of the outcome of an inflammatory response. In order to assure the survival of a host, it is imperative that its immune system does not over-react to innocuous insult. For example, a small number of dead bacteria entering a wound should not elicit the same response as invasion of that wound by millions of live, replicating microbes. The dead bacteria can easily be disposed of by tissue-resident macrophages without the need for a full-blown inflammatory response, which could cause serious damage to the tissue. Similarly, the commensal relationship many bacteria have with higher organisms would be impossible were those hosts to mount a significant inflammatory response to otherwise benign microbes. As such, macrophages will not induce such a response unless they reach a particular threshold of signal density from their PRRs. This is supported by recent observations that different signaling outputs are disparately induced depending on the extracellular concentration of bacterial LPS (79). While NF-κB signaling was induced by very low concentrations of LPS ($\leq$0.1 nM lipid A), many of the inflammatory genes associated with the NF-κB pathway, such as *Tnf*, were not transcribed. Only stimulation above a certain ligand threshold induced the bulk of NF-κB-related genes, which correlated with input from the MAPK pathway. Therefore, these two signaling pathways, which are stimulated via the same receptor, have separate thresholds of activation which will, in turn, affect the transcriptional outcome of both of their gene programs. Importantly, this demonstrates again that NF-κB signaling cannot be viewed in a vacuum, separate from other signaling events occurring within a cell.

There are likely many other factors affecting NF-κB activation thresholds in macrophages, including the activity of separate receptors. For example, stimulation of a macrophage with cytokines such as IFNs or TNF has profound effects on the stoichiometry of NF-κB pathway components and PRRs (33, 80), which will affect their relative availability to the signaling cascade. Inflammatory disease states (including aging), autoimmunity, and chronic infection will also alter signaling thresholds in macrophages, possibly exacerbating the underlying condition. In the case of aging, chronic stimulation of the NF-kB pathway leads

to a shift in the inflammatory baseline of an individual, making it more difficult to mount an effective immune response against pathogens (81–83). The effects of aging on NF-κB processing and nuclear occupancy is an important topic for future investigation.

Thresholds will likely also be altered upon stimulation of a cell with a more complex ligand, such as a live bacterium, due to the cooperation of multiple signaling pathways. For example, it has been shown that signaling outcomes in macrophages are significantly altered when stimulated with live vs. dead bacteria (84–86). While these studies do not look specifically at NF-κB signaling, the effects of "vita-PAMPs" (or PAMPs that are only found in live pathogens) can be easily linked to alterations in signaling thresholds of common NF-κB readouts. For example, bacterial mRNA, which is not found in dead bacteria preparations, is necessary for the activation of IRF3 and subsequent type I IFN responses (87). As mentioned above, IFN signaling can have a profound effect on NF-κB signaling outcomes. Vita-PAMPs are also vital for the stimulation of certain inflammasomes and the subsequent initiation of cell death (87), which also has important implications for NF-κB signaling, especially at the population level.

The threshold for activation of a macrophage also allows for sub-tissue microenvironments to limit immune damage. The probability that an individual macrophage reaches its threshold for signaling is directly proportional to the concentration of receptor ligand in its immediate vicinity (88). As such, cells that are further away from a focus of damage or infection are less likely to reach their inflammatory thresholds. If a small amount of ligand were capable of instigating a maximal inflammatory response from these cells, the immune-mediated damage to an infected tissue would be uncontrollable, leading to death of host tissues. Indeed, knocking out negative regulators of NF-κB signaling, such as the A20 deubiquitinase which deactivates the IKK complex, leads to lethal inflammatory diseases due to uncontrolled inflammation associated with TNFR signaling (89).

Finally, thresholds for a cellular response and the transcription of particular gene sets could also be dependent on the activation state of an individual unstimulated cell. It has been shown in HeLa cells that the amplitude and gene profile of a particular transcriptional response to TNF can be determined by the fold-change of nuclear NF-κB after stimulation, and not just on the total nuclear occupancy (90). It will be interesting to see how the nuclear NF-κB occupancy in a naïve macrophage may alter its signaling threshold.

OTHER INNATE IMMUNE CELLS

Reports on NF-κB signaling dynamics in other innate immune cells remain rare, though some work has been performed looking at cell-specific outcomes of NF-κB activation in neutrophils and dendritic cells, as briefly outlined here.

Dendritic Cells

Dendritic cells (DCs) represent the bridge between innate and adaptive immune responses, presenting antigens to T and B cells in order to activate immunity to specific foreign invaders. The maturation of DCs into professional antigen presenting cells requires the NF-κB protein RelB (91). RelB-deficient DCs are unable to induce antigen-specific T cell responses both *in vitro* and *in vivo* (92). RelB-deficient mice suffer from spontaneous allergic airway inflammation, though the adoptive transfer of RelB+ DCs reverses this phenotype (93). RelB is considered a "non-canonical" NF-κB protein, however studies have shown that canonical stimuli and pathway components are essential for RelB activity in DCs (94, 95). RelB activity in DCs has been shown to be negatively regulated by the canonical IκB proteins IκBα and IκBε, and a canonical pathway activation mechanism is responsible for RelB-specific DC immune activity (91). Interestingly, this study also provided evidence that RelB may act as a downstream regulator of cRel. Whether cRel's importance in DCs is linked to their common lineage with macrophages remains to be seen, and how activation dynamics of the different Rel proteins in DCs compares to other cell types is another important topic for future investigation.

Neutrophils

Neutrophils are tightly-regulated leukocytes that enter tissues during inflammatory responses in order to seek out and destroy pathogens. These highly inflammatory cells recognize PAMPs and subsequently activate NF-κB by the canonical pathway (96). In order to limit the inflammatory potential of neutrophils, they are very quick to undergo apoptosis after their activation. It has been shown that neutrophils have abnormally high levels of nuclear IκBα, which is responsible for dampening NF-κB-mediated gene expression and inducing apoptosis more quickly than in mononuclear cells (97). While this has not been tested directly, this study would suggest that NF-κB dynamics in neutrophils be characterized by cytosolic IκBα degradation, a single NF-κB translocation event, followed by re-synthesis of IκBα and subsequent apoptosis of the cell.

THE FUTURE OF NF-κB SIGNALING PATHWAY ANALYSIS IS BRIGHT

A common thread running through current signaling research is that we are now working in an era with greatly expanded capabilities in regards to analyzing smaller cellular populations in greater detail than ever before. To this end, new technologies are being harnessed in order to gain highly granular insights into signaling dynamics in single cells and in real-time. Signaling dynamics and their effects on gene regulation cannot be appropriately studied at the population level, nor with traditional time course experiments, so it is imperative that we keep harnessing new technologies to increase the resolution of these readouts. CRISPR-based gene manipulation, coupled with the single-cell transcriptional measurements and high-content imaging already in use, should permit more direct determinations of the causal relationships between NF-κB activation and transcriptional outcomes in the same single cell. Using these more accurate tools, single or multiple NF-κB, IκB, and IKK genes could be monitored or perturbed to better investigate how they interact to control gene expression in macrophages and other immune cells. This can also be extended

to other modulators of this pathway, such as deubiquitinases and chromatin regulators. CRISPR can also be used to modify genes at their endogenous loci with fluorescently-tagged versions for imaging purposes. These fluorescent proteins can be tracked *in situ* to gain a better understanding of their dynamics through space and time, or to identify novel binding partners, filling in gaps in our understanding of pathway regulation. Using these techniques in multiple contexts (e.g., dose responses of ligand, multi-ligand stimulation, acute infection) will help us tease apart how signaling changes with these different parameters, creating a more complete model of NF-κB signaling responses and transcriptional regulation.

Another thing that is clear, based on the studies reviewed here, is that studying NF-κB requires the concurrent study of the many other signaling pathways that interact with it. Studying how and when RelA enters and exits the nucleus has provided us with a great deal of insight into the roles of this pathway, but has also uncovered the need to study additional signaling events simultaneously. To this end, the use of fluorescently-tagged reporter molecules will permit measurement of the temporal activity of multiple kinases, transcription factors, and gene promoters in a single cell (98). By increasing the amount of information we can capture from single cells, researchers can more accurately model the signaling dynamics of members of several signaling pathways concurrently. These models can then be used to better predict how cells will react to single or multiple stimuli or in different contexts. While we may never achieve a "unified theory of inflammation," more accurate models of cell behavior will be a great benefit to drug discovery and personalized medical interventions.

Mathematical modeling of NF-κB activation and translocation dynamics is far more advanced than for most other signaling pathways, with some of the earliest models appearing almost 20 years ago (99). These models have improved over time as we have gained further understanding of the roles of the various NF-κB and IκB isoforms (100). Later, modeling studies combined with knock-outs for various NF-κB signaling proteins began to describe multiple feedback loops via multiple IκB proteins (101) as well as autocrine signaling through TNF receptors (14). Currently, models exist that encapsulate an enormous amount of information, including differences in NF-κB dimer identity, positive and negative feedback loops, and interactions between canonical and non-canonical signaling pathways (102–105). Unfortunately, there has been little published regarding NF-κB dynamic modeling in the past few years, despite the amount of data we have on NF-κB signaling increasing at pace. We hope that the current models of NF-κB-mediated responses will be updated to encapsulate these important studies expediently. Also, it will be important for future studies to integrate these models with other technologies like those described above so that we can see how applicable they are to *in vivo* systems. With a combination of mathematical modeling, genetic perturbation, and *in vivo* imaging, the power of these tools to predict transcriptional regulation, druggable targets, and the potential effects of genetic variation will increase exponentially.

Finally, due to the multitude of endogenous and exogenous factors that modulate NF-κB signaling in innate immune cells, it is vital that the effects of tissue microenvironments on inflammation are studied in much greater detail in the coming years. With the advent and growth of *in situ* genetic perturbations (106), multi-valent reporters (98), and *in vivo* imaging (107, 108), researchers can now, theoretically, look in real-time at signaling processes happening in animal models of inflammation and infection. Though there remain significant technical challenges in combining these technologies, the ability to look at specific cell populations *in vivo* will provide us with an enormous leap forward in how we understand signaling dynamics and their outcomes in truly relevant contexts, making the necessary investment worthwhile.

CONCLUSIONS

NF-κB is a master regulator of innate immune responses, and vital to many of the roles that macrophages and other innate immune cells play in orchestrating the inflammatory response to pathogens. In this review, we have outlined the many variables that influence the outcomes of NF-κB signaling, including those that are cell-, tissue-, and stimulus-specific. Over 30 years of research has illuminated the dynamics of this signaling pathway and the genes that are regulated by it, leading to many breakthroughs in how we understand NF-κB function. However, much of this information has come from studying non-hematopoietic cells or pathway components in cell-free conditions. As new technologies and techniques have been developed over the past decade, it has become feasible to study NF-κB signaling in less tractable cell models such as primary macrophages as well as *in vivo*. Recent studies, outlined above, have highlighted differences in signaling dynamics in these contexts which act to support the goals of the innate immune system—that is, to regulate and tailor the inflammatory response to pathogens in order to balance the destruction of invaders with the limitation of potentially harmful hyperinflammation.

Macrophages are capable of integrating an impressive amount of information regarding the identity and virulence of pathogens, as well as endogenous cues present in their microenvironment, in order to modulate the immune response to best protect the host. Central to this ability are the many ways in which NF-κB signaling is modulated based on shifting thresholds of activation, the integration of information from various classes of PRR, and tight regulation of transcription through rigorous positive and negative feedback loops. How these components fit together in different contexts, and how we may be able to modulate or interfere with them to the benefit of patients, is an important field of future research.

The differences between inflammatory signaling in fibroblasts and macrophages (and other innate immune cells) allow for the host to survive most infectious threats, and it is important that we, as researchers, continue to study signaling in different contexts in order to gain a more thorough understanding of how these processes contribute to the immune response. By combining new technologies, we now have the ability to study these phenomena in greater resolution than ever before, even *in vivo* or in primary human cells. The future of NF-κB signaling research is bright—and perhaps fluorescent!

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The writing strategy for this review was devised by MD and IF. The initial draft was written by MD with subsequent drafts edited by MD and IF collaboratively.

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REVIEW
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Techniques for Studying Decoding of Single Cell Dynamics

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Cells must be able to interpret signals they encounter and reliably generate an appropriate response. It has long been known that the dynamics of transcription factor and kinase activation can play a crucial role in selecting an individual cell's response. The study of cellular dynamics has expanded dramatically in the last few years, with dynamics being discovered in novel pathways, new insights being revealed about the importance of dynamics, and technological improvements increasing the throughput and capabilities of single cell measurements. In this review, we highlight the important developments in this field, with a focus on the methods used to make new discoveries. We also include a discussion on improvements in methods for engineering and measuring single cell dynamics and responses. Finally, we will briefly highlight some of the many challenges and avenues of research that are still open.

Keywords: dynamics, decoding, encoding, single cell, signaling, live cell microscopy

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INTRODUCTION

Specificity requires a cell to be able to recognize heterogeneous signals as inputs and reliably compute heterogenous outputs in response. Cells receive signals that can be derived from the organism itself—autocrine, paracrine, and endocrine signals—from the environment, or from other organisms, for example, during infection. Frequently, several signals are present simultaneously and in rapidly changing amounts and durations. Despite this, cells must be able to reliably differentiate signals and generate a specific response based on the signal identity, intensity (i.e., the amount of signal present), frequency (i.e., the duration the signal is present for), and context (i.e., the other signals present and the cellular state).

Accordingly, cells have evolved myriad mechanisms to receive, transmit, and process information reproducibly in a fluctuating and noisy environment. Cells generally first "encode" the signal they receive, by transmitting information about the signal into the activation of specific signaling pathways. Cells are then able to "decode" this information into phenotypic responses and changes in gene and protein expression. Notably, stochastic fluctuations in the concentration of signaling molecules, the numbers of intracellular signaling proteins, and the composition of the microenvironment can be substantial at the single cell level (1). Therefore, the pathways have evolved to be robust to this unavoidable biological noise. The signaling pathways are also frequently redundant or overlapping; many cellular signaling pathways are able to transmit information from a variety of signals to produce heterogeneous outcomes, while many signals can affect multiple pathways (2–4).

Over the past couple decades, it has become increasingly clear that cells use a variety of signaling architectures to encode detailed information about the signals they encounter as temporal patterns of activation of transcription factors, kinases, calcium ions, and other signaling molecules (5–7).

Cells must therefore also possess mechanisms to interpret this dynamic information and translate it into a transcriptional or phenotypic response. One classic example is the discrimination of nerve growth factor (NGF) and epidermal growth factor (EGF) by rat neuronal precursors. NGF stimulation produces sustained activation of the ERK pathway that prompts the cell to differentiate, while EGF stimulation engenders transient activation of the ERK pathway that is decoded as a proliferative cue (**Figure 1A**) (8–10). Another classic example is the nuclear factor (NF)-κB innate immune signaling pathway, which exhibits oscillatory activation patterns when stimulated with tumor necrosis factor (TNF)-α, but sustained activation when stimulated with lipopolysaccharide (LPS), resulting in different gene expression patterns (11–13).

The importance of dynamic decoding of stimulus dose and identity has also been described in several other pathways. For example, p53 is known to use dynamics to differentiate doses of gamma radiation, and between gamma and UV radiation (14, 15). The Msn2 pathway in yeast uses dynamics to differentiate between osmotic and oxidative stress, as well as the severity of glucose starvation (16). Moreover, the Notch pathway has been recently discovered to differentiate between some Delta-like ligands using dynamic patterns (17). Finally, recent studies have described how individual cells interpret multiple, simultaneous stimulants (18, 19). These are only a few examples in a large space; we strongly encourage readers to reference some of the excellent reviews published on cellular encoding and decoding for a more complete overview, especially of classic examples (20–22).

Clearly, dynamic encoding and decoding are widespread in biology, but studying how cells interpret cellular dynamics can be challenging, because population measurements occlude the behavior of individual cells (**Figure 1B**), and making targeted perturbations in signaling pathways is difficult. In order to understand how individual cells encode and decode dynamics, often multiple different measurements have to be made in the same single cell at multiple timepoints. Features of the signaling dynamic patterns (**Figure 1C**) can then be correlated to other measurements of cell behavior to understand how the cell is using dynamics. Recently, the portfolio of high-throughput and single-cell technologies has expanded greatly and become accessible to a wider spectrum of labs, allowing signaling dynamics to be studied in myriad systems.

For example, optogenetics has enabled precisely targeted activation of signaling pathways allowing for greater understanding of the role of dynamics in development and cancer signaling (23–26). Advances in microfluidics have enabled new precision in stimulation timing and dosage (19, 27–29), as well as studies of single cell protein secretion or transcriptomics (30–33). Finally, new reporters have created opportunities to make measurements in novel signaling pathways and contexts (29, 34–41). In this review, we will highlight various experimental strategies that have been successfully used to study cellular dynamic decoding, with an emphasis on single cell studies. We will also discuss recent technological developments that have enabled the field to grow rapidly, and end by discussing some potential future avenues of study and technological challenges that still persist.

DYNAMIC DECODING OF CELLULAR INFORMATION

Population-level studies have revealed some of the connections between signaling dynamics and cellular responses (12, 13, 42, 43). However, as discussed previously, population measurements are not necessarily indicative of single cell behavior (**Figure 1B**). In order to understand how single cells decode dynamic signals into a phenotypic response, it is necessary to make combined measurements of the signaling dynamics and the downstream cellular response in the same single cell. Microscopy has proven to be an invaluable tool for these studies, given the versatility of measurements that it can make, including not only live-cell fluorescence for measuring the signaling dynamics themselves, but also single molecule fluorescence *in situ* hybridization (smFISH) for measuring gene expression (44), and immunofluorescence or other antibody-based methods for measuring protein expression. In addition, there has been recent work to combine other modalities, such as RNA-seq and microfluidics, with live-cell imaging, expanding the repertoire of possible measurements. Here we present a collection of recent studies that demonstrate effective strategies for probing the connection between dynamics and cellular responses on a single cell level.

Live-Cell Imaging Coupled With Measurements of Physical Phenotypes

The most straightforward way to interrogate how cells decode dynamics is to measure signaling dynamics and clear phenotypic responses, such as cell death, cell migration, or cell division. These measurements are well-adapted to live-cell microscopy, as measurements of cellular dynamics and the phenotypic response can be made using the same measurement modality with few technical limitations.

For example, p53 is a transcription factor with a critical role in regulating cell growth and apoptosis in response to DNA damage (45). Previous population-level studies suggested cells with p53 activation below a specific threshold would initiate growth arrest, while cells above that threshold would undergo apoptosis (46). However, single cell studies using a fluorescent p53 reporter showed that in order to undergo apoptosis, p53 levels in the cell must indeed reach a threshold, but that this threshold increases over time (**Figure 2A**) (47). Therefore, the decision of apoptosis or cell growth arrest is determined by the dynamics of p53 activation, as opposed to a static threshold. This observation could only have been made using a single cell dynamical approach.

A similar study revealed aspects of TNF signaling that are correlated with apoptosis. TNF signaling initiates a pro-apoptotic cascade, as well as induction of pro-survival genes by NF-κB (48). Using a microfluidic device to precisely control stimulus timing and dosage, Lee et al. showed that short pulses—as short as 1 min—of TNF-α can be more effective at inducing apoptosis than longer pulses. Single-cell measurements of NF-κB activation showed that longer pulses of TNF sustained longer residence times of NF-κB in the nucleus, suggesting that NF-κB dynamics

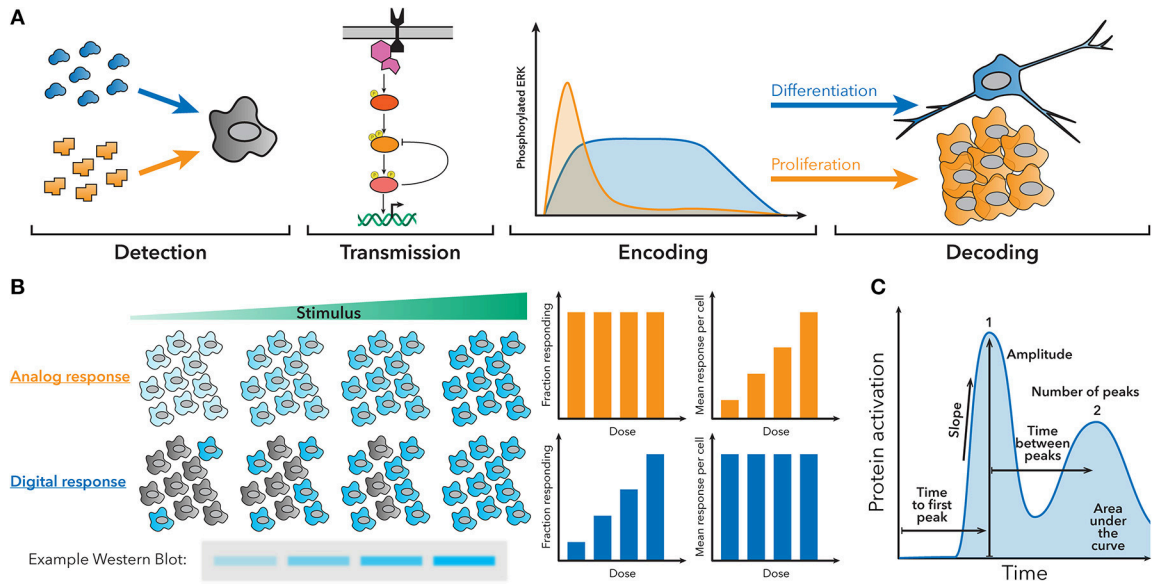

FIGURE 1 | Fundamentals of dynamic encoding and decoding. **(A)** Cells can encode information about the signals they encounter as dynamic patterns of signaling pathway activation. These patterns can then be decoded to produce a specific response. For example, NGF creates sustained ERK activation, which leads to differentiation, while EGF creates transient ERK activation, which leads to proliferation. **(B)** Population-level measurements, such as a western blot, can hide the behavior of single cells in the underlying system. For example, an analog or digital response could produce similar western blots, despite having different amounts of active cells and activity per cell. **(C)** Examples of some features of dynamic traces that can be used to encode information.

are correlated with the relative balance of pro-apoptotic and pro-survival signaling (49). The pro-apoptotic arm of the pathway is initiated on a slower time-scale than, and is inhibited by, the pro-survival arm of the pathway (48, 50, 51). Therefore, the authors propose a model where the sustained NF-κB activation caused by longer TNF pulses, maintains inhibition of the pro-apoptotic signaling arm, leading to greater relative pro-survival signaling (49).

Live-cell microscopy has also been used to understand how the spatiotemporal dynamics of the mitogen-activated protein kinases (MAPKs) regulate mating processes in single yeast cells. In order to undergo successful mating and fusion, individual yeast cells must remodel their cell walls, arrest the cell cycle, and polarize their growth (52, 53). A Förster resonance energy transfer (FRET) reporter of the activity of two MAPKs, Fus3 and Kss1, coupled with visual observation of cell shape and growth, revealed that elevated Fus3 activity at the sites of polarized growth were required for initiating polarity and fusion between mating cells, showing that the spatiotemporal patterning of Fus3 activity, and not just Fus3 levels, are required for the correct mating phenotype (54).

Reporters of multiple different pathways can also reveal how the context of an immune stimulus can affect how an immune cell will respond (55). Innate immune cells use pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), to detect molecules that are indicative of an infection. A cell line expressing both an NF-κB and a c-Jun N-terminal kinase (JNK) reporter was challenged with increasing levels of immune stimulation, from only LPS to infection with *Salmonella typhimirium*, allowing for measurements of the cell's signaling response in each case (35). This work showed how an individual cell uses TLR signaling to discriminate similar signals in a variety of different contexts. For example, in a population of cells exposed to *S. typhimirium,* uninfected cells typically activated only NF-κB, while those cells that were infected with bacteria typically activated both JNK and NF-κB (35).

Multiple live-cell reporters exist that broaden the types of physical phenotypes that can be directly measured using live-cell microscopy. For example, fluorescent probes have been developed that allow for quantitative measurements of single cell kinase activation (34, 37), visualization of various modes of cell death and caspase activation (36, 56), as well as cell cycle progression and measurements of proliferation rate (57). This proliferation reporter was used in conjunction with a FRET-based reporter of ERK activity to show that cells use ERK pulse duration and overall activity to regulate entry into S-phase and cell cycle timing. Subsequent experiments using high content immunofluorescence (HCIF) suggested that the main quantitative factor controlling steady-state proliferation in single cells is ERK output (58).

These experiments are clear examples of ways that we can begin to understand how signaling dynamics generate physical phenotypic responses. Furthermore, the development of new biosensors, improvements in our ability to regulate cellular dynamics (see "Experimental strategies for engineering or modulating dynamic signaling patterns"), and improvements in high-throughput phenotype characterization (59), should allow for even more insight in this area of research.

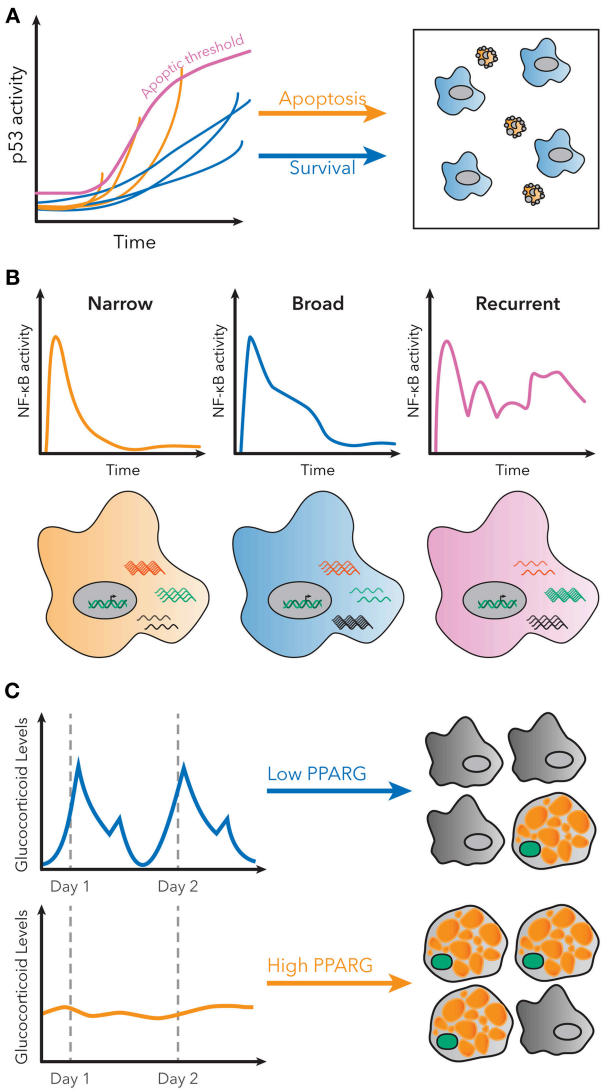

FIGURE 2 | Examples of decoding dynamic signaling patterns. **(A)** Apoptosis due to p53 signaling is not determined by a static threshold, but by a dynamic, increasing threshold. Some cells do not undergo apoptosis, even though they have higher p53 levels than some cells that do undergo apoptosis. Figure adapted from Paek et al. (47). **(B)** Subpopulations with distinct patterns of NF-κB activity exist in single cells stimulated with LPS. These patterns are correlated with different gene expression patterns for known NF-κB targets. Figure adapted from Lane et al. (32). **(C)** Basal rates of adipocyte differentiation are low *in vivo*, despite large pulses of glucocorticoid production daily. However, continuous glucocorticoid inputs of similar total magnitude induce more stabilization of PPARG, indicated in green, and higher differentiation rates. Figure adapted from Bahrami-Nejad et al. (73).

Live-Cell Imaging Coupled With Measurements of Gene Expression and Transcription

Signaling pathways often exert control over the cell by making changes to the expression of specific target genes. In such cases, dynamic signaling patterns are likely to be decoded as quantitative changes in gene expression. However, unraveling this connection can be more challenging than measuring physical phenotypes because it requires measuring signaling dynamics and gene expression in the same single cell. Notwithstanding this complication, many recent studies have demonstrated experimental strategies to successfully combine measurement modalities to uncover interesting results.

For example, the nuclear abundance of NF-κB varies significantly in both stimulated and unstimulated cells, suggesting that NF-κB transcription might also be highly variable (60). However, smFISH measurements in single cells show that transcript levels for many NF-κB targets vary less than the activity of NF-κB (61). Measurements of a fluorescent NF-κB reporter, combined with end-point smFISH quantification of NF-κB regulated transcripts, showed that dynamic quantities, such as fold change of NF-κB, were better predictors of transcriptional levels than static quantities, such as NF-κB nuclear abundance. Subsequent mathematical modeling revealed a potential mechanism based on an incoherent feed-forward loop generated by transcription factor competition (61). Recent work, also using a smFISH-based strategy, further showed that fold change of NF-κB was an accurate predictor of transcript levels at promoters with both high and low levels of TNF-induced transcription (62).

A similar example involved studying the responses of NF-κB to simultaneous LPS and TNF-α stimulation to understand how cells respond to multiple stimuli. A fluorescent reporter was used to measure NF-κB activation in 3T3 cells across a range of LPS and TNF-α concentrations. For most concentrations, the response could be classified as an LPS-only or TNF-α-only response, but for a number of intermediate concentrations, there was a synergistic response that had characteristics of both TNF- and LPS-stimulated cells. Subsequent smFISH measuring the mRNA of a number of relevant chemokines and cytokines revealed that dual-responding cells had, on average, higher expression of Cxcl10 and Csf3 (18). Similar strategies have also been successfully used in other signaling systems (63–65).

Single-cell dynamic imaging can also reveal new phenomena that can be further studied using other techniques. For example, the HIV virus is known to integrate into the host genome and lay dormant in some T lymphocytes, which presents a major obstacle for treatment with antiretroviral therapy (66). Although these latent reservoirs of virus exhibit stochastic, low level activation, the molecular regulators controlling viral activation are still incompletely understood (67). NF-κB is a known regulator of the HIV long terminal repeat (LTR) promoter; however, a recent study using fluorescent reporters of HIV and NF-κB activation revealed that NF-κB activation is not predictive of levels of viral activation across different clones (68). Instead, using smFISH and chromatin immunoprecipitation (ChIP), the authors found that the chromatin environment regulates transcriptional bursting and can explain clone-to-clone variability. These findings revealed that NF-κB-chromatin interactions are required to explain transcriptional bursting and viral activation (68).

FISH-based strategies have also been used to elucidate the role dynamics can play *in vivo* during development. For example, it had been shown that myogenesis requires transient, not

sustained, activation of Notch, but the mechanism of transient Notch activation was not clear (69). Recently, it was revealed that the Notch pathway also uses dynamics to encode and decode information about the identity of the activating stimulus (17). A creative experimental system, using engineered "sender" cell lines that produce either Dll1 or Dll4 and a "receiver" cell line with a chimeric Notch receptor driving expression of a fluorescent protein allowed measurements of the dynamics of Notch activation in the presence of either ligand. These experiments revealed that Dll1 stimulation leads to pulsatile Notch activation, while Dll4 creates sustained Notch activation, with differences in gene expression as a result. The results were reproduced in an *in vivo* model by electroporating either Dll1 or Dll4 into one side of the neural crest of a chick embryo and then using hybridization chain reaction (HCR) FISH to stain for MyoD1, a muscle regulatory factor. Their results revealed that MyoD1 is upregulated by Dll1, which creates pulsatile dynamics, while Dll4, which creates sustained dynamics, downregulated MyoD1 (17).

In systems where endpoint measurements of gene expression are insufficient, multiple fluorescent reporters can be used to measure signaling and transcriptional output simultaneously. For instance, TNF-α is a known regulatory target of NF-κB, that can subsequently regulate downstream responses through paracrine and autocrine signaling (11, 32, 70). A cell line with reporters for both NF-κB activity and transcription from the TNF-α promoter was used to simultaneously measure the signaling and transcriptional dynamics in real-time. Measurements revealed low correlation between many measures of NF-κB activity and the output from the TNF-α promoter. However, the time-integrated NF-κB activity was well correlated with the total output from the TNF-α promoter, demonstrating that continuous measurements of transcriptional activity can reveal more information than endpoint measurements in some systems (71).

Finally, it is now also possible to measure signaling dynamics and genome-wide transcriptional responses in the same single cell. RNA-seq provided a method to measure the entire transcriptome of a single cell, but it remained unsolved how to connect those data with measurements of transcription factor activation dynamics. Lane et al. used microfluidics to isolate cells for live-cell imaging and single-cell RNA-seq to connect the identity of the cell in both datasets. Their results revealed that distinct patterns of NF-κB signaling in response to the same stimulus correlated to different global transcriptional responses (**Figure 2B**) (32). The ability to measure global gene expression resultant from heterogeneous dynamics is exceedingly useful, because it allows for phenotypic characterization of single cell dynamics without a need for *a priori* knowledge of the target genes.

Live-Cell Imaging Coupled With Protein Expression Measurements

Frequently, a cellular response to signals that it receives is to differentially regulate the expression or secretion of proteins. For example, a large part of the immune response is coordination of cytokine and chemokine secretion by immune cells at the site of infection. Therefore, another promising avenue for research in cellular dynamics is to study changes in protein expression and secretion in conjunction with measurements of dynamics. Immunofluorescence can be used to measure intracellular protein expression, similar to measurements of gene expression using smFISH. Alternatively, microfluidic devices or microwell-based assays can be used to measure protein secretion from single cells.

For example, protein quantification can be used to understand how signaling pathways in cells control differentiation. Hormones such as glucocorticoids strongly induce adipogenesis *in vivo* and *in vitro*, but basal rates of preadipocyte differentiation are low in living animals, despite large daily spikes in glucocorticoid hormone production (72). This raises the question of how the differentiation pathway in preadipocytes is able to filter daily, pulsatile signals. Live cell imaging of endogenous adipogenic transcription factors CEBPB and PPARG, and staining for markers of fat cell differentiation, revealed that the transcriptional circuit in preadipocytes effectively filters out pulsatile signals, but responds to continuous signals of the same total magnitude (**Figure 2C**). A model predicted that such a response could be achieved if the system had both fast and slow feedback loops, and further protein and mRNA staining revealed FABP4 as a potential slow-feedback partner in the pathway (73).

Most often, protein expression is measured at the experiment's endpoint, but sometimes more frequent measurements of downstream protein expression changes are required. ERK signaling has been described as both a "persistence detector," which drives approximately digital expression of target genes based on the duration of ERK activity (74–76), and also as a system where peak amplitude qualitatively regulates gene induction (77). Simultaneously measuring ERK activity and induction of Fra-1, a target of ERK, using live-cell reporters instead revealed that linear integration of ERK activity was the primary determinant of downstream responses (78).

Single-cell protein secretion is more challenging to measure, because of the low amounts of protein secreted and the need for isolating individual cells. One strategy for studying single cell protein secretion is to use total internal reflection microscopy to measure secreted proteins in a microwell by a sandwich immunoassay (79). This approach was used to make concurrent measurements of caspase-1 activation using a FRET reporter and IL-1β secretion in single cells. Further analysis revealed that caspase-1 activation is digital and controls a burst of IL-1β secreted from dead macrophages (33). Alternatively, multiple microfluidic strategies to combine live-cell imaging and antibody-based detection of secreted proteins have been developed (30, 31). Cells are initially captured in single cell wells, where they can be exposed to precise doses and durations of stimuli and imaged using a fluorescent microscope. The media from each cell's well can be sampled and measured with antibodies for secreted proteins at various time points during the experiment. Depending on the device, it is also possible to stain cells using immunofluorescence, allowing for both secreted and intracellular proteins to be measured (30).

Finally, changes in protein expression can also be controlled by chromatin regulators, which impart histone and DNA modifications (80, 81). It is now possible to study how single cells use different chromatin regulators to produce varying dynamics of gene expression. Bintu et al. used a doxycycline-inducible system to recruit individual chromatin regulators to regulate the expression of a fluorescent protein. They showed that epigenetic silencing and reactivation are digital processes in single cells and that different chromatin regulators modulate the fraction of cells silenced. Further, using a stochastic model, they describe the different dynamics for both silencing and reactivation, created by each chromatin regulator (82). How cells use chromatin modifications to process signal information is still poorly understood (83), but studies of single cell chromatin regulation dynamics provide a promising avenue for future research.

EXPERIMENTAL STRATEGIES FOR ENGINEERING OR MODULATING DYNAMIC SIGNALING PATTERNS

Studying cellular decoding is more challenging than studying cellular encoding, for technical and biological reasons. The primary biological challenge is that cells have complicated signaling pathways that interact with each other and control heterogeneous outputs. Thus, it is technically difficult to prove that the dynamics are the causative factor in the phenotypic measurement. It has also been difficult to perturb signaling dynamics in ways that would help to establish causality of phenotype, especially in single cells. Here we summarize strategies that have been successfully used to modulate signaling dynamics, as well as significant technical advances that have enabled novel ways of controlling signaling dynamics in single cells.

Engineered Dynamics Using Optogenetics and Other Synthetic Systems

Recent advancements in synthetic biology have opened exciting new ways of precisely and selectively controlling dynamic signaling. One such advancement is the field of optogenetics, which exploits light to control protein function and cell activities with high spatio-temporal resolution (**Figure 3A**). Optogenetic tools are generally faster and more selective than pharmacological stimulation, and their ability to generate flexible temporal patterns brought us a concept of engineering system identification to study the characteristics of cellular signaling pathway in a more direct manner. Here we introduce a few examples of optogenetic strategies applicable to signaling dynamics, but more detailed information about limitations and other applications have also been recently reviewed (84–86).

One commonly used system is Phy-PIF, an optogenetic system with a fast deactivation rate. Both binding and dissociation are induced by light stimulation; red light induces binding and infrared light induces dissociation (87). OptoSOS adopts this system to activate the Ras-ERK signaling pathway by inducing translocation of SOS to the plasma membrane. It can be used to reproducibly activate the pathway using very short pulses (<1 min) and high frequencies of activation, enabling one to measure the frequency response of the pathway (88). This system was recently used to show that a mutation in B-Raf in a human cancer line led to slower decay kinetics of the pathway, meaning that a larger space of input frequencies and strengths are interpreted as growth signals in this cell line (23).

Cry2 is another widely used optogenetic system. It has a slower activation and deactivation rate compared to other systems, but it has the unique property that it exhibits both hetero- and homo-dimerization upon blue light stimulation. Cry2 binds to the N-terminal domain of CIB1 in a blue-light dependent manner. Similarly to OptoSOS, cRaf fusion to Cry2 was used to activate the ERK pathway by inducing translocation to the plasma membrane (89) (**Figure 3B**). Using this system, it was shown that pulsatile ERK activation led to higher cell proliferation than sustained activation. Several genes that are induced better by pulsatile ERK activation were also identified. Photoactivation by recruiting a partner to a membrane comprises many examples such as PKA (90), AKT (26, 91) and TrkA (92). In addition to this heterodimerization between Cry2 and CIB1, Cry2 is known to have a propensity for oligomerization. This property of oligomerization was later improved with a small change in sequences (93, 94). As many signaling events are initiated by homo-oligomerization, particularly receptor signaling, they have been widely applied to many signaling pathways (95–100).

A light-oxygen-voltage-sensing (LOV) domain is a photosensory motif found in many proteins across diverse species. Blue light stimulation induces covalent bond formation between the LOV domain and its flavin cofactor, leading to a partial unfolding between the LOV domain and C-terminal α-helix. LOV domains have been engineered for many applications due to the small size of this domain (∼110 amino acids). For example, a light-switchable gene promoter system was developed by fusing a fungal LOV domain and the Gal4 transcription factor lacking the dimerization domain (101). This system was also applied to control temporal patterns of proneural gene Ascl1 expression in neural progenitor cells (24), and the oscillatory and sustained expression of Ascl1 were shown to induce proliferation and differentiation, respectively.

Contrary to the examples above exploiting translocation or recruitment, there are many optogenetic tools that can directly control allostery and fragment complementation. This includes Dronpa-based strategies (25, 102), LOV domain-based proteins utilizing photo-uncaging (103–106), and a number of light-sensitive channels and receptors. For example, Hannanta-Anan and Chow used melanopsin to generate a wave of calcium release (107). By systematically controlling the calcium oscillation amplitude, frequency, and duty cycles, they found downstream NFAT integrates total elevated calcium concentrations due to its slow export rate.

Dimerization can also be induced chemically; the dimerization of FKBP and FRB with rapamycin is a classic example that has been used in a wide variety of applications for many years (108–110). Though many tools are essentially irreversible due to high affinity binding, acute induction of dimerization or translocation

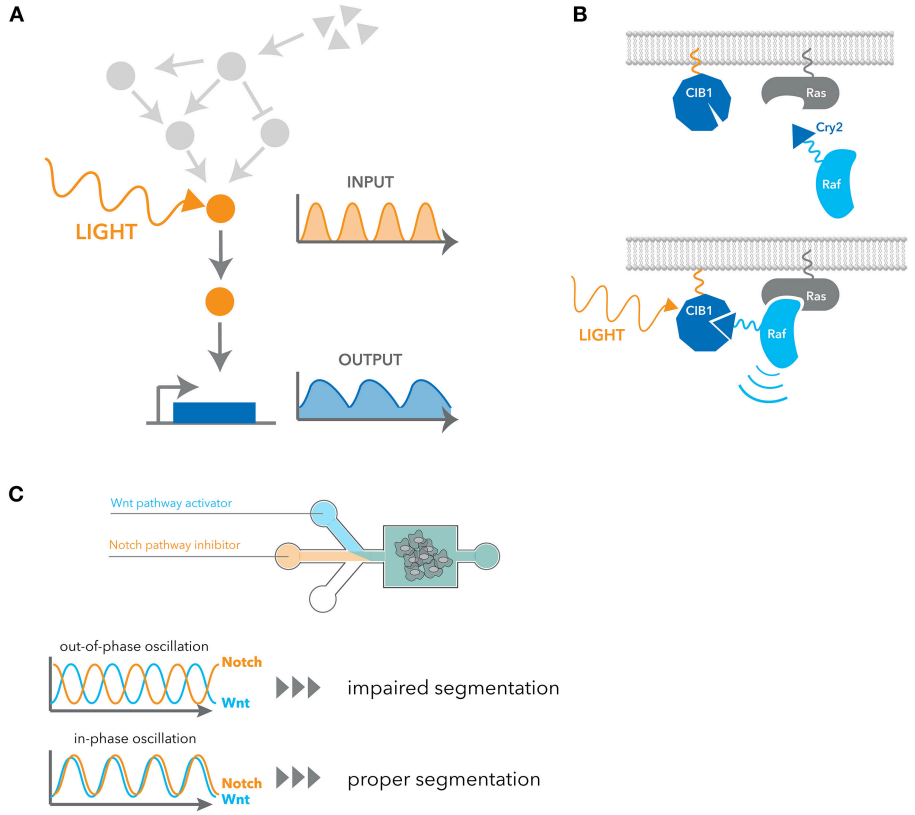

FIGURE 3 | Engineering approaches for manipulating dynamic signaling patterns. **(A)** Optogenetic tools can dynamically and selectively activate a pathway in isolation from endogenous receptor signaling contexts. **(B)** Blue light induces dimerization between the N-terminal CIB1 and Cry2 domain fused to cRaf, leading to the recruitment of cRaf to the membrane. Ras activates cRaf at the membrane, and thus it activates the downstream ERK pathway. **(C)** Microfluidic devices were used to control the flux of small-molecule inhibitors of the Notch and Wnt signaling pathway. In-phase oscillations of these two pathways led to proper mesoderm segmentation, whereas out-of-phase oscillation impaired segmentation.

has been an effective strategy to investigate causation of signaling events. For instance, Santos et al. constructed the nuclear Cdk1-FKBP and cyclin B1-FRB reporters to test the spatial positive feedback regulation of Cdk1-cyclin B1 (111). The chemical dimerization of these complexes in nuclei triggered cyclin B1 nuclear translocation, which was confirmed by translocation of a fluorescent protein fused to cyclin B1, but not fused to FRB.

Another potential approach to control dynamics is to engineer a fully synthetic version of the pathway in an orthogonal cellular environment. As an example, the ERK pathway was reconstructed in yeast, and this minimal cascade itself was shown to generate ultrasensitivity (112). Recently, a synthetic NF-κB signaling pathway was introduced in yeast to study its oscillatory behavior (113). The amplitude and period of the oscillatory response to α-factor can be experimentally regulated by tuning the level of RelA from an inducible promoter, the stability of the protein, and/or the promoter strength driving the expression of the negative feedback component, IκBα. Synthetic systems provide an easy way to manipulate pathway parameters and circuit structures with small-molecule inputs.

Microfluidics Can Precisely Control Dose and Timing of Stimulus

Fluidic control was a standard approach to dynamically manipulate a stimulation pattern before genetic or synthetic approaches became popular. A simple fluidic setup with a pump has been used for several decades to control input flux, including early studies of glucagon signaling (114) and calcium signaling (115). Microfluidic devices now represent a dramatic improvement, providing us with more precise control to generate virtually any kind of temporal pattern, to study both dynamic encoding and decoding.

NF-κB activation in single cells has been well studied using such devices (116, 117). One study showed that cells respond to TNF-α in a probabilistic manner, meaning that only a fraction of cells activates the NF-κB pathway when TNF-α concentrations are low. Microfluidics-based temporal control of TNF stimulation enabled multiple discrete pulses of TNF-α to be delivered to the media. These experiments showed that variability in the NF-κB response depends not only on pre-existing cellular variability, but also on a previously unknown stochastic element (118).

In terms of dynamic decoding, the filter characteristics of the yeast stress pathway has been extensively studied using microfluidic devices (16, 119, 120). By modulating the amplitude, frequency, and duration of periodic Msn2 nuclear translocation, it was shown that each promoter transcribed by Msn2 has a distinct sensitivity to amplitude and pulse frequency. These differences can differentially regulate at least four classes of genes downstream of Msn2 (121).

Microfluidics have also been used to manipulate cellular phenotypes by controlling signaling dynamics. For example, EGF and NGF stimulation in PC12 cells activate the ERK pathway in a transient and sustained manner, respectively, leading to different cellular outcomes. With a microfluidic device, Ryu et al. inverted the outcomes from each growth factors simply by changing the stimulus patterns (28). As another example, Sonnen et al. observed that the segmentation of the presomitic mesoderm is dependent on relative timing between Wnt and Notch signaling oscillations (29). They used microfluidics to generate either in-phase or out-of-phase oscillations of these two pathways and showed the out-of-phase oscillations impair segmentation (**Figure 3C**).

TECHNICAL IMPROVEMENTS IN HIGH THROUGHPUT SINGLE CELL MEASUREMENTS

Many of the examples above show the versatility and capability of microscopy to interrogate relationships between signaling dynamics and downstream phenotypes in single cells. It is becoming clear that signaling dynamics can be decoded to distinct gene expression programs leading to diverse cellular phenotypes; yet most studies were only able to measure a few genes due to technical challenges. Here we will go over some of the recent technical advancements that potentially expand the throughput and accessibility of this measurement modality.

As we saw in the examples above, FISH and immunofluorescence are commonly used techniques for capturing downstream responses. FISH can be implemented in a high-throughput manner, as one can strip or bleach probes and thus iterate detection (122–124). The downside of these techniques is their cost and sensitivity, since many probes are required to bind one species of mRNA and thereby amplify a specific signal. Two recently developed methods utilize different signal amplification schemes, enabling higher sensitivity and gain. The first method is proximity ligation *in situ* hybridization technology (PLISH) (125). PLISH amplifies a target region by rolling circle amplification after generating closed circle probe oligonucleotides by RNA-templated proximity ligation. The second technique, called click-amplifying FISH (clampFISH), uses non-enzymatic click chemistry to generate closed circle oligonucleotides (126, 127). Both techniques were shown to provide better fluorescence signals in both cell culture and tissue samples (126).

Similar to high-throughput FISH approaches, there are methods which attempt to determine the amount of many specific proteins via immunofluorescence over multiple cycles. For example, Lin et al. developed cycIF by bleaching a fluorophore conjugated with a primary antibody with hydrogen peroxide (128). They were able to detect 60 proteins in a tumor tissue sample by repeating the bleaching and staining steps for each protein of interest (129). Another group developed the antibody elution method called 4i which elutes antibodies with a mix of reducing agent, low pH and chaotropic salts, and a blocking buffer (130). This is compatible with indirect immunofluorescence, and 40 proteins were detected with this approach. A similar approach, co-detection by indexing (CODEX), uses oligonucleotide conjugated antibodies (131). Instead of repeating antibody binding steps, this method first carries out the binding process, followed by repeated detection of the barcoded antibodies by incorporating fluorophore-labeled nucleotides by polymerase.

These methods rely on fluorescence detection and thereby the number of detections at a time is limited by the spectral overlap. In contrast to these approaches, imaging mass cytometry and multiplexed ion beam imaging use metal-labeled antibodies (132–134). The signal from the metal isotopes are measured via mass spectrometry, allowing simultaneous detection of more proteins than would be possible by fluorescence. Both methods were able to measure more than 30 proteins from tumor samples (135, 136), and it can be also combined with high-throughput FISH methods (137).

For live-cell image analysis, computational automation is increasingly a requirement due to the amount of data that can readily be acquired. One of the recent breakthroughs in this area involves deep learning. Convolutional neural networks were first applied to classification of histopathologic images for a diagnostic purpose (138–140). In 2016, a software tool called DeepCell employed this type of classification task for automated image segmentation of cells (141). In addition to a higher segmentation accuracy for fluorescent images, this deep learning-based approach also allows us to segment objects using a non-labeled image such as phase-contrast or DIC images (142–144).

CONCLUDING REMARKS

The capability and compatibility of single-cell measurement techniques has advanced significantly in the last several years. It is now possible to measure signaling pathway activation and RNA, protein or metabolite levels in single cells, often in real time. The throughput of these measurements is also increasing rapidly, especially with the development of better computational techniques for image analysis and iterative FISH and immunofluorescence approaches. Moreover, our ability to engineer single cell dynamics is also rapidly improving with the development of techniques such as optogenetics. As a result, studies describing how individual cells respond to inputs using dynamic patterns have expanded into new systems and levels of detail.

Practically, these new discoveries may reveal ways to target signaling dynamics in disease contexts, potentially leading to novel treatments (145). Pharmacologically altered signaling dynamics have only been demonstrated in a few studies, but

this may change with the development of better tools and knowledge (64, 146). Additionally, better understanding of dynamic cellular responses can help lead the way to cells with functional engineered signaling circuits, which have large potential as possible therapeutics and scientific tools (147).

Nonetheless, this field is still in the early stages and many challenges remain to be addressed. Reporter development remains a challenging problem, and thus reporters currently exist for only a small subset of pathways. Furthermore, most measurements of signaling pathways continue to rely on exogenous reporters, which can differ from the responses seen with endogenous proteins. While work has been done to make it easier to directly measure endogenous proteins, these methods still remain more difficult than using exogenous reporters. Finally, large-scale genetic screens have enabled new levels of understanding in many fields through the ability to search for important effectors across the entire genome. However, using microscopy in conjunction with genome-wide screens is still exceedingly challenging because of the need to connect the measurements made using microscopy to the genetic perturbation in each cell. Thus, there is still much room for improvement in both the techniques available to the study of dynamics, as well as the number of systems that these techniques can be applied to.

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Immune Differentiation Regulator p100 Tunes NF-κB Responses to TNF

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Tumor necrosis factor (TNF) is a pleiotropic cytokine whose primary physiological function involves coordinating inflammatory and adaptive immune responses. However, uncontrolled TNF signaling causes aberrant inflammation and has been implicated in several human ailments. Therefore, an understanding of the molecular mechanisms underlying dynamical and gene controls of TNF signaling bear significance for human health. As such, TNF engages the canonical nuclear factor kappa B (NF-κB) pathway to activate RelA:p50 heterodimers, which induce expression of specific immune response genes. Brief and chronic TNF stimulation produces transient and long-lasting NF-κB activities, respectively. Negative feedback regulators of the canonical pathway, including IκBα, are thought to ensure transient RelA:p50 responses to short-lived TNF signals. The non-canonical NF-κB pathway mediates RelB activity during immune differentiation involving p100. We uncovered an unexpected role of p100 in TNF signaling. Brief TNF stimulation of p100-deficient cells triggered an additional late NF-κB activity consisting of RelB:p50 heterodimers, which modified the TNF-induced gene-expression program. In p100-deficient cells subjected to brief TNF stimulation, RelB:p50 not only sustained the expression of a subset of RelA-target immune response genes but also activated additional genes that were not normally induced by TNF in WT mouse embryonic fibroblasts (MEFs) and were related to immune differentiation and metabolic processes. Despite this RelB-mediated distinct gene control, however, RelA and RelB bound to mostly overlapping chromatin sites in p100-deficient cells. Repeated TNF pulses strengthened this RelB:p50 activity, which was supported by NF-κB-driven RelB synthesis. Finally, brief TNF stimulation elicited late-acting expressions of NF-κB target pro-survival genes in p100-deficient myeloma cells. In sum, our study suggests that the immune-differentiation regulator p100 enforces specificity of TNF signaling and that varied p100 levels may provide for modifying TNF responses in diverse physiological and pathological settings.

Keywords: TNF, pulsatile, NF-kappaB, p100, temporal control, gene-expression specificity

INTRODUCTION

Tumor necrosis factor (TNF) is a pleiotropic cytokine whose primary physiological function involves coordinating innate and adaptive immune responses (1). TNF engages the canonical NF-κB pathway to activate RelA:p50 NF-κB heterodimers that are sequestered in the cytoplasm of unstimulated cells by inhibitor of κB (IκB) α, β, and ε proteins (2). In the canonical pathway, TNF treatment induces the IκB kinase (IKK) complex consisting of NEMO and IKK2 (or IKKβ), which phosphorylates IκBs leading to their degradation and nuclear translocation of RelA:p50. In the nucleus, RelA:p50 mediate the expression of specific pro-inflammatory and immune response genes.

Typically, TNF briefly stimulates tissue resident cells due to its short half-life *in vivo* (3). Previous studies demonstrated that the NF-κB system, in fact, distinguishes between brief and chronic TNF signals for a wide range of TNF concentrations (4–6). Brief TNF stimulation induces a transient RelA:p50 activity peak persisting in the nucleus for about an hour. In contrast, chronic TNF stimulation triggers an additional second wave of protracted RelA:p50 activity, which lasts in the nucleus for more than 8 h. This late RelA:p50 activity displays oscillatory behavior at single-cell resolution (7). Importantly, chronic TNF treatment activates a distinct set of late-acting NF-κB target genes that are not induced upon brief TNF stimulation (4, 8). Regardless of the duration of TNF treatment, RelA:p50 induce rapid synthesis of the inhibitors of the canonical pathway, including IκBα, IκBε, and A20 (9, 10). A series of elegant studies suggested that coordinated functioning of these negative feedback regulators determines dynamical RelA:p50 responses to time-varied TNF inputs (6, 11–13). It is thought that RelA:p50 regulation by the canonical NF-κB pathway largely provides for distinct transcriptional outputs to brief and chronic TNF stimulations (14). On the other hand, deregulated TNF signaling has been implicated in several human ailments, including inflammatory bowel disorders and neoplastic diseases (1).

The non-canonical NF-κB pathway mediates a separate RelB-containing NF-κB activity. In resting cells, p100 encoded by *Nfkb2* retains RelB and other NF-κB proteins in the cytoplasm (15). Non-canonical signaling induced by B-cell activating factor (BAFF) or lymphotoxin $\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) activates a complex consisting of NF-κB inducing kinase (NIK) and IKK1 (or IKKα), which phosphorylates p100. Subsequently, the C-terminal inhibitory domain of p100 is removed by proteasome resulting in the release of RelB:p52 NF-κB heterodimers into the nucleus. In comparison to the canonical RelA activity, the non-canonical pathway elicits a weak but sustained RelB activity, which induces genes involved in immune cell differentiation and immune organ development. In the absence of p100, RelB appears in the nucleus as a minor RelB:p50 NF-κB activity (16, 17). Notably, this constitutive RelB:p50 activity partially compensated for the absence of immune-organogenic RelB:p52 functions in $Nfkb2^{-/-}$ mice (18).

Previous mechanistic analyses have identified molecular connections between the canonical and non-canonical NF-κB pathways. For example, canonical signaling induces the expression of genes encoding RelB and p100 from the respective NF-κB target promoters (15). A subpopulation of RelA binds to p100 and is activated by non-canonical signaling (16, 19–21). Conversely, IκBα retains a fraction of RelB and liberates a weak RelB NF-κB activity during canonical signaling in wild type MEFs (22, 23). More so, RelA and RelB heterodimers possess overlapping DNA binding and gene-expression specificities (23–26). Because NF-κB pathways are interlinked, we asked if constituents of the non-canonical pathway influence dynamical TNF signaling.

Here, we demonstrate that p100, a component of the immune-differentiating non-canonical pathway, is critical for the NF-κB system to discriminate between brief and chronic TNF signals. Brief TNF treatment, akin to chronic simulations, induced a biphasic NF-κB response in p100-deficient cells. However, the late NF-κB DNA binding activity induced in p100-deficient cells consisted of RelB:p50, which modified TNF-mediated gene controls in MEFs. Our study further revealed that RelA and RelB heterodimers bound to largely overlapping chromatin locations despite differences in the RelA-dependent and the RelB-mediated gene controls in p100-deficient cells. Mechanistically, NF-κB-driven RelB synthesis strengthened the basal RelB:p50 activity in p100-null cells upon TNF stimulation and produced this lasting NF-κB response. Finally, myeloma cells lacking p100 owing to genetic aberrations produced a long-lasting pro-survival RelB response to brief TNF stimulation. In sum, the NF-κB system engages distantly related molecular species with seemingly distinct biological functions for enforcing dynamical and gene controls of TNF signaling. Our work suggests that varied cellular abundance of p100 may also provide for a mechanism of tuning TNF responses in diverse physiological and pathological settings.

RESULTS

A Mathematical Model of the Integrated NF-κB System Predicts a Role of p100 in TNF Signaling

Mathematical reconstructions of cellular networks offer insights on the underlying signal-processing mechanisms (27, 28). We developed a mathematical model (see **Supplementary Materials** for details), which depicted the NF-κB system consisting of interlinked canonical and non-canonical modules (**Figure 1A**), for probing dose-duration control of TNF signaling *in silico*. In this model, IκBs and inhibitory p100 complexes both regulated nuclear NF-κB (NF-κBn) activities. For varying dose and treatment duration, TNF activates IKK2 with diverse kinetic profiles. Accordingly, we used theoretical IKK2 activity profiles of varying peak amplitude or duration as model inputs (**Figure 1B**; **Figures S1A,B**). Our computational simulations broadly captured the previously described NF-κB dynamics (4, 6). For example, the duration of NF-κBn response was insensitive to changes in the amplitude of IKK2 signal but proportionately increased as a function of the duration of IKK2 input (**Figure 1C**). Simulating mutant cell systems devoid of one or the other NF-κB regulators, we examined their role in this dynamical control. Remarkably, our computational analyses suggested an aberrant NF-κB control in the *Nfkb2*-deficient system where

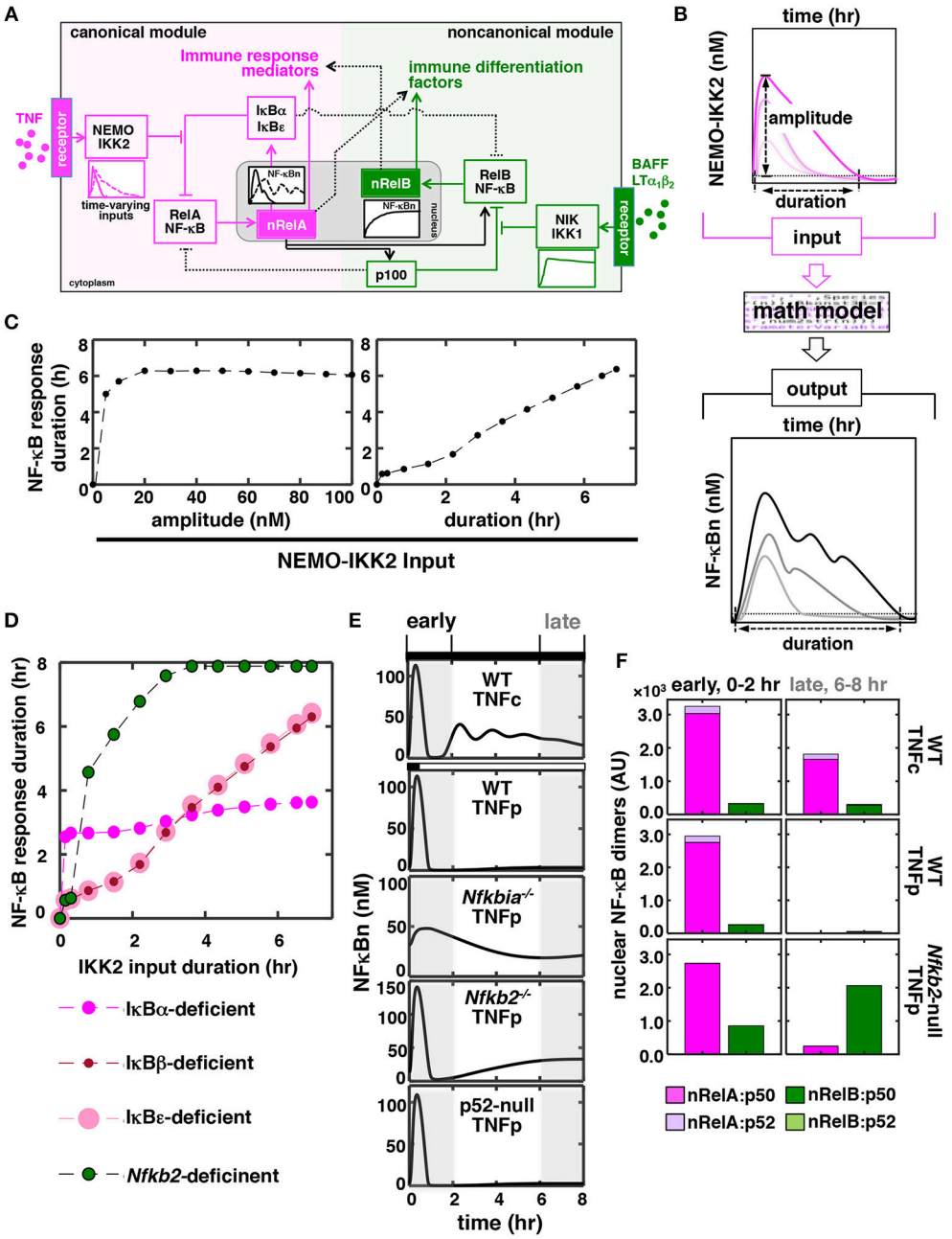


FIGURE 1 | *In silico* studies identify a role of p100 in discriminating between time-varying TNF inputs. **(A)** A graphical depiction of the NF-κB system. TNF through the canonical pathway (magenta) dynamically regulates the activity of RelA:p50 heterodimers, which mediate the expression of immune response genes. BAFF or $LT\alpha_1\beta_2$ induces a distinct RelB NF-κB activity via a separate non-canonical pathway (green) for driving the expression of immune differentiation factors. However, these two NF-κB pathways are molecularly connected and display certain overlap in relation to gene expressions. Solid and dotted black lines represent major cross-regulatory mechanisms and those involving less-preferred biochemical reactions, respectively. NF-κBn, nuclear NF-κB activity. nRelA and nRelB represent corresponding nuclear heterodimers. **(B)** Schema describing *in silico* production function analyses. Briefly, theoretical IKK2 activity profiles of various peak amplitudes and durations were fed into the mathematical model, and NF-κBn responses were simulated in a time-course. Durations were estimated as the time elapsed above a specific threshold value, which was determined as the sum of the basal NF-κB or IKK activity and 5% of the corresponding basal-corrected peak activity, in the corresponding activity curves. **(C,D)** Graph plot of the duration of simulated NF-κBn responses as a function of the peak amplitude or the duration of theoretical IKK2 inputs. IKK2 activities of various peak amplitude but with invariant 8 h of duration (**C**, left) or with various durations but identical 60 nM peak amplitude (**C**, right and **D**) were used. Computational simulations involved **(C)** the WT system and **(D)** the indicated mutant systems. **(E)** *In silico* studies revealing NF-κBn responses in a time-course in WT and various mutant systems. Experimentally derived IKK2 activity profiles, obtained using MEFs treated with TNF either chronically (TNFc) or for 0.5 h (TNFp), were used as model inputs. Early (0–2 h) and late (6–8 h) phases have been marked using gray boxes. **(F)** Computational modeling predicting TNFp-induced nuclear activities of RelA and RelB heterodimers in WT and *Nfkb2*-null systems. Early and late activities were determined as the area under the corresponding activity curve between 0 and 2 h and 6–8 h, respectively, subsequent to correction for basal values. AU, arbitrary unit.

even short-duration IKK2 inputs produced prolonged NF-κBn responses (**Figure 1D**).

To investigate further dynamical TNF signaling *in silico*, we fed experimental IKK2 activity profiles obtained using MEFs treated with TNF into our mathematical model as inputs (**Figure S1C**) (6, 20). Indeed, long-lasting IKK2 activity associated with chronic TNF treatment (TNFc) triggered a prolonged, biphasic NF-κBn response consisting of RelA:p50 heterodimers in our simulation studies (top panels, **Figures 1E,F**). Short-lived IKK2 input related to brief 0.5 h of TNF treatment (TNF pulse, TNFp) produced only a transient 1 h of NF-κBn response. As expected, a weakened negative feedback extended the TNFp-induced NF-κB response beyond 1 h in the IκBα-deficient system. Corroborating our studies involving theoretical IKK2 inputs, computational simulation of the TNFp regime in the *Nfkb2*-deficient system produced a prolonged NF-κBn response, whose temporal profile was somewhat comparable to that of the TNFc-induced NF-κBn activity (**Figure 1E**). The prolonged activity induced in the *Nfkb2*-deficent system was biphasic where the late phase lasted for more than 8 h. However, this late activity was absent in the p52-null system, where p100 was expressed but its conversion into p52 was not permitted. Because p100 deficiency triggers also canonical RelB:p50 activation, we probed the dimer composition of this late-acting NF-κB response. Our mathematical model included the description of four NF-κB heterodimers, namely RelA:p50, RelA:p52, RelB:p50, and RelB:p52. Recapitulating previously published experimental data, our simulation studies revealed that the TNF-induced NF-κBn activity consisted of mostly RelA:p50 in the WT system with only a minor amount of RelA:p52 and RelB:p50 heterodimers (**Figure 1F**). Our computational model further indicated that primarily signal-induced nuclear accumulation of RelB:p50 heterodimers generated the late-acting NF-κBn response to TNFp in the *Nfkb2*-deficient system (**Figure 1F**). Therefore, our mathematical modeling studies predicted a role of the non-canonical signal transducer p100 in producing appropriate NF-κBn responses to time-varying TNF inputs.

p100 Restrains Late-Acting RelB:p50 NF-κB Response to Brief TNF Stimulation

To verify experimentally the predictions of our mathematical model, we treated MEFs, immortalized using NIH 3T3 protocol, with TNF and measured the resultant NF-κBn activities in a time-course using the electrophoretic mobility shift assay (EMSA). TNFc treatment of WT cells induced a biphasic NF-κBn response comprising of an early peak, which lasted for ∼1 h, and a gradually weakening second phase between 3 and 8 h (**Figure 2A**). TNFp treatment of WT MEFs produced the early peak activity, which was substantially broadened in TNFp-treated *Nfkbia*$^{-/-}$ cells lacking IκBα (**Figures 2A,B**). TNFp indeed induced a prolonged NF-κBn response in *Nfkb2*$^{-/-}$ MEFs that consisted of an early peak and a progressively strengthening second phase (**Figure 2B**). Of note, TNFc generated a similar biphasic activity in *Nfkb2*$^{-/-}$ cells (**Figure S2A**). Our shift-ablation assay confirmed that the late NF-κBn DNA binding activity induced in WT cells in response to TNFc was composed of mostly RelA:p50 heterodimers (**Figure 2C**). Similarly, the late NF-κBn activity induced by TNFp in *Nfkbia*$^{-/-}$ cells consisted of RelA:p50. It was earlier shown that p100 deficiency alters the RelB homeostasis, where a subpopulation of RelB translocate into the nucleus, and yet another fraction is sequestered by IκBα and activated upon canonical signaling (16, 17, 20, 22, 23). Our shift-ablation assay corroborated these studies. We noticed in *Nfkb2*$^{-/-}$ MEFs a low level of basal RelB:p50 activity; targeting IκBα-bound complexes, TNFp further augmented this RelB activity at 0.5 h post-stimulation that was diminished to the basal level by 1 h (**Figure S2B**). In addition, brief TNF stimulation produced a robust late-acting RelB response, which persisted in the nucleus of *Nfkb2*$^{-/-}$ MEFs even 16 h after stimulation (**Figure 2C**; **Figure S2B**). Furthermore, IL-1β, which induces NF-κB signaling transiently in WT cells (6), produced a similar late RelB:p50 activity in *Nfkb2*$^{-/-}$ MEFs (**Figure 2D**; **Figure S2C**). Our studies suggested that p100 imparted dynamical NF-κB control by preventing late-acting RelB:p50 response to short-lived IKK2 signals generated by pro-inflammatory cytokines. However, deficiency of p100 and that of the well-articulated negative feedback regulator, IκBα caused distinct aberrations with respect to the temporal profile and the composition of the signal-induced nuclear NF-κB activity.

Of note, we relied on bulk measurements of transcription factors present in the nuclear extracts. Therefore, our study does not rule out that p100 deficiency triggers an asynchronous, oscillatory RelB:p50 response to TNFp at the single-cell level. Cellular heterogeneity may also amount to two distinct cell population with only one sustaining an elevated RelB:p50 activity—this may in fact lead to an underestimation of late RelB:p50 response in our bulk measurement based analyses.

Dissecting Molecular Mechanism Underlying Late RelB:p50 Response to Brief TNF Stimulation in the Absence of p100

Sensitivity analysis provides information on regulatory mechanisms governing the functioning of the modeled network (28). In local sensitivity analyses, rate parameters are individually altered; multiple parameters are changed simultaneously in multiparametric analyses. By estimating the effect of parameter perturbation on the model output, relative importance of the associated biochemical reaction in signal processing is determined. Utilizing a variance-based, multiparametric sensitivity analysis method (29), we investigated the biochemical mechanism underlying late-acting RelB:p50 response to TNFp in the *Nfkb2*-deficient system. We assembled the large number of model parameters into 48 distinct groups (**Figure 3A**). Each of these groups consisted of functionally related biochemical parameters associated with a specific molecular species (see **Table S5** for a detailed description on parameter grouping). For instance, kinetic rate parameters associated with the synthesis of IκBα, including constitutive and NF-κB-responsive transcriptions as well as translation, were grouped together. Using Monte Carlo sampling, we explored the

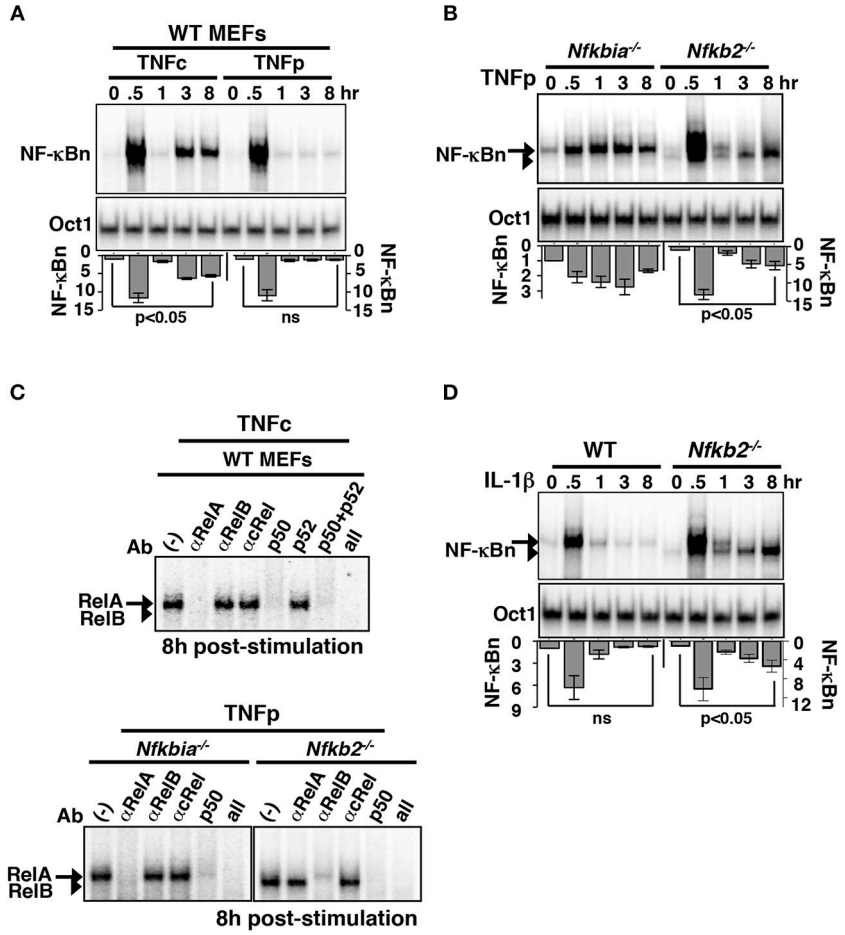


FIGURE 2 | Brief TNF stimulation of *Nfkb2*$^{-/-}$ MEFs induce an additional late NF-κB activity composed of RelB:p50 heterodimers. **(A)** WT MEFs were subjected to TNFc or TNFp treatments, cells were harvested at the indicated time-points after the commencement of stimulations, and NF-κBn DNA binding activities were resolved in EMSA (top panel). DNA binding activity of Oct1 served as a loading control (middle panel). Bottom: signals corresponding to NF-κBn were quantified from four independent experiments and presented in relation to the basal activity in a bargraph. Statistical significance was evaluated using Student's *t* test. **(B)** EMSA comparing NF-κBn induced in a time-course in *Nfkbia*$^{-/-}$ and *Nfkb2*$^{-/-}$ MEFs upon TNFp treatment (top panel). As determined in **(C)**, the arrow and the arrowhead indicate RelA-containing and RelB-containing NF-κB complexes, respectively. Bottom: quantitative analysis of the total NF-κBn activities from four experimental replicates. **(C)** Composition of the NF-κBn activities that persisted after 8 h of TNFp treatment in *Nfkbia*$^{-/-}$ and *Nfkb2*$^{-/-}$ MEFs, was determined in the shift-ablation assay. Antibodies against the indicated NF-κB subunits were used for ablating the respective DNA binding complexes in EMSA. Data represents two independent experiments. **(D)** Time-course analysis of NF-κB DNA binding activity induced upon IL-1β treatment of WT or *Nfkb2*$^{-/-}$ MEFs (top panel). Bottom: quantified NF-κB signal intensities; data represent four experimental replicates. Quantified data presented in this figure are means ± SEM.

parameter space surrounding the nominal values simultaneously among the different parameter groups. The effect of parameter uncertainty for individual parameter groups on the late RelB:p50 activity was summarized as the total effect index (29). Group-V showed a substantially high total effect index indicating that parameters belonging to this group played a dominant role in determining the late RelB:p50 response (**Figure 3B**; **Figure S3A**). Group-V consisted of rate parameters associated with NF-κB-driven and constitutive syntheses of *Relb* mRNA as well as translation of *Relb* mRNA. In a local sensitivity analysis, we then distinguished between these Group-V parameters for their relative contributions in eliciting late RelB:p50 activity. We introduced a 10% increase in the individual rate parameters and the resultant effect on the late RelB:p50 response was scored subsequent to data normalization. Our analysis indicated that particularly NF-κB-mediated transcription of *Relb* promoted the late RelB:p50 response to TNFp in the *Nfkb2*-deficient system (**Figure 3C**). Both RelA and RelB heterodimers are capable of inducing the expression of *Relb* mRNA from the endogenous NF-κB target promoter (17, 23). Indeed, our computational model included the description of RelA, as well as RelB, mediated synthesis of RelB. To understand the contribution of these individual processes in late-acting RelB:p50 response, we abrogated either RelA-dependent or RelB-mediated RelB transcriptions in our model. Our computational simulations revealed that disruption of either RelA-mediated or RelB-driven transcription of RelB diminished the late RelB:p50 response in p100-deficient cells (**Figure 3D**). Therefore, our computational

studies suggested that RelA-mediated transcription of *Relb* mRNA was not sufficient and autoregulatory synthesis of RelB was important for modifying dynamical TNF controls.

We tested these computational predictions experimentally. We observed that TNFc activated delayed expression of *Relb* mRNA, which is encoded by a NF-κB target gene, in WT MEFs (**Figure 3E**). Consistent with the lack of late NF-κBn activity in TNFp-treated WT MEFs, TNFp-induced expressions of *Relb* mRNA were less prominent in these cells (**Figure 3E**). However, TNFp treatment of $Nfkb2^{-/-}$ MEFs led to heightened synthesis of *Relb* mRNA and protein at 6 h post-stimulation that temporally coincided with the late RelB activity observed in these cells (**Figures 3E,F**). Using retroviral constructs, we then expressed RelB from either a constitutive or a NF-κB responsive transgenic promoter in $Relb^{-/-}Nfkb2^{-/-}$ MEFs. TNFp treatment induced the accumulation of RelB mRNA in $Relb^{-/-}Nfkb2^{-/-}$ cells expressing RelB from the NF-κB-driven, but not constitutive, promoter (**Figure S3B**). Furthermore, TNFp triggered the late RelB:p50 activity only in engineered cells expressing RelB from the NF-κB responsive promoter, but not in cells expressing RelB from the constitutive promoter (**Figure 3G**). These results suggested that NF-κB-induced synthesis of RelB was required for triggering the late-acting RelB:p50 response to TNFp in the absence of p100. Therefore, our combined mathematical and biochemical analyses established that NF-κB-driven sustained RelB production promoted progressive nuclear accumulation of RelB:p50 heterodimers in response to brief TNF stimulation of p100-deficient cells.

RelB:p50 Modify the TNF-Activated Gene-Expression Program in $Nfkb2^{-/-}$ MEFs

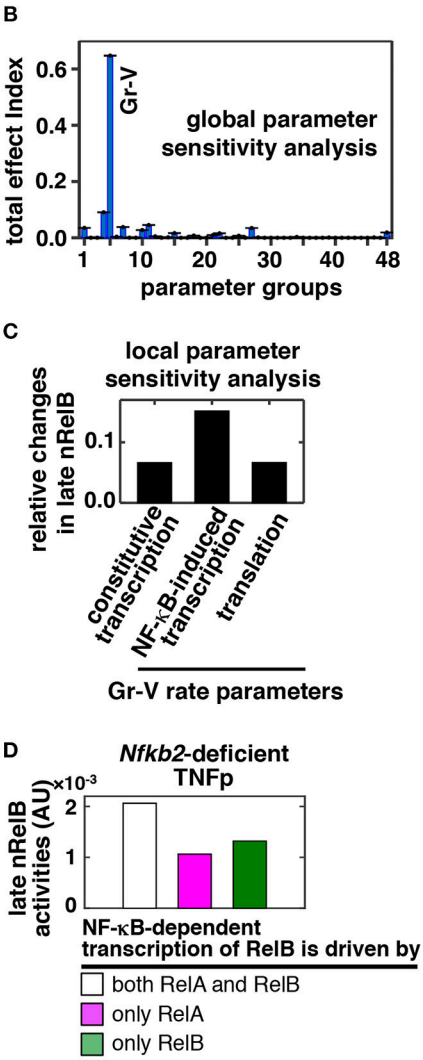
Next, we sought to determine the gene-expression specificity of RelB:p50 in microarray mRNA analysis (Materials and Methods, **Supplementary Materials**). For side by side comparison of gene controls by RelA:p50 and RelB:p50, we focused on TNFc regime, which produced equivalent nuclear activity of these two heterodimers at 6 h post-stimulation in $Nfkb2^{-/-}$ MEFs (**Figure 4A**) (23). To dissect genetically heterodimer-specific gene expressions, we additionally examined $Relb^{-/-}Nfkb2^{-/-}$ MEFs, which activated exclusively RelA:p50 upon TNFc treatment, and $Rela^{-/-}Nfkb2^{-/-}$ cells, which elicited solely RelB:p50 response (**Figure 4A**). As controls, we used WT MEFs, which induced RelA:p50 activity in response to TNFc, and NF-κB-deficient cells, which lacked all three transcription-competent NF-κB subunits RelA, RelB, and cRel. In our microarray mRNA analysis, we first considered genes whose expression was induced at least 1.3 fold at 6 h post-TNFc treatment in $Nfkb2^{-/-}$ MEFs, but not in NF-κB-deficient cells. Accordingly, we arrived at a list of 304 NF-κB dependent genes. Based on their differential expressions in $Relb^{-/-}$ $Nfkb2^{-/-}$ and $Rela^{-/-}Nfkb2^{-/-}$ MEFs, we cataloged these NF-κB-dependent genes into six distinct clusters, which were arranged further into four gene-groups (Gr-I to Gr-IV; **Figure 4B**; **Supplementary Materials**, Materials and Methods). Gr-I genes were induced in WT, $Nfkb2^{-/-}$ and $Relb^{-/-}Nfkb2^{-/-}$ MEFs that possessed the RelA:p50 activity (**Figures 4A,B**). Gr-II genes were activated either in the presence of RelA:p50 in WT, $Nfkb2^{-/-}$ and $Relb^{-/-}$ $Nfkb2^{-/-}$ MEFs or in RelB:p50-containing $Rela^{-/-}$ $Nfkb2^{-/-}$ cells. Genes belonging to Gr-III required RelB:p50 for their expressions; they were induced in $Nfkb2^{-/-}$ or $Rela^{-/-}Nfkb2^{-/-}$ MEFs, but not in WT or $Relb^{-/-}$ $Nfkb2^{-/-}$ cells. Gr-IV genes were activated only in $Nfkb2^{-/-}$ MEF possessing both RelA:p50 and RelB:p50 activities. Our analyses of knockout cells suggested that RelB:p50 heterodimer could mediate the expression of a subset (Gr-II) of NF-κB-target genes activated by TNF in WT cells involving RelA:p50. Of note, previous studies also reported that RelA:p50 and RelB:p50 function redundantly in mediating the expression of certain pro-inflammatory genes, such as those encoding RANTES, as well as pro-survival genes, such as those encoding cFLIP (20, 23). Intriguingly, RelB:p50, either alone (Gr-III) or in collaboration with RelA:p50 (Gr-IV), activated additional genes in $Nfkb2^{-/-}$ MEFs that were not normally induced in WT MEFs. Therefore, p100 modified transcriptional responses to TNF in MEFs involving both RelA- as well as RelB-dependent mechanisms.

We then subjected these gene-groups to gene ontology (GO) analyses. Consistent with the well-articulated role of the canonical pathway in immune-activating TNF signaling, Gr-I and Gr-II comprising TNFc-induced RelA-important genes were enriched for GO terms associated with innate and adaptive immune responses (**Figure 4C**). Gr-II also scored highly for terms linked to cellular respiration. Gr-III and Gr-IV consisting of RelB-important genes activated in p100-deficient cells were instead enriched for terms associated with cellular differentiation, aging, and cell death as well as metabolic processes. These RelB-important genes scored poorly for immune response related GO terms.

We asked if overlapping and distinct gene controls by RelA and RelB heterodimers were mediated at the level of chromatin binding. To address this, we subjected $Nfkb2^{-/-}$ MEFs to TNFc treatment for 6 h and subsequently performed chromatin immunoprecipitation using anti-RelA or anti-RelB antibodies followed by deep-sequencing (ChIP-seq) analysis (see Materials and Methods, **Supplementary Materials**). We then assessed the RelA as well as the RelB ChIPed-tag density around (± 2 kb) the center of the top 2077 RelA binding peaks (top panels, **Figure 5A**). Similarly, RelA and RelB binding surrounding the top 2241 RelB binding peaks were charted (bottom panels, **Figure 5A**). These top-ranking peaks were selected basing on their intensity as well as their rank in the irreproducible discovery rate test (30). Our peak-centered heatmap revealed that RelA and RelB bound to mostly overlapping chromatin sites and with almost similar proficiency. Next, we focused our analyses on Gr-I, Gr-II, Gr-III, and Gr-IV genes, which showed distinct requirements of NF-κB subunits for their expressions. We considered chromatin locations up to 50 kb from the transcription start site for assigning peaks to a given gene. Our analyses revealed that RelA or RelB recruitments to chromatin sites in TNFc-stimulated $Nfkb2^{-/-}$ cells were equivalently enriched for all four gene-groups (see bargraphs **Figure 5B**). We indeed noticed a substantial overlap between RelA- and RelB- associated genes globally and in the individual gene-groups

A

Groups (Gr)	Respective Biochemical Reactions
Gr-I to Gr-VI	synthesis (constitutive and NF-κB-induced transcription, translation) and degradation (constitutive and signal-induced) of IκBα (Gr-I, VII), IκBβ (II, VIII), IκBε (III, IX), RelA (IV, X), RelB (V, X!) and p50 (VI, XII). Gr-I to VI correspond to synthesis and Gr VII to XII correspond to degradation related parameters groups. Similar numbering formalism has been used for the rest of the table -> elementary species; elementary species -> deg
VII to XII	
XIII to XIV	generation of RelA:p50 and RelB:p50 dimers from monomers. $monomer_1 + monomer_2$ -> NF-κB dimer
XV to XIX	generation of IκB-inhibited NF-κB dimers, viz.IκBα:RelA:p50, IκBβ:RelA:p50, IκBε:RelA:p50, IκBα:RelB:p50 and IκBε:RelB:p50. IκB + NF-κB -> IκB:NF-κB
XX to XXI	constitutive degradation of RelA:p50 and RelB:p50 dimers. NF-κB ->
XXII to XXVI	constitutive and signal-induced degradation IκBs present in IκBα:RelA:p50, IκBβ:RelA:p50, IκBε:RelA:p50, IκBα:RelB:p50 and IκBε:RelB:p50 complexes. IκB:NF-κB -> NF-κB
XXVII	constitutive degradation NF-κBs present in IκBα:RelA:p50, IκBβ:RelA:p50, IκBε:RelA:p50, IκBα:RelB:p50 and IκBε:RelB:p50 complexes. IκB:NF-κB -> IκB
XXVIII to XXX, and XXXIV to XL	nuclear import and export of IκBα, IκBβ, IκBε, RelA:p50, RelB:p50, IκBα:RelA:p50, IκBβ:RelA:p50, IκBε:RelA:p50, IκBα:RelB:p50 and IκBε:RelB:p50. Gr-XXVIII to XXX, and XXXIV to XL correspond to nuclear import related parameter groups.
XXXI to XXXIII, and XLI to XLVII	
XLVIII	TNF induced modification of RelB



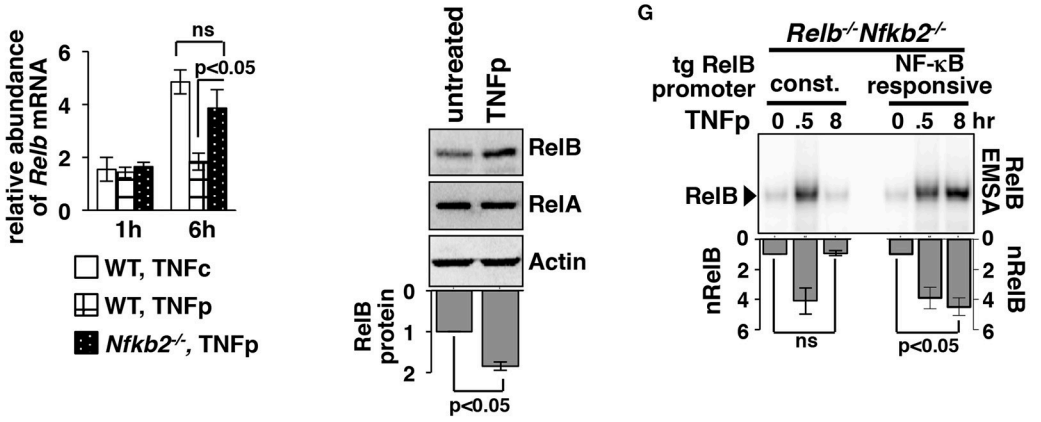


FIGURE 3 | NF-κB-dependent RelB synthesis promotes the late RelB:p50 activity induced upon brief TNF treatment of *Nfkb2*$^{-/-}$ cells. **(A)** Parameter groups analyzed in the variance-based, multiparametric sensitivity analysis. **(B)** Variance-based multiparametric analysis revealing the total effect index, which represent the effect of the parameter uncertainty on the late (6–8 h) RelB:p50 activity induced by TNFp in the *Nfkb2*-deficient system for the individual parameter groups. Standard bootstrapping was used for estimating error ranges. Gr, group. Gr-V consists of parameters related to RelB synthesis; including constitutive and NF-κB induced transcriptions as well as translation. **(C)** Local sensitivity analysis revealing the effect of 10% increase in the indicated parameters belonging to Gr-V on the late

(Continued)

FIGURE 3 | RelB:p50 response to TNFp in the *Nfkb2*-deficient system. Differences in the basal-corrected, RelB:p50 activity between the unperturbed system and perturbed systems were scored. **(D)** Computational simulations of the late nRelB response to TNFp involving *Nfkb2*-deficient systems, where the expression of RelB mRNA is mediated by either RelA as well as RelB heterodimers or by RelA alone or by exclusively RelB. **(E)** WT and $Nfkb2^{-/-}$ MEFs were treated with TNFp before being subjected to qRT-PCR analysis of *Relb* mRNA abundances normalized to that of *Actb* mRNA. WT MEFs treated with TNFc were used as control. Bargraphs demonstrate the abundances of mRNAs in TNF-treated cells relative to those measured in the untreated cells. Data represent four biological replicates. **(F)** $Nfkb2^{-/-}$ MEFs were treated with TNFp and harvested at 6 h post-stimulation before being analyzed by Western blotting. Actin served as a loading control. Bottom: densitometric analysis of the relative abundance of RelB protein in whole cell extracts; data represent five biological replicates. **(G)** TNFp-induced nRelB activity in $Relb^{-/-}Nfkb2^{-/-}$ MEFs stably expressing RelB from a retroviral transgene (tg) either constitutively (const.) or from an NF-κB responsive promoter. Ablating RelA DNA binding with an anti-RelA antibody, residual nRelB activities were revealed by RelB-EMSA. Data represent four independent experiments. Quantified data presented in this figure are means ± SEM.

(see Venn diagrams, **Figure 5B**). Finally, we examined browser tracks of a select set of genes belonging to these gene-groups (**Figure 5C**). *Tlr2* belonging to Gr-I and *C3* belonging to Gr-II were bound by both RelA and RelB in TNFc-treated $Nfkb2^{-/-}$ MEFs. Among the Gr-III genes, *Psmc4* did not recruit these NF-κB subunits but *Bcl10* engaged both RelA and RelB. Similarly, either RelA or RelB was not recruited to *Me2* belonging to Gr-IV, but both bound *Bcl3*. Therefore, despite genetic analyses revealing distinct sets of RelA- and RelB-important genes, our ChiP-seq analyses suggested that RelA and RelB bound to largely overlapping chromatin sites, and that a subset of RelB-important genes circumvented NF-κB binding for their expressions.

Taken together, our genome-scale analyses implied that RelB:p50 were capable of modifying the TNF-induced gene-expression program in MEFs. RelB:p50 activated by TNFc in $Nfkb2^{-/-}$ cells induced a distinct set of genes, which were not induced by RelA:p50 in WT cells and encoded functions unrelated to immune processes. However, this distinct gene control was not attributed to specific chromatin binding by RelB:p50 heterodimers.

p100 Determines Specificity and Dynamical Control of TNF-Mediated Gene Expressions

We further validated our microarray data for a select set of genes representing different gene-groups using quantitative real time-polymerase chain reaction (qRT-PCR) analyses. TNFc treatment for 6 h triggered the RelA-dependent expression of Gr-I gene *Tlr2* in WT, $Nfkb2^{-/-}$ as well as $Relb^{-/-}Nfkb2^{-/-}$ MEFs and not in $Rela^{-/-}Nfkb2^{-/-}$ cells (**Figure 6A**). TNFc induced *Csf1* belonging to Gr-II, whose members were activated redundantly by RelA:p50 and RelB:p50 in our microarray studies, not only in WT and $Nfkb2^{-/-}$ MEFs but also in $Relb^{-/-}Nfkb2^{-/-}$ and $Rela^{-/-}Nfkb2^{-/-}$ cells. As expected, RelB was both necessary and sufficient, and mediated the expression of Gr-III gene *Klf5* in $Nfkb2^{-/-}$ and $Rela^{-/-}Nfkb2^{-/-}$ MEFs but not in WT and $Relb^{-/-}Nfkb2^{-/-}$ cells. Consistent to the proposed requirement of both RelA:p50 and RelB:p50 for the expression of Gr-IV genes, *Me2* and *Mras* was induced selectively in $Nfkb2^{-/-}$ MEFs. NF-κB-deficient cells did not activate these genes in response to TNF. Therefore, our qRT-PCR analyses substantiated our genome-scale data highlighting distinct gene controls by RelA and RelB heterodimers.

Sustained expression of NF-κB-dependent genes require prolonged RelA:p50 nuclear activity, such as those produced in WT cells by TNFc (4, 8, 31, 32). On other hand, it has been found that transient RelA:p50 activity elicited by TNFp is inadequate for the continued expression of NF-κB-target genes. Because TNFp stimulated a prolonged nuclear activity of RelB:p50 in $Nfkb2^{-/-}$ MEFs, we asked if TNFp triggered persistent expression of NF-κB-dependent genes in p100-deficient cells. Our time-course analyses demonstrated that TNFc induced progressive accumulation of mRNAs encoding Gr-I and Gr-II genes in WT as well as $Nfkb2^{-/-}$ MEFs between 1 and 6 h post-treatment (**Figures 6B,C**). As expected, TNFp failed to sustain the expression of these RelA-important genes in WT cells. $Nfkb2^{-/-}$ MEFs upheld the expression of Gr-II genes, which could be activated by either RelA or RelB factors, in response to TNFp at 6 h post-stimulation. Akin to TNFc, TNFp additionally stimulated delayed expressions of RelB-important genes, which included Gr-III as well as Gr-IV genes, in $Nfkb2^{-/-}$ MEFs at 6 h post-stimulation (**Figures 6D,E**). These genes were not activated in WT MEFs even upon TNFc stimulation. Collectively, p100 enforced both dynamical control and the specificity of the TNF-induced gene-expression program. Brief TNF stimulation of p100-deficient cells triggered a prolonged RelB:p50 activity, which not only sustained the expression of a subset of RelA-important genes but also induced delayed expressions of metabolic and immune-differentiation related genes, which were not normally activated by RelA:p50 in WT MEFs.

Repeated Pulses of TNF Strengthen Late-Acting RelB:p50 Response in $Nfkb2^{-/-}$ Cells

Within tissue microenvironment, macrophages secrete TNF in repeated bursts. Accordingly, effect of periodic TNF pulses on the nuclear NF-κB activity has been investigated *ex vivo*. When administered at short intervals, repeated TNF pulses produce a refractory state in WT cells leading to a diminishing RelA:p50 response (12, 33). Because p100 deficiency provoked an additional RelB:p50 response to brief TNF stimulation, we set out to examine mathematically as well as experimentally NF-κB activation in response to periodic TNF pulses in $Nfkb2^{-/-}$ MEFs. Corroborating earlier studies, our computational simulations suggested that two consecutive TNF pulses separated by 1 h would lead to a weakened RelA:p50 response to the succeeding TNF pulse in both WT and *Nfkb2*-deficient systems (**Figures 7A,B**). Interestingly, our simulation studies also predicted that for a pulse separation of 1–4 h,

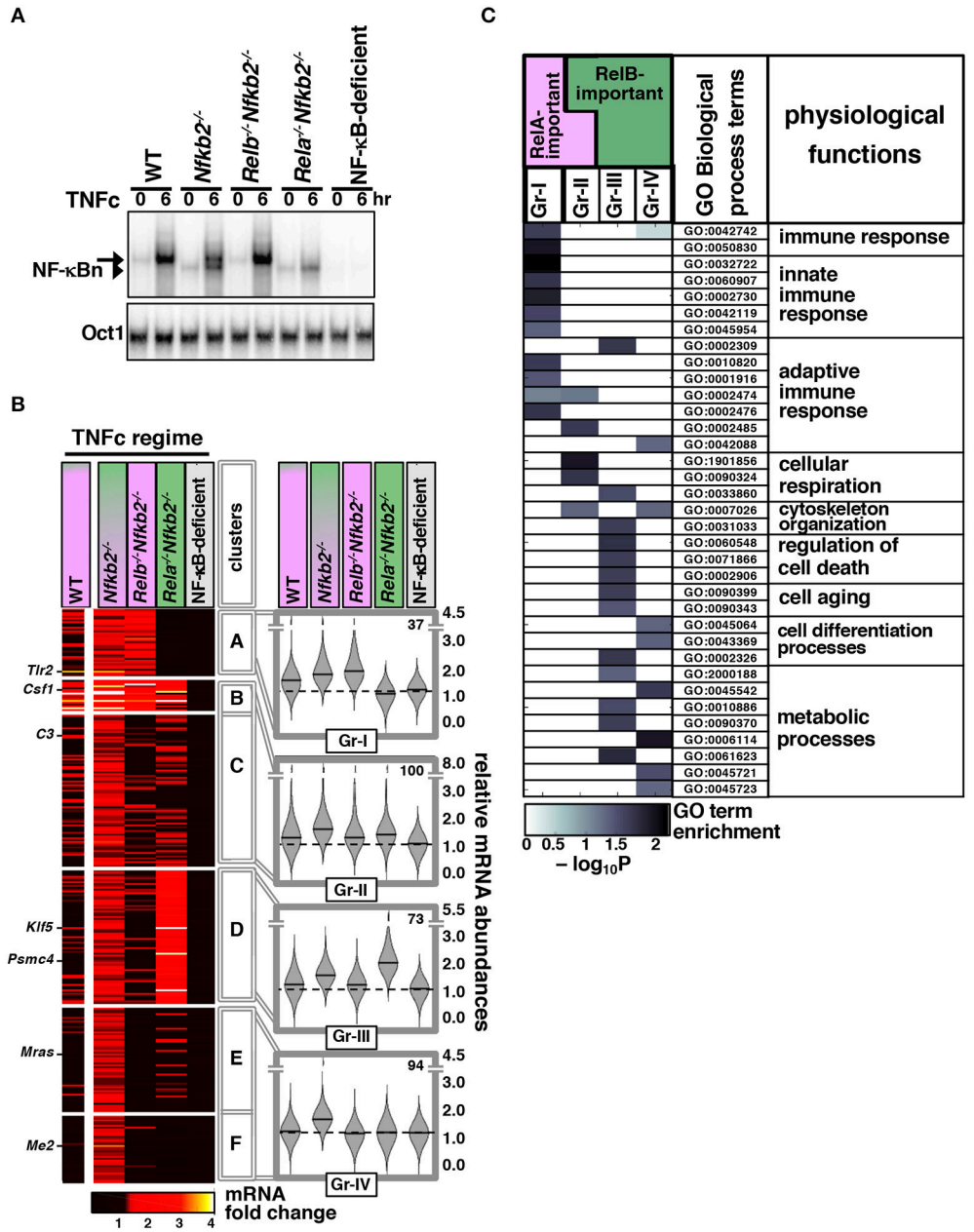


FIGURE 4 | Global analyses reveal distinct gene controls by the TNF-activated RelB:p50 heterodimer. **(A)** MEFs of the indicated genotypes were treated with TNFc for 6 h before being subjected to EMSA. The data represents three independent experiments. **(B)** In our microarray mRNA analysis, we considered genes with high confidence detection in biological replicates across various knockout MEFs, and at least 1.3 fold increase in the average expression upon 6 h of TNFc treatment in *Nfkb2*$^{-/-}$ cells, but <1.3 fold average induction in NF-κB-deficient cells to arrive onto a list of 304 genes. Heatmap demonstrates TNF-induced fold changes in the expressions of these genes in the indicated knockout cells clustered using the partition around medoids algorithm. A representative data using WT MEFs has been indicated in the left column. The resultant six gene-clusters were arranged into four gene-groups. Representative genes belonging to different groups have been indicated. Right: violin plots show relative frequency distributions of fold change values and corresponding medians for various genotypes as well as the number of members in each gene-group. **(C)** Functional enrichment of various Gene Ontology for Biological Process terms in the indicated gene-groups was determined by topGO. A subset of highly enriched terms in either of the gene-groups is highlighted. Broad physiological functions associated with these GO terms have been also indicated.

a succeeding TNF pulse would augment the late RelB:p50 activity induced at 8 h by the preceding TNF pulse in the *Nfkb2*$^{-/-}$deficient system. In our computational model, this heightened late RelB activity was accompanied by an increased abundance of *Relb* mRNA and protein (**Figure S4A**). Our experimental analyses substantiated that as compared to a single pulse, two or three consecutive TNF pulses augmented the late RelB activity as well as the abundance of *Relb* mRNA and

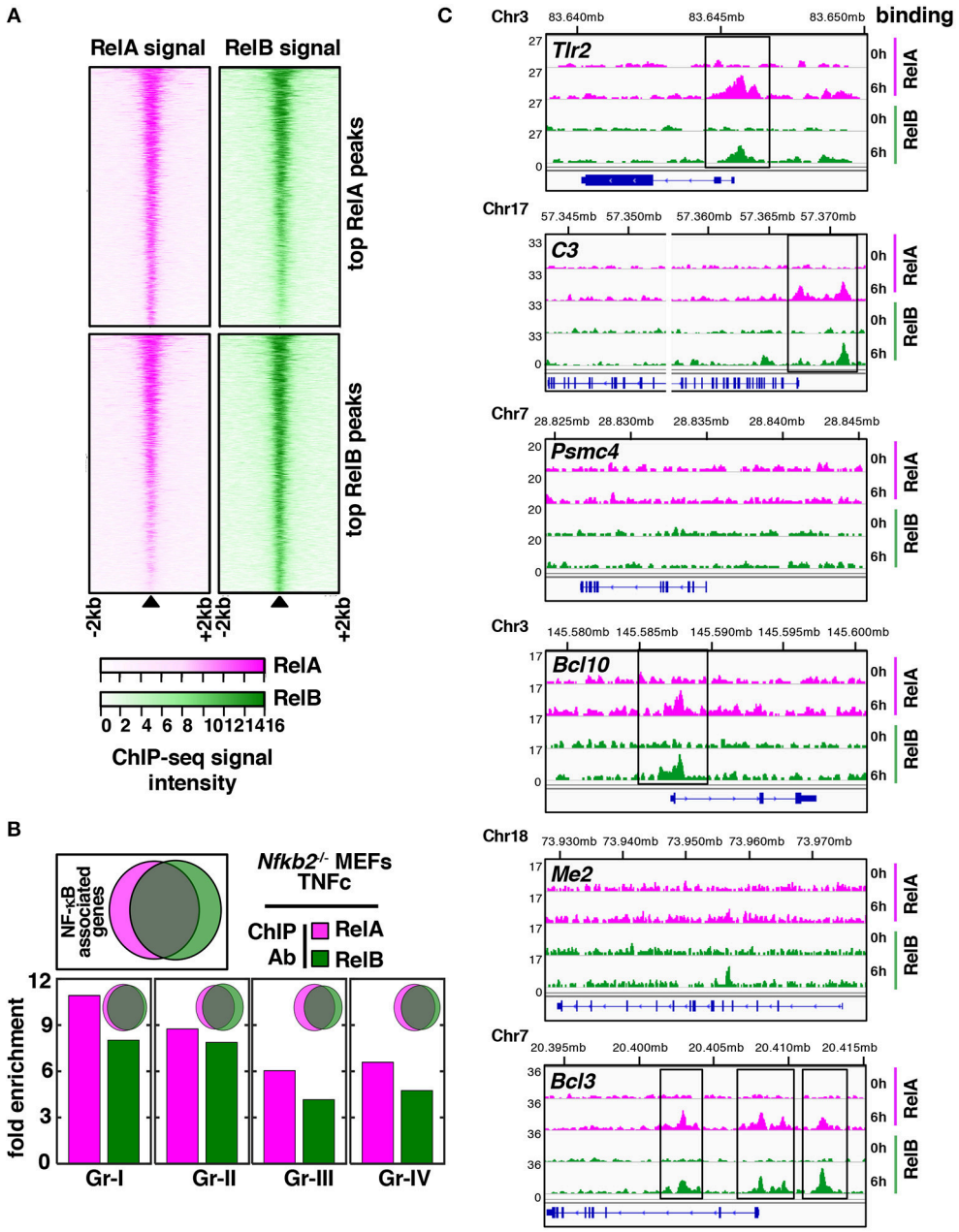


FIGURE 5 | Overlapping genome-wide distributions of RelA and RelB in TNF-stimulated *Nfkb2*$^{-/-}$ MEFs. **(A)** Using ChIP-seq analyses, we captured the genome-wide distribution of RelA and RelB in *Nfkb2*$^{-/-}$ MEFs subjected to TNFc treatment for 6 h. A peak centered heatmap revealing RelA and RelB binding to chromatin locations surrounding top RelA or top RelB binding peaks. **(B)** Venn diagrams reveal overlap between RelA- (magenta) and RelB-associated (green) genes globally (top, left) or for the individual gene groups. Bar graphs reveal relative enrichment of RelA-associated and RelB-associated genes for various gene groups. **(C)** Representative browser tracks of RelA and RelB binding to genes belonging to various gene groups. The y-axis represents normalized reads per 10 million. Chromosomal locations of each gene in mm9 are shown above ChIP-seq tracks.

protein in *Nfkb2*$^{-/-}$ MEFs (**Figure 7C**; **Figure S4B**). Finally, double or triple TNF pulses enhanced the delayed expression of RelB-important genes in *Nfkb2*$^{-/-}$ cells (**Figure 7D**). These studies identified an important role of p100 in the pulsatile TNF regime; although p100 did not participate in attenuating the RelA activity, it prevented escalating RelB:p50 response to periodic TNF pulses.

Brief TNF Stimulation Triggers a Late-Acting, Pro-Survival NF-κB Response in p100-Deficient Myeloma Cells

The non-canonical NF-κB pathway often accumulates gain-of-function mutations in multiple myeloma and these genetic aberrations were shown to completely degrade p100 in myeloma cells (34). It has been also suggested that TNF, which has a very

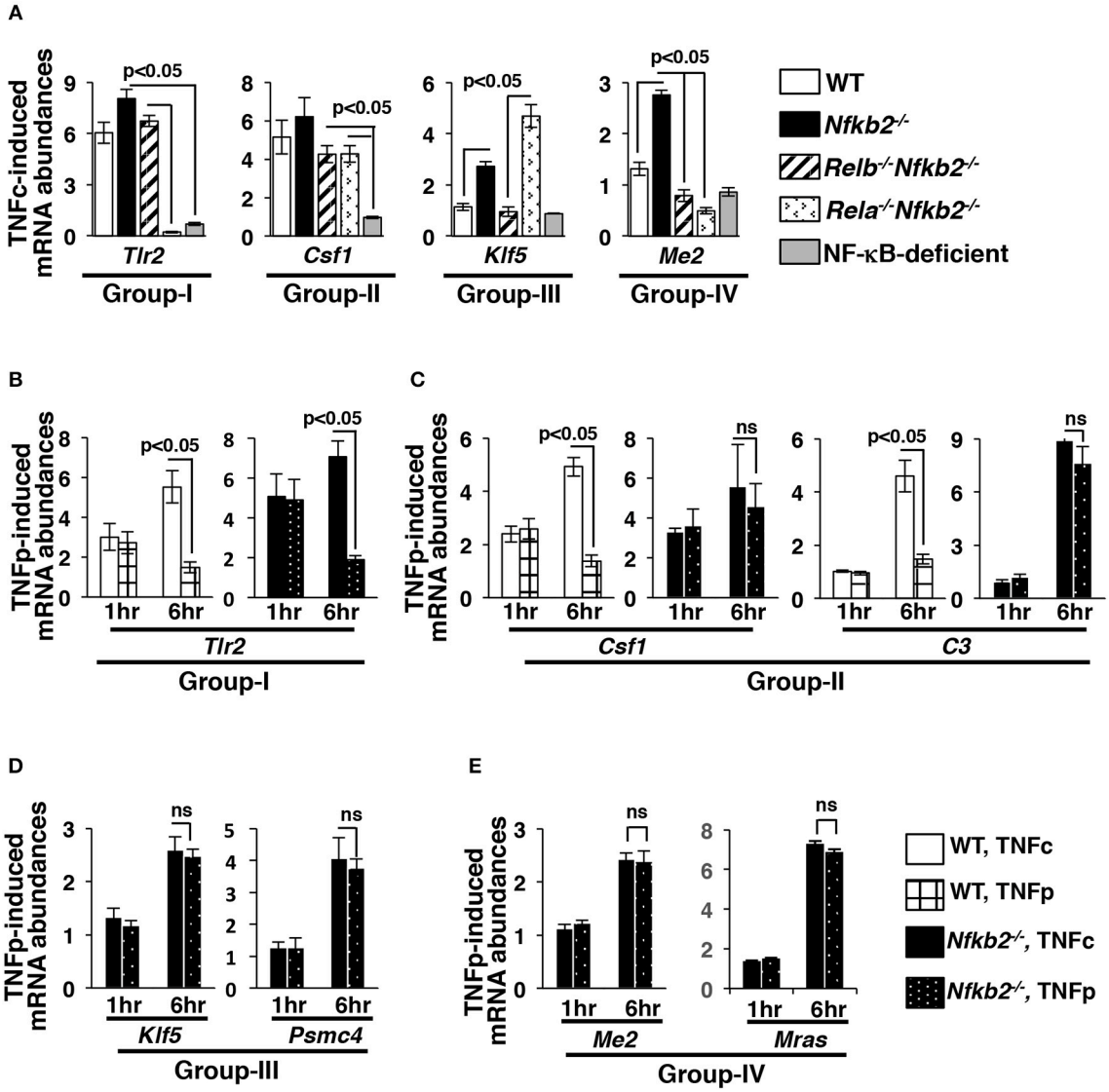


FIGURE 6 | Brief TNF stimulation induces delayed, RelB-dependent gene expression in *Nfkb2*$^{-/-}$ MEFs. **(A)** WT and knockout MEFs were subjected to TNFc treatment for 6 h, and the expressions of the indicated genes belonging to different gene-groups were measured by qRT-PCR. Bargraphs demonstrate the abundances of the corresponding mRNAs in stimulated cells relative to those measured in untreated cells. Data represent three biological replicates. **(B–E)** WT and *Nfkb2*$^{-/-}$ MEFs were subjected to TNFc or briefly stimulated with TNF for 0.5 h (TNFp), and subsequently cells were harvested at the indicated time-points before being subjected to qRT-PCR analyses. Bargraphs demonstrate TNF-induced expressions of the indicated genes, representing various gene-groups, in relation to untreated cells. Data represent four independent experiments. Quantified data presented in this figure are means ± SEM.

short serum half-life, promotes survival of myeloma cells within the tumor microenvironment. We have earlier demonstrated that KMS28PE human myeloma cell-line was devoid of p100 because of non-canonical pathway mutations (23). Furthermore, chronic TNF treatment of these p100-depleted myeloma cells induced RelA:p50 as well as RelB:p50 complexes, both of which activated the expression of pro-survival factors. We asked if p100 deficiency modified the NF-κB response of myeloma cells to short-lived cytokine signals, such as those generated by TNFp. To this end, we compared KMS28PE cells with control OciMy5 cells, which preserved p100 expressions, in our biochemical studies. Brief TNF treatment induced a transient RelA NF-κB activity in OciMy5 cells that lasted about an hour (**Figures 8A,B**). In KMS28PE cells, TNFp induced a similar transient RelA activity that was mostly attenuated at 8 h post-stimulation. These p100-depleted myeloma cells also possessed constitutive RelB activity. Indeed, TNFp further induced progressive nuclear accumulation of RelB in KMS28PE that produced a strong RelB NF-κB DNA binding activity at 8 h post-TNFp treatment. Finally, our gene-expression studies revealed that TNFp induced late-expressions of mRNAs encoding pro-survival factors Bcl2 and cFLIP in KMS28PE cells (**Figure 8C**); these gene activities temporally

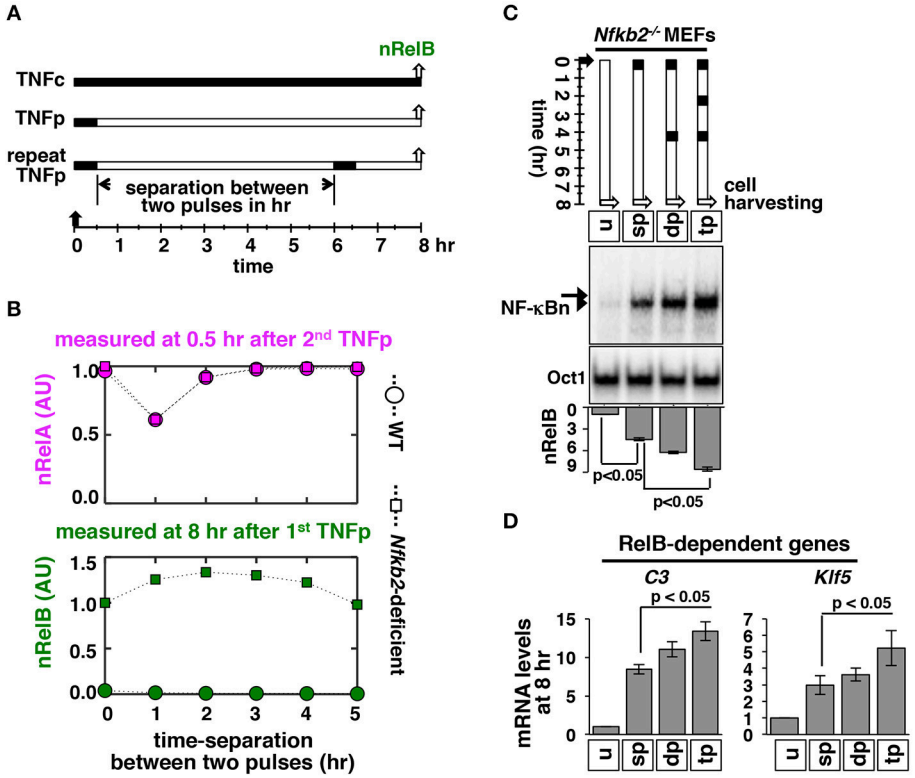


FIGURE 7 | Repeated TNF pulses of *Nfkb2*$^{-/-}$ cells strengthen late RelB:p50 signaling. **(A)** Schema describing repeated TNFp regime: the short-lived IKK2 input associated with the TNFp regime was fed into the model successively with varied separation time between two TNF pulses and corresponding NF-κBn was simulated. **(B)** Computational studies revealing the early nRelA activity induced 0.5 h after the second TNFp (top) and the late nRelB activity induced 8 h after the first TNFp (bottom) as a function of the separation time between two successive pulses in WT and *Nfkb2*-deficient systems. The early and late activities were normalized to those induced in response to a single pulse in the *Nfkb2*-deficient system. **(C)** *Nfkb2*$^{-/-}$ MEFs were treated with either a single TNFp (single pulse, sp) or two successive TNFp separated by 4 h (double pulse, dp) or three pulses at 2 h intervals (triple pulse, tp). Cells were harvested 8 h after the first pulse and analyzed for NF-κBn by EMSA. *u* denotes untreated. Bottom: quantitative analysis of the nRelB activities; data represent three experimental replicates. **(D)** *Nfkb2*$^{-/-}$ MEFs were subjected to the indicated treatments; cells were harvested 8 h after the first pulse and expressions of the indicated genes were measured by qRT-PCR. Bargraphs represent three biological replicates. Quantified data presented in this figure are means ± SEM of three biological replicates.

coincided with the robust late-acting RelB response observed in these cells. TNFp stimulation did not induce pro-survival gene expressions in OciMy 5 cells. Our studies suggested that short-lived cytokine signals triggered a late-acting, pro-survival NF-κB response in human malignancies in the absence of p100.

DISCUSSION

Our study suggested that by insulating RelB heterodimers from the canonical NF-κB pathway, p100 enforced dynamical and gene controls of TNF signaling (**Figure 9**). As such, TNF engages the canonical pathway for activating RelA:p50 heterodimers, which induce the expression of specific immune response genes (2). Brief and chronic TNF treatments induce transient and long-lasting RelA:p50 activities, respectively, and produce distinct transcriptional responses. It is thought that the IκBα-mediated negative feedback hardwired in the canonical module enables the NF-κB system to distinguish between time-varied TNF inputs. p100 is rather known for transducing non-canonical NF-κB signals, which mediate nuclear activation of RelB heterodimers during immune differentiation (15). We found that the absence of p100 provoked a prolonged, biphasic NF-κB response to brief TNF stimulation. However, this late-phase NF-κB activity was composed of RelB:p50, and not RelA:p50, heterodimers. In *Nfkb2*$^{-/-}$ cells subjected to brief TNF stimulation, RelB:p50 sustained the expression of a subset of immune response genes and also activated additional RelB-important genes, which encoded immune differentiation and metabolic functions. In response to periodic TNF pulses, the NF-κB system produces a refractory state that exerts a detrimental effect on the signal-induced RelA response and prevents unchecked RelA:p50 activity. In contrast to its inhibitory effect on the signal-induced RelA response, repeated TNF pulses strengthened the late-phase RelB:p50 activity in p100-deficient cells and augmented the expression of RelB-important genes.

TNF and other canonical pathway inducers do not cause degradation of p100, which is proteolyzed during non-canonical signaling. However, the TNF-activated canonical pathway induces the expression of *Nfkb2* mRNA, and the non-canonical signal transducer p100 interacts with RelA (19, 21). Indeed,

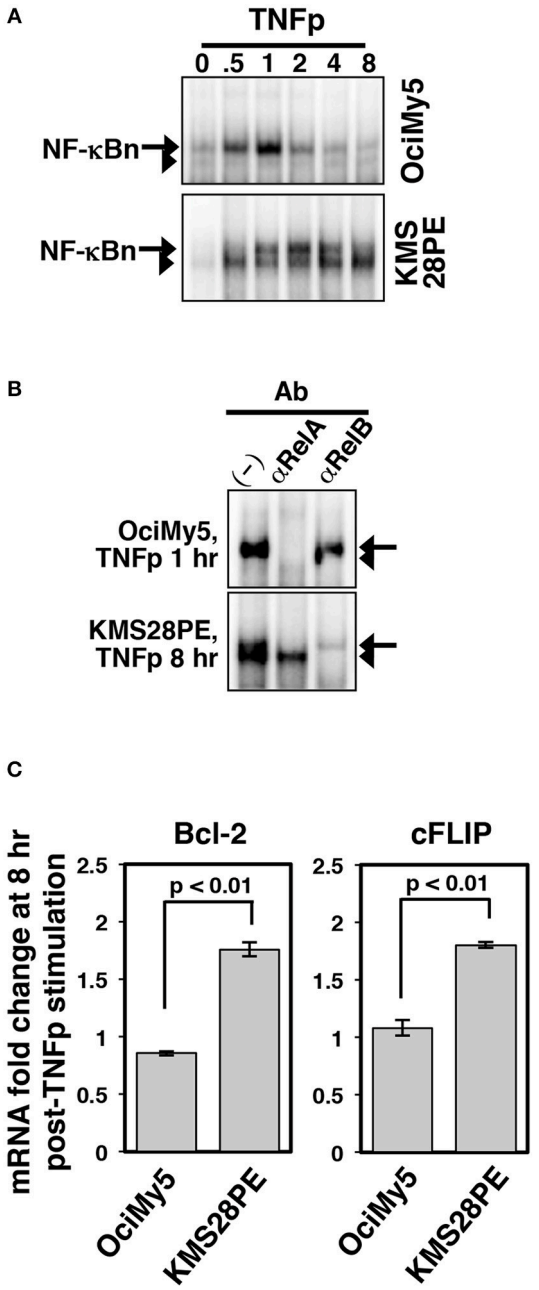


FIGURE 8 | Altered dynamical NF-κB control in p100-deficient myeloma cells. **(A)** EMSA demonstrating NF-κBn activation upon brief TNF stimulation of the indicated human myeloma cell-lines. **(B)** The composition of the NF-κBn activities induced at 8 h post-TNFp stimulation in these myeloma cell-lines was determined by shift-ablation assay. **(C)** qRT-PCR revealing the expression of the indicated pro-survival genes in myeloma cells at 8 h post-TNFp treatment. The data represent means ± SEM of three biological replicates.

a plausible role of p100 in TNF signaling was investigated earlier (20). Shih et al. (20) observed that p100 functions rather redundantly with IκBα in mediating post-induction attenuation of the RelA:p50 activity induced by chronic TNF treatment. Their study instead identified an important role of the p100-mediated negative feedback in regulating LPS-induced canonical RelA activity (20). In the absence of p100, however, a subpopulation of RelB is sequestered in the cytoplasm by IκBα, while the remainder translocates into the nucleus and produces a minor RelB:p50 NF-κB activity (17, 22, 23). It was shown that chronic TNF treatment, which degrades IκBα, strengthens this constitute RelB:p50 activity present in *Nfkb2*$^{-/-}$ MEFs (16, 20, 23). The RelB:p50 activity induced in p100-deficient cells by TNFc paralleled the signal-induced RelA:p50 activity; it consisted of an early 0.5 h peak followed by an attenuated activity at 1 h and a late-acting response prevailing between 3 and 8 h (23). Our brief TNF stimulation regime instead generated contrasting temporal profiles of these two NF-κB heterodimers in *Nfkb2*$^{-/-}$ cells; it induced a transient RelA:p50 activity but a prolonged RelB:p50 response (**Figure 9**). Our mechanistic studies suggested that NF-κB-driven RelB synthesis augmented the constitutive RelB:p50 activity present in *Nfkb2*$^{-/-}$ MEFs in response to TNFp stimulation. In the absence of sequestration by p100, newly-synthesized RelB produced by TNFp translocated into the nucleus as RelB:p50 heterodimers, which generated enduring NF-κB response to short-lived TNF signal in *Nfkb2*$^{-/-}$ MEFs.

Despite the established role of RelB in immune organogenesis, gene regulation by RelB heterodimers remain poorly understood. *In vitro* DNA interaction studies and ChIP-seq analyses showed that RelB and RelA heterodimers in fact bind to largely similar κB sequences (24–26). More so, genome-scale investigation indicated significant overlap between RelA and RelB with respect to the gene-expression specificity (22, 23, 25, 26). Our own global analyses involving *Nfkb2*$^{-/-}$ MEFs revealed both overlapping and distinct gene functions of RelA:p50 and RelB:p50. We identified a subset of TNF-activated genes, whose expressions were induced redundantly by these two heterodimers. Indeed, expressions of these NF-κB-dependent genes were sustained by RelB:p50 in TNFp-stimulated *Nfkb2*$^{-/-}$ MEFs. In addition, we characterized a distinct set of RelB-important genes, which were not normally activated by TNF and required RelB:p50 for their expressions. Surprisingly, our ChIP-seq analyses demonstrated equivalent binding of RelA and RelB heterodimers to the chromatin loci associated with RelB-important genes or genes that were activated redundantly by RelA or RelB. Our study, which involved the well-orchestrated MEF-based cell system subjected to a uniform cell-stimulation regime, indicated that DNA-protein interactions played a rather insignificant role in determining the gene-expression specificity of NF-κB heterodimers. In line with an earlier proposal (35), we speculate that the gene-expression specificity is largely contingent upon the interaction of NF-κB heterodimers with other transcription factors. Because certain RelB-important genes did not show NF-κB binding at their promoters, we do not rule out possible engagement of the RelB-driven transcriptional feedforward loop in mediating the expression of a subset of RelB-important genes (36). Future studies ought to elaborate the regulatory mechanism driving expressions of these RelB-important genes in immune cells.

In addition to modulating immune response, the pleiotropic cytokine TNF also contributes to immune differentiation, for example osteoclastogenesis (37). Interestingly, genetic

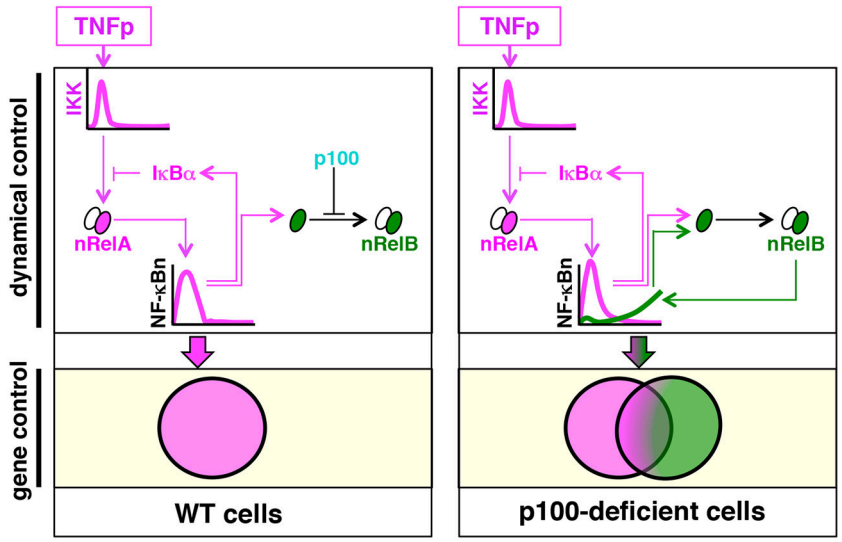


FIGURE 9 | The proposed model explaining the role of p100 in the dynamical control and the gene expression of specificity of TNF-induced NF-κB signaling. Brief TNF stimulation elicits transient NF-κB activity composed of RelA:p50 heterodimers, which mediate the expression of immune response genes. An absence of p100 triggers a late RelB:p50 NF-κB activity in response to TNF that induces also the expressions of genes involved in metabolic and cellular differentiation processes.

studies often implicated non-canonical signal transducers RelB and p100 in TNF-dependent biological processes, including osteoclastogenesis (38–41). More so, it was reported that the abundance of p100 is subject to cell-type specific regulations with immature dendritic cells possessing only a minor amount (22). We propose that varied cellular abundance of p100 may provide for a mechanism of tuning TNF responses involving RelB:p50 in diverse physiological settings. Moreover, *Nfkb2* was shown to be frequently mutated in cancers and aberrant TNF signaling has been implicated in neoplastic diseases (42, 43). In particular, we have previously demonstrated that non-canonical pathway mutations completely degrade p100 in a subset of myeloma cell-lines. Our current study indicated that p100 depletion might enable RelB-dependent, late-acting expressions of pro-survival genes in myeloma cells subjected to brief TNF stimulation. Interestingly, a synthesis-dependent IRF4 activity was shown to protect myeloma cells in human patients (44). Furthermore, recent studies suggested that altered metabolism exacerbates malignant growth in human cancers (43). In this context, it will be important to determine if the synthesis-driven RelB activity, which was augmented upon periodic TNF pulses, caused abnormal metabolism in human malignancies with dysfunctional p100. In other words, our mechanistic studies, which involved MEF-based model cell culture system, should be further extended to analyze immune cells and disease-associated cells for unraveling physiological and patho-physiological significance of p100-mediated control of TNF signaling. Interestingly, previous single-cell studies demonstrated that asynchronous, oscillatory RelA activities shape the late NF-κB response to TNF in WT cells (7, 12, 13). This oscillatory control was later implicated in NF-κB-driven gene expressions. Our bulk measurement based experimental analyses involving p100-deficient cells likely masked plausible cell-to-cell variations of the late-acting RelB:p50 response to TNFp. We argue that our study will further motivate single-cell analyses addressing the role of p100 in producing cellular heterogeneity at the level of NF-κB responses.

In sum, we show that brief TNF stimulation produces a long lasting RelB:p50 NF-κB activity in the absence of p100 that not only sustains the expression of a subset of RelA target, immune-response genes, but also activates genes with biological functions separable from immune responses. Stimulus-specific cellular responses are often achieved through distinct dynamical control of shared signaling kinases and transcription factors. For example, neuronal growth factor (NGF) induces the sustained activity of extracellular signal-regulated kinase (Erk) for promoting cell differentiation. In contrast, transient Erk activation by epidermal growth factor (EGF) causes cell proliferation (45, 46). Genome-wide knockdown studies indicated that a vast regulatory network, and not a handful of components belonging to specific pathways, controls the amplitude of the activity of these signaling molecules (47). The NF-κB system is comprised of interlinked canonical and non-canonical modules and controls the activity of multiple transcription factors, which have overlapping as well as distinct gene functions. Our study offered evidence that an interconnected NF-κB system, and not the individual NF-κB modules, directs dynamical activity of the specific NF-κB transcription factors in response to extracellular stimuli, and that the abundance of the immune-differentiation regulator p100 may inform cell-type specific biological responses to pro-inflammatory cytokines.

MATERIALS AND METHODS

Mice, Cells, and Plasmids

WT and gene-deficient C57BL/6 mice were used in accordance with the guidelines of the Institutional Animal Ethics Committee of the National Institute of Immunology (approval no. #258/11). MEFs generated from E13.5 embryos were used subsequent to immortalization by the 3T3 protocol. Some key data have been also reproduced using primary MEFs. *Rela*$^{-/-}$*Rel*$^{-/-}$*Relb*$^{-/-}$ 3T3 MEFs, which lacked the expression RelA, cRel, and RelB, were utilized as NF-κB-deficient cells. *Relb*$^{-/-}$*Nfkb2*$^{-/-}$ MEFs expressing transgenic RelB from retroviral constructs were reported earlier (23). Human-derived myeloma cell-lines OciMy5, KMS28PE, and JK6L used in this study were a kind gift from Dr. Michael Kuehl, NCI.

Biochemical Analyses

In the TNFp regime, cells were treated briefly for 30 min with 1 ng/ml of TNF (Roche, Switzerland). Subsequently, TNF-supplemented media was substituted with TNF-free media, and cells were harvested at the indicated times after the commencement of the TNF treatment. In certain instances, cells were subjected to repeated pulses of TNF at the specified time intervals. Alternately, cells were treated chronically with 1 ng/ml of TNF (TNFc) or stimulated with 10 ng/ml IL-1β (Biosource, USA). As described (48), nuclear and whole cell extracts were analyzed by EMSA and Western blotting, respectively. The gel images were acquired using PhosphorImager (GE Amersham, UK) and quantified in ImageQuant 5.2.

Gene Expression Analyses

Total RNA was isolated from cells, stimulated either briefly or chronically with 10 ng/ml of TNF, using RNeasy kit (Qiagen, Germany). qRT-PCR was performed as described earlier (23); see **Table S1** for the description of primers. A detailed description of microarray mRNA analyses is available in the **Supplementary Materials**. The partition around medoid-based clustering analysis (49) was implemented in the Cluster package in R; the heatmap and violin plots were generated in MATLAB. For determining the significance of gene-expression differences between various genotypes within a given gene-group, we conducted multiple hypotheses testing and computed the effect sizes (**Supplementary Materials**). See **Table S2** for a description of genes belonging to different gene-groups. The enrichment of the Gene Ontology terms was determined by Fisher's exact test using the "weight algorithm" available in topGO (50) and the entire Illumina MouseRef-8 v2.0 gene-array was used as the background. As described (51), ChIP experiments were performed using MEFs treated chronically with 10 ng/ml TNF (also see **Supplementary Materials**). Anti-RelA (sc-372) and anti-RelB antibodies (sc-226) were from Santa Cruz Biotechnology. Fold enrichment of RelA- or RelB-associated genes for a given gene group was computed against a list of randomly chosen 1,000 genes as control. We used Integrated Genome Viewer (IGV) to generate the browser tracks of individual genes.

Computational Modeling

We utilized a previously published mass action kinetics-based NF-κB mathematical model (23) subsequent to necessary refinements (**Supplementary Materials**). These refinements improved the performance of the model with respect to the *Nfkbia*-deficient system, but preserved the model behavior observed earlier in WT and *Nfkb2*-deficient systems (23). The model was stimulated using Ode15s in MATLAB (2014b, Mathworks, USA). The abundances of various molecular species during early signaling was determined as the area under the respective timecourse curves between 0 and 2 h, and those during late signaling was estimated between 6 and 8 h. Variance-based, multiparametric sensitivity analysis has been described (29). Using iterative Monte Carlo sampling (1,000 simulations), we simultaneously explored a predetermined range (±10%) of parameter space around the initial values for the indicated parameter groups. The parameters belonging to a specific group were altered by the same factor for a given simulation.

See the **Supplementary Materials** and additional references (52–55) for the details of computational analyses on pathway modelling and microarray gene expression data.

Statistical Analysis

Error bars were shown as S.E.M. of 3–6 experimental replicates. Quantified data are means ± SEM, and two-tailed Student's *t*-test was used for verifying statistical significance unless otherwise mentioned. Statistical tests associated with global gene-expression analyses have been detailed in the **Supplementary Materials**.

ETHICS STATEMENT

WT and gene-deficient C57BL/6 mice were used in accordance with the animal usage guideline and recommendations of the Institutional Animal Ethics Committee of the National Institute of Immunology. The protocol was approved by the Institutional Animal Ethics Committee and the approval no is approval no. #258/11.

AUTHOR CONTRIBUTIONS

BC carried out *in silico* studies under the supervision of SB and JG. PR conducted cell-based analyses with the help from US, YR, and MC and the guidance from SB and RS. ChIP-seq experiments were performed by MZ and AS and analyzed by SD and BC with the guidance from RS and SB. BC and PR wrote the manuscript with SB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00997/full#supplementary-material

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REVIEW
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Cellular Specificity of NF-κB Function in the Nervous System

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Nuclear Factor Kappa B (NF-κB) is a ubiquitously expressed transcription factor with key functions in a wide array of biological systems. While the role of NF-κB in processes, such as host immunity and oncogenesis has been more clearly defined, an understanding of the basic functions of NF-κB in the nervous system has lagged behind. The vast cell-type heterogeneity within the central nervous system (CNS) and the interplay between cell-type specific roles of NF-κB contributes to the complexity of understanding NF-κB functions in the brain. In this review, we will focus on the emerging understanding of cell-autonomous regulation of NF-κB signaling as well as the non-cell-autonomous functional impacts of NF-κB activation in the mammalian nervous system. We will focus on recent work which is unlocking the pleiotropic roles of NF-κB in neurons and glial cells (including astrocytes and microglia). Normal physiology as well as disorders of the CNS in which NF-κB signaling has been implicated will be discussed with reference to the lens of cell-type specific responses.

Keywords: NF-κB, gene expression, Plasticity, Neurons, transcription, synapse, glia, central nervous system (CNS)

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BACKGROUND

Mammalian NF-κB functions as a dimer composed of five potential Rel/NF-κB family subunits that can be divided into two classes. Rel A (p65), c-Rel, and Rel B are synthesized as mature proteins which contain transcription transactivation domains (TADs). The remaining subunits, p50 and p52, are post-translationally cleaved from the precursor proteins p105 (NFKB1) and p100 (NFKB2), respectively, and lack TADs (1). NF-κB subunits can hetero- or homo-dimerize; while all subunits have been reported to be expressed in different brain cell types, the p50:p65 dimer appears widely abundant to date. In unstimulated conditions, NF-κB dimers are held latent mainly in the cytoplasm by binding to a set of proteins from the "inhibitor of kB" (IκB) family. Following cellular stimulation by growth factors, excitatory synaptic transmission, immune-modulatory factors, or other activators, the IκB inhibitors undergo post-translational modification and ultimately degradation through well-studied steps which have been reviewed in detail elsewhere (2). These steps may be broadly categorized into either an alternative pathway or the "cannonical pathway" (CP) in which IκB phosphorylation is mediated by the IκB kinase complex (IKK). In the CP, NF-κB activation occurs when incoming cellular stimuli connect to signaling assemblies mediating IKK activation. IKK activation leads to IκB inhibitor phosphorylation on critical serine residues, followed by ubiquitination and proteasomal degradation, which frees the NF-κB dimer to undergo stable translocation into the cellular nucleus and regulate transcription of genes containing consensus κB binding sites in the DNA of their enhancers or promoters. NF-κB-mediated transcription has also been reported to be enhanced through phosphorylation of the Rel proteins (3).

Studies in the nervous system have assessed several aspects of the pathway as proxies to monitor NF-κB activation, including accumulation of Rel proteins in the nucleus and Rel phosphorylation, as well as the classical reduction in IκB protein levels and accompanying induction of NF-κB-DNA binding activity (e.g., by electromobility shift assay, EMSA). Multiple approaches have also been used to test roles for NF-κB in molecular and behavioral studies of brain function. Mutation of the serine residues critical for IκBα phosphorylation and degradation has been used to engineer a dominant-negative "super-repressor" (DN-IκB) transgene that exerts blockade of the NF-κB activation pathway (4), and constitutively active variants of IKK (CA-IKK) have been used to produce genetically controlled NF-κB pathway activation. Viral expression constructs and mouse lines enabling selective expression of the DN-IκB and CA-IKK have also been effective tools in dissecting cell-type specific roles of NF-κB in the CNS. Mouse lines engineered to allow site-specific recombination using Cre-lox systems should become particularly useful in allowing neuroscientists to define endogenous gene function for members of the Rel family in discrete brain regions, cell types, and developmental periods. In the following sections, we will review studies which incorporate multiple approaches to define our current understanding of NF-κB function in the mammalian nervous system during both health and disease, with a particular focus on the complexity of cell-type and subunit-specific roles.

Normal Physiology

The heterogeneous cellular composition of the brain has presented a major challenge to understanding the effects of NF-κB pathway activation in single cells as well as its role in cognitive function of the brain as a whole. Comparative studies of NF-κB in the many rare cell types of the CNS remains uncharted territory. However, to appreciate the overall impacts of signaling through the NF-κB pathway on brain health and function it is nonetheless instructive to consider more general assessments of activation and function in neurons vs. glia. Effective promoters for driving cell-type selective manipulations of the NF-κB signaling pathway exist for neurons but have until very recently hampered studies of glial-specific roles, as discussed below. Partially due to this limitation, more of the existing evidence for discrete cell-type and subunit-specific roles of NF-κB activation has emerged from selective pathway manipulations in neurons.

NEURONS

There are likely hundreds of different types of neurons within the mammalian CNS, depending upon the classification method (5). Broad neuronal categories in which NF-κB function has been investigated include both excitatory (glutamatergic) and inhibitory (GABAergic) neurons, as well as the neuronal subcompartment of the synapse, the sites where connections between neurons are made. A broad range of stimuli are documented to activate neuronal NF-κB, ranging from well-known inflammatory mediators, to stimuli whose action may participate more selectively in NF-κB signaling in neurons, such as the growth factors neuronal growth factor (NGF) and Brain-derived growth factor (BDNF) and excitatory neurotransmitters (6).

In addition to selective induction pathways, there are also multiple functional readouts of NF-κB activation on both the cellular and behavioral level which appear to be specific for the neuronal population of NF-κB. Three members of the NF-κB family, p50 (7–9), c-Rel (10–12), and p65/RelA (13, 14), as well as IKK (15), have been implicated in regulating cognitive behaviors in mice, including learning and memory. However, most of the studies examining NF-κB subunit-specific effects on behavior have not been carried out in a cell-type selective manner, and have instead used mouse models ubiquitously lacking particular subunits which could have phenotype contributions from loss of NF-κB outside the nervous system, such as immunological deficits. Neuron-selective disruption of NF-κB signaling [by DN-IKK expression predominantly in either forebrain excitatory neurons (16) or GABAergic interneurons] (17) has confirmed essential roles for NF-κB in a variety of assays of synaptic plasticity as well as mammalian cognitive behavior tasks. However, such NF-κB pathway manipulation does not deliver subunit specific information. Several informative reviews on cognitive effects of NF-κB signaling have been published (18–21), and we will not dwell further on this topic here.

Activation of the NF-κB signaling pathway by excitatory neurotransmission and its participation in multiple forms of structural and synaptic plasticity is likely a basis for the function of this transcription factor in cognitive behaviors. Deficits in NF-κB signaling have been shown to produce impairments in *in vitro* assays of long-term plasticity, including long-term potentiation (LTP) (9, 10, 17, 22) and long-term depression (LTD) (12). Activation of the NF-κB pathway in murine excitatory glutamatergic neurons promotes dendritic spine and excitatory synapse formation (23), while diminished NF-κB activity (loss of RelA/p65) reduces dendritic spine size and density as well as miniature excitatory post-synaptic currents (mEPSCs), during developmental periods of synapse formation, or in mature neurons responding to increased synaptic demand (23). Collectively, these effects are consistent with a role for NF-κB in enhancing excitatory synaptic function. While these cell-autonomous effects were observed with manipulation of RelA/p65 in excitatory neurons, it is unknown if they are specific only for the RelA subunit of NF-κB. Diminishing NF-κB activity in inhibitory GABAergic neurons (through selective DN-IκB expression) has been reported to produce a distinct phenotype of diminished inhibitory tone and enhanced excitatory firing (17). NF-κB is also the first transcription factor to be implicated in the feedback mechanisms that regulate the endpoint of homeostatic synaptic plasticity to elevated excitatory activity (24). During the homeostatic response to chronic elevated excitatory activity, NF-κB activation by polo-like kinases (Plks) opposes Plk-mediated degradation of the synapse stabilizing protein, spine-associated RapGTPase-activating protein (SPAR), by transcriptionally upregulating SPAR in hippocampal excitatory neurons *in vitro* and *in vivo*. Neurons which are deficient in NF-κB (RelA/p65) fail to limit homeostatic adjustments in the context of chronic elevated neuronal excitation, producing exaggerated

homeostatic reductions in dendritic spines and excitatory synaptic currents (24).

Excitatory neurotransmitters were first demonstrated to activate NF-κB in cultured cerebellar granule neurons (25, 26) and in the developing cerebellum *in vivo*, where NF-κB activation was shown to be sensitive to antagonism of receptors for the glutamate excitatory neurotransmitter (27). The ability of excitatory glutamatergic stimuli to mediate rapid induction of NF-κB through the cannonical pathway appears to be specific to neurons, compared to glial cells. Prolonged (24 hr) exposure of cultured primary astrocytes to glutamate has been reported to generate a toxic, oxidative stress-mediated activation of NF-κB (28), and glutamate stimulation of glioma cell lines can produce secondary non-cannonical NF-κB activation through epidermal growth factor receptor (EGFR) signaling (29). In contrast, rapid activation of neuronal NF-κB downstream of excitatory stimulation occurs predominantly through the NMDA glutamate receptor subtype and L-type voltage sensitive calcium channels in a variety of neurons from distinct brain regions including the cerebellum, hippocampus, and cortex[as reviewed in (30)]. Stimulation through the glutamate metabotropic receptors can also produce neuronal NF-κB activation of p50, p65, and c-Rel subunits as reported through ELISA of area CA1 lysates from hippocampal slices (12).

The gating of NMDA receptors and L-type calcium channels generates the majority of glutamate-mediated calcium influx, which has been implicated in NF-κB activation through the cannonical pathway in neurons from multiple brain regions (13, 26, 31, 32). Consistent with a critical role for calcium elevation in mediating excitatory NF-κB activation, elevating calcium through use of a calcium ionophore was found sufficient to produce IKK activation in studies using hippocampal or striatal neurons (13, 33). Further, calcium-responsive signaling cascades, including transduction through the calcium calmodulin dependent protein kinase II (CaMKII) are critical for glutamate-mediated activation of IKK and NF-κB. CaMKII-dependent activation of the IKK complex appears not to be specific to neurons, however, as CaMKII isoforms have been linked to NF-κB activation in a variety of cells, including T cells, cardiac myocytes, and fibroblasts (34–36). The CaMKIIα isoform has been specifically linked to NF-κB activation where studied in hippocampal neurons and purified retinal ganglion cells (13, 37). CaMKIIα activation downstream of elevated calcium leads directly or indirectly to IKK activation. Activated NF-κB has been shown to undergo dynein-dependent active transport resulting in nuclear accumulation of the transcription factor (38–40).

Calcium elevations in response to excitatory glutamate stimulation are particularly high in the subcompartment of the neuronal synapse. CaMKIIα is also highly enriched in both the synapse and the synaptic region of the post-synaptic density (PSD, a specialized region attached to the post-synaptic membrane opposite presynaptic terminals), where it is well-positioned to respond to incoming calcium signals and plays prominent roles in synaptic plasticity. Like CaMKII, NF-κB, and the IKK and IκB signaling components are also located within synapses. Immunohistochemical evidence of NF-κB in neuronal processes provided a first suggestion of its presence at synapses (26, 41), that was supported by the presence of NF-κB, as p65:p50 dimers, in biochemically isolated synapses (13, 41–43) from wildtype but not p65-deficient mice (13). In excitatory (pyramidal) neurons the post-synaptic side of glutamatergic synapses are located upon small specialized protrusions from the neuronal dendrites, which are known as dendritic spines. NF-κB dimers composed of either p65:p50 or p65:p65 are selectively enriched within dendritic spines in hippocampal pyramidal neurons (23, 44), and are also found in the PSD (45). Analysis of a series of truncation and deletion mutants narrowed the region of the p65 subunit critical for enrichment within dendritic spines to a 30 amino acid section in the mid-region of p65 protein, located between the amino-terminal Rel homology domain (RHD) and the C-terminal transactivation domain (TAD) (44). A p65 minimal mutant lacking spine enrichment but retaining transcriptional activity was selectively deficient in transcriptional responses to stimuli incoming through the excitatory synapses on dendritic spines, in comparison to cellularly diffuse stimuli. The region of p65 implicated in synaptic enrichment bears little conservation across other Rel family subunit proteins and largely lacks previously characterized functional domains, indicating that these have either not yet been defined in other cell types or that the region has a unique functional significance in neurons. The region of p65 implicated in synaptic enrichment does include a Src homology 3 (SH3) poly-proline binding motif as well as an intrinsically disordered region predicted with high confidence (44). Mature dendritic spines are connected to the parent dendrite of neurons by relatively thin spine necks that can constrict cytoplasmic ionic and biochemical fluxes in response to incoming stimuli. Consequently, subcellular restriction of signaling pathways could confer value to co-compartmentalization of the NF-κB transcription factor at excitatory synapses in neurons. Evaluation of hippocampal pyramidal neurons lacking dendritic spine enrichment of NF-κB revealed that they had less mature dendritic spines and a reduced density of dendritic spines compared to wildtype hippocampal neurons (44). Interestingly, the p65 subunit of NF-κB is also reported to be enriched at the axon initial segment in cortical neurons, where it is proposed to be sequestered by binding to ankyrin G (46); it is not yet clear if these effects are selective only for the p65 subunit.

Broadly summarized, work from many labs indicates that neuronal NF-κB functions under normal physiological conditions to promote synapse growth and to enhance synaptic activity and enduring forms of plasticity. In addition to gene targets previously characterized in the immune and cancer fields, NF-κB has also been shown to regulate downstream targets with particular relevance for synaptic plasticity, including PSD-95, SPAR, PKA, nNOS, and growth factors, such as BDNF and IGF-2(18–21, 23, 24, 47–49). Functions in neuronal plasticity may underlie the requirements for NF-κB in behavioral readouts of cognition documented in many investigations, however some behavioral experiments have not utilized neuronal-specific manipulations of the NF-κB pathway so NF-κB in other cell types could participate in observed phenotypes. The enrichment of NF-κB at excitatory

synapses and its activation by excitatory synaptic activity are also key to the unique roles of the NF-κB pathway in mammalian neurons.

GLIA

Glial cells within the CNS are comprised of four basic types: astrocytes, oligodendrocytes, microglia, and ependymal cells (epithelial lining which produce cerebral spinal fluid). For the purposes of this review, we will focus primarily on astrocytes and microglia. In contrast to neurons, NF-κB in glial cells has not been reported to be activated by excitatory neurotransmitters or under basal conditions. Reporter assays of NF-κB-dependent gene expression in primary glial cultures from normal murine cortex or in cryosections showed no evident NF-κB activity under basal conditions in glial cells that were identified through glial fibrillary acidic protein (GFAP) staining (50, 51). GFAP serves to mark many astrocytes and ependymal cells, as well as some oligodendrocytes and precursor cells in the mature CNS. Due to the limited basal NF-κB activity in glia, many studies have investigated the glial functions of NF-κB in settings of inflammation, injury or disease. As in neurons, the predominant activated form of NF-κB in mature glial cells appears to contain the RelA/p65 subunit, rather than utilizing non-cannonical signaling mediated by RelB:p52 heterodimers. However, evidence for the involvement of p52 and RelB has been reported in neural stem cells from the adult mouse nervous system following lymphotoxin β receptor-mediated signaling (52). RelB and p52 are also reported to function in driving tumor progression in glioma cell lines and is correlated with invasive potential (53). In addition, roles for RelB in reactive astrocytes (54) and microglia (55, 56) have been reported in the setting of chronic inflammation, such as can occur following brain injury or infection.

Microglia cells are the specialized population of resident macrophages in the CNS responsible for immune defense. Under normal conditions, these cells comprise an estimated 10–15% of all cells within the CNS. Brain microglia are typically in a resting state, but in the context of injury or disease they can become activated and aid in clearing cellular debris or innate immunity functions. Under chronic or extreme conditions this activation can lead to an overproduction of cytotoxic factors, such as excess nitric oxide, IL-1β and TNFα (57). In this setting, activation of NF-κB signaling pathways in microglia and consequent production of inflammatory mediators can exacerbate neuronal cell death. In primary cultures from mice with reduced microglial IKK activity (by conditional loss of IKKβ exon 3 in the myeloid lineage), the production of inflammatory mediators and hippocampal neuronal cell death in response to kainic acid exposure was reduced, in comparison to wildtype cultures with an intact microglial NF-κB pathway (58). NF-κB signaling in microglia may also play a role in the healthy brain by exerting homeostatic regulation of neuronal excitability and synaptic plasticity. Mice with selective depletion of IKKβ in microglial cells (by conditional loss of IKKβ exon 3 in the myeloid lineage), are reported to exhibit reduced brain expression of the NF-κB target genes, IL-1β, IL-6, and inducible NOS, and also display behavioral defects in hippocampal-dependent associative learning (22). In the same model system, *in-vitro* assays of plasticity, including long-term potentiation and excitatory field potentials, were consistent with the microglial NF-κB pathway participating in the down-regulation of neuronal excitability (22). This is an interesting contrast to the pro-excitation cis-regulatory role supported for NF-κB within neurons. Conditional deletion of an NF-κB regulatory protein, the A20 deubiquitinase, in microglia also supports roles for microglial NF-κB signaling in both neuronal homeostasis as well as in response to injury (59). Microglial deficiency in A20 resulted in increased numbers of microglia and an increase in synaptic excitation (59). Collectively, these studies are also consistent with the previously established roles of microglia in developmental and learning-associated synapse formation within the CNS (60, 61).

Astrocyte lineages are found throughout the CNS and have long been appreciated for their function in forming the blood brain barrier as well as signaling in the support and repair of neurons. While astrocytes are the most numerous and diverse glial cells with multiple astrocyte subtypes described, the understanding of astrocyte NF-κB function currently lacks this depth and is best characterized for astrocytes as a whole. Multiple studies have demonstrated that signaling through NF-κB in astrocytes contributes to pro-inflammatory responses following injury and that inhibition of NF-κB in astrocytes can promote functional recovery. For example, expression of a DN-IκBα driven by the GFAP promoter, has been shown to reduce cytokine expression, prevent damage to neurons and nerves, and to improve recovery after spinal cord or optic nerve injury (62–64). A pro-inflammatory role of glial NF-κB is also well-documented in disease settings, several of which are discussed below. Astrocytic NF-κB has also been shown to have roles apart from promoting the expression of pro-inflammatory genes. In the healthy CNS, astrocytes play a critical role in effective termination of excitatory signals by clearing glutamate released from synapses in part through the glutamate transporter-1 (GLT-1). The dynamic induction of astrocyte GLT-1, which is dependent upon the presence of neurons and neuronal activity-dependent activation of NF-κB in astrocytes, has been shown to be largely ablated by inhibition of astrocyte NF-κB using DN-IκB expression in culture (65). Critical NF-κB regulatory sites on the GLT-1 gene were identified (65). Recently, the astrocyte NF-κB pathway has also been implicated in the central control of metabolism, including regulation of blood sugar, blood pressure, and body weight (66). Astrocytes undergo dynamic structural plasticity of their processes, which can be modulated in the hypothalamus in response to metabolic information regarding the fed or unfed state (66). Mice expressing CA-IKKβ under control of the GFAP promoter were found to have impaired astrocyte plasticity with sustained astrocyte process shortening in the hypothalamic region of the brain, a phenotype also observed with chronic overnutrition. While transient modulation of this astrocyte NF-κB signaling pathway could participate in

metabolic responses, the experimental setting of chronic CA-IKKβ expression was observed to lead to metabolic disease including glucose intolerance and obesity.

It should be noted that a general caveat to many studies that use genetic manipulations to investigate glial-specific roles of the NF-κB pathway is the lack of suitable promoters and identifying markers for glial cells. GFAP is a common marker used to identify astrocytes and to drive manipulations in glial cells, but GFAP has also been shown to be present in certain neuronal subtypes as well as in some precursor cells (67). For example, a transgenic mouse line expressing the DN-IκB under control of the GFAP promoter, displays expression in precursor cells and a deficit in adult neurogenesis (68), as well as expression in adult astroglial cells which complicates determining the origins of observed learning and memory deficits (69). GFAP-promoter driven expression of DN-IκB in cultured neural stem cells is reported to promote glial lineage differentiation at the expense of neuronal lineage differentiation (52). Recently a new astrocyte-selective marker has been identified, Aldh1I1, which is reported to show little or no detection in neuronal populations or precursors (67). In the future, fruitful investigations of NF-κB function in glia will hopefully have the opportunity to make use of increasingly selective tools for glial subtype expression, such as the Aldh1I1 promoter for astrocytes.

Disease

As in the immune system, numerous roles for NF-κB in disorders of the CNS have been documented. A chief controversy has been whether NF-κB plays primarily a protective effect on neuronal health, or whether its pro-inflammatory actions exacerbate neuronal apoptosis in settings of CNS disease. Early evidence using cell-type specific expression of the dominant negative IκB (DN-IκB) to selectively inhibit NF-κB in neurons indicated that NF-κB within neurons played an anti-apoptotic role (16). This is consistent with evidence that, in addition to regulating immune and inflammatory genes, NF-κB also regulates the expression of growth factors as well as genes that antagonize cell death. NF-κB has been shown to regulate anti-apoptotic genes including caspase inhibitors, TNF-receptor associated factors, TRAF1 and TRAF2, and the Bcl-2 family (Bcl-2, Bcl-x_L, Bfl-1) (70–75). Cis-regulation of anti-apoptotic genes in neurons can confer resistance to death-inducing signals under adverse conditions and enhance survival in response to growth factors (75–78). With some exceptions and subunit dependence [as reviewed elsewhere (79)], substantial evidence now supports an anti-apoptotic role for NF-κB within neurons while prolonged NF-κB activation in reactive glial cells, has been associated with detrimental outcomes, inflammation, and neuronal cell death. Overall In this section, we will focus on selected disease examples to highlight differential roles of NF-κB in glia vs. neurons in disorders of the CNS. **Table 1** shows functions of NF-κB in the CNS and how these functions are altered in disease states.

TABLE 1 | Summary of NF-κB roles in neurons and glia, both in normal physiology and disease.

Cell type	Overview of NF-κB functions	
	Normal physiology	**Disease**
Neuron	* Synaptic plasticity * Learning and memory * Synapse to nuclear communication * Developmental growth and survival in response to trophic cues	* Aberrant synapse to nuclear communication * Protective from death-inducing signals associated with injury or inflammatory mediators * Protective against apoptosis in neurodegenerative models
Glia	* Immune response * Injury response * Glutamate clearance * Central control of metabolism	* Chronic NF-κB activation elevates neuroinflammation and neuronal cell death * Prolonged NF-κB induction increases activated microglia * c-Rel loss increases microglial activation

The pleiotropic functions of the NF-κB signaling pathway coupled with the cellular diversity of the nervous system mean that this table reflects generalizations, while more specific details are in the text of this review.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by progressive loss of memory and other cognitive functions, changes in behavior, difficulty completing basic tasks, and confusion. An accumulation of amyloid-β (Aβ) plaques and neurofibrillary tangles in the brain, as well as neuroinflammation and vascular alterations, are hallmarks of AD. The link of plaques and neurofibrillary tangles to disease pathology remains uncertain and is an area of active investigation. Aberrant neural network activity, synaptic dysfunction and synapse loss correlate strongly to decline in cognitive function and neurodegeneration, but the molecular mechanisms are not fully understood and some have been difficult to recapitulate in mouse models of the disease (80). Genetic studies link multiple genes to AD development; including amyloid precursor protein (APP), Presenilin 1 (PSEN1), Presenilin 2 (PSEN2), apolipoprotein E (ApoE), and Triggering Receptor Expression on Myeloid cells 2 (TREM2), along with others (81). Promoter analysis and functional studies link expression of each of these genes to regulation by NF-κB (82–85). In some cases, products from AD-associated genes, such as presenilin 1, have also been shown to mediate reciprocal activation of NF-κB (RelA/p65 containing dimers) in putative pro-inflammatory cascades (86).

A prominent role for neuroinflammation associated with neurodegenerative changes has been documented, but the complexity of immune cell types in the brain has contributed to conflicting reports attributing the aberrant inflammation to either systemic immunity, brain-recruited monocytes, or brain-resident microglia. It has also been debated whether the recruitment of microglia might be beneficial (albeit insufficient) in combatting neurodegenerative processes in AD, or whether microglial activation might be a contributing factor to neurodegeneration. TREM family proteins are part of a neuroinflammatory cascade, and evidence supports NF-κB as a central player governing expression of both TREM1 and TREM2(84). Microglial activation driven by NF-κB (RelA) and cytokine signaling is reported in data from microglia

isolated from a tau transgenic mouse model (rTg4510) of AD where downstream differential expression of TREM2 and APOE was also observed (87). In microglia, TREM2 functions as a surface receptor required for responses associated with activation, including survival, proliferation, and phagocytosis. A hypomorphic variant of TREM2 is associated with elevated risk of late-onset AD in humans while TREM2 loss of function mutations as associated with dementia, and mice deficient in TREM2 develop AD-associated pathologies (88, 89). Recently, single cell sequencing approaches have allowed finer resolution of the immune cell heterogeneity in AD and have shed light on a potential protective microglial type associated with neurodegeneration. This research revealed that a TREM2-dependent program activated in a unique disease-associated microglia (DAM) type was associated with restricting the development of AD and was beneficial to mitigating the disease likely through phagocytosis (90). Findings of this study are in-line with a second recent report, from different investigators, which showed that TREM2 intracellular signaling functions to maintain the metabolic fitness and phagocytic responses of microglia operating to defend the brain in AD (91, 92). Since microglia are active participants in the formation, remodeling, and elimination of synapses, this research may also shed light on the mechanisms which underlie synapse loss in AD (93, 94). This research also raises the issue of whether NF-κB-dependent regulation of microglia TREM2 expression might also play an as-yet unexplored role in the synaptic plasticity associated with learning and memory.

The role of NF-κB in AD was recently covered more broadly in a dedicated review (6) which discussed the regulation of NF-κB by Aβ as well as giving a comprehensive overview of NF-κB targets with potential implications in AD development or cognitive symptoms, including CREB, MnSOD, CAMKII, and PSD95. NF-κB has also been linked to regulation of ApoE, of which the ApoE4 variant is the strongest genetic risk factor for development of late onset AD, while ApoE3 is neutral and ApoE2 is protective. Gene promoter analysis identified NF-κB binding sites upstream of the ApoE transcription initiation region (95) and characterization of ApoE4 transcriptional regulation through the use of luciferase reporter assays in glial cells stimulated with Aβ confirmed functional regulation of expression through NF-κB signaling (82). Further studies are needed to determine the extent to which NF-κB may regulate ApoE4 and other ApoE variants in the brain and whether these signaling pathways are impacted in AD. The dual functions of NF-κB in cognitive processes and inflammatory cascades have highlighted interest in NF-κB as a therapeutic target for early intervention in treatment of AD. Interestingly, not only genetic but also environmental risk factors for AD, as well as protective factors, such as diet, anti-inflammatory medications, and exercise, show correlative relationships to NF-κB (96). Aging, the most significant risk factor for AD, is also associated with elevated levels of brain NF-κB activation and tissue-specific inflammation with relevance to AD and other neurodegenerative processes (96). While putative cell-type specific roles for NF-κB in microglia have emerged with its function in TREM2 regulation, continued exploration will be needed to explore whether other risk factors exhibit cell-type specific roles for NF-κB in AD.

HUNTINGTON'S DISEASE

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder characterized by changes in mood and a decrease in coordination and mental abilities, and is progressive and fatal. HD is caused by mutations expanding the CAG triplet repeat region of the Huntington gene that encodes a polyglutamine tract (polyQ) in the amino-terminus of the Huntingtin protein (HTT). Normal HTT has <26 repeats, while the mutated version leading to disease can typically have >35 repeats, with a higher repeat number correlated to increased severity and an earlier disease onset. Research from several investigators suggests that the polyglutamine expansion may alter the conformation of HTT protein (97). HTT is expressed throughout the body, but the normal function of HTT and why mutated HTT is most disruptive to neurons remain incompletely understood. Normal HTT has been shown to interact with various neuronal proteins, including both the p50 and p65 subunits of NF-κB (45, 98). HTT has also been shown to enhance intracellular transport through interaction with the cytoplasmic dynein molecular motor (99, 100), and this function of HTT is disrupted by the polyQ expansion associated with HD (100, 101). Dynein and the dynactin complex move cargo toward the minus-ends of microtubules, facilitating retrograde transport in neuronal dendrites, and nuclear transport of activated NF-κB following stimulation requires dynein and the dynactin complex (38, 40). Interestingly, HTT is enriched at the post-synaptic density of neuronal synapses, along with the p50 and p65 NF-κB subunits, and has been reported to preferentially associate with activated NF-κB and to enhance the movement of p65-containing NF-κB dimers out of dendritic spines. The polyQ expansion of HTT associated with HD impairs the enrichment of HTT in the PSD and reduces the movement out of dendritic spines and nuclear accumulation of NF-κB (45). This work suggests that aberrant synapse-to-nucleus transport of NF-κB in neurons could participate in the etiology of HD.

While the majority of studies have linked NF-κB to HD specifically in neuronal cell types, mutant HTT has also been found to affect neuroinflammation, which could suggest a role in glia. Astrocytes from the caudate nucleus brain region of human patients with HD and from the cortex of a mouse model of HD exhibit increased activation of NF-κB (nuclear localization of RelA/p65) (102). Under basal conditions, the increased activation of NF-κB only occurred in astrocytes and not neurons or microglia. The increased activation of NF-κB was reported to be due to elevated astrocyte IKK activity which agreed with a previous study showing higher IKK activities in the brains of a mouse model of HD (103). Blockade of IKK alleviated neurotoxicity caused by the HD astrocytes and ameliorated symptoms of HD (102). NF-κB has also been shown to regulate HTT at the transcription level; analysis of the HTT promoter identified an NF-κB binding

site that regulates HTT transcription, as well as a SNP in this binding site which impaired NF-κB binding and lowered HTT transcription (104). Importantly, this SNP was shown to impact development of HD in an allele-specific manner; when the SNP was present on the HTT mutant allele a protective effect of delayed onset was observed in HD patients while early onset HD was associated with the presence of the SNP on the wildtype HTT allele. While this study was primarily conducted at the genomic, rather than cell-type specific, level, effects of the NF-κB binding site and SNP in the HTT promoter were validated in ST14A cells, which are derived from the striatal brain region and display features of medium spiny neurons (104). These findings highlight the importance of the NF-κB pathway in regulating HTT gene expression and progression to HD.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is caused by death of motor neurons which leads to worsening ability to move voluntary muscles and eventually leading to difficulty speaking and breathing. While most cases of ALS are sporadic, about 10% of ALS cases are inherited. Aberrant expression or mutation of multiple genes have been associated with ALS, with chromosome 9 open reading frame 72 (C9ORF72), superoxide dismutase-1 (SOD1), NIMA-related kinase 1 (NEK1), FUS RNA binding protein (FUS), and TAR DNA-binding protein (TDP-43) collectively being amongst the most common genes in the familial fraction of ALS cases. NF-κB has been shown to be involved in the regulation or interaction with several of these genes. An NF-κB binding site identified in the promoter of human SOD1 was reported to mediate increases in SOD1 levels in response to PI3K/Akt signaling (105). The p65 subunit of NF-κB has been shown to undergo protein-protein interaction with TDP-43, an association that is increased in ALS. TDP is proposed to play a co-activator role for NF-κB and inhibition of NF-κB reduces inflammation and neuron death (106). Mutations in optineurin (OPTN) have also been shown to be associated with ALS. While wildtype OPTN has been shown to negatively regulate TNFα-induced activation of NF-κB, familial ALS-associated mutations in OPTN abolish this inhibition of NF-κB activation (107).

Neuroinflammation and the activation of microglia are hallmarks of ALS. Activated NF-κB in glial cells with both inherited and sporadic forms of ALS has been demonstrated by immunohistochemistry (106). Increased microglial activation of NF-κB (by EMSA and phospho-p65 immunoblot) in spinal cord of both human patients with ALS and in the SOD1-G93A mouse model of ALS has been shown to parallel disease progression (108). In this mouse model, the death of motor neurons could be rescued through selective NF-κB inhibition (IKKβ deficiency or DN-IκB expression, using colony stimulating factor receptor (CSF-1R) promoter driver which is microglia selective within the post-natal mouse brain) in microglial cells, while NF-κB inhibition in the astrocyte glial subtype was without effect (108). Recent work suggests that NF-κB activation in astrocytes may also play a role in ALS, in part by regulating the proliferation and immune response in microglia (109), albeit using the GFAP promoter which is also active in neural precursors to drive CA-IKKβ. While astrocyte NF-κB activation and corresponding microglial proliferation was shown to be neuroprotective during the pre-symptomatic phase, astrocyte NF-κB activation in later symptomatic phases worsened disease progression by increasing pro-inflammatory microglial activation (109); it should be noted for inferences from this study that the GFAP promoter which is also active in neural precursors was used to drive CA-IKKβ. In summary, in addition to the targeted motor neurons, NF-κB activation in non-neuronal cells plays a crucial role in the pathogenesis of ALS.

PARKINSON'S DISEASE

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting the motor system and frequently eventually accompanied by dementia. Early symptoms can include tremor, muscle rigidity, slowed movement, and difficulty walking and can also include mental and emotional problems and psychosis. The causes of PD are not fully understood but genetic and environmental factors, as well as inflammatory mechanisms are associated with the disease. Symptoms are thought to arise primarily due to death of dopaminergic neurons in the midbrain substantia nigra, which has been attributed to cellular disturbances including protein aggregation, ER stress, mishandling of calcium, and mitochondrial dysfunction (110). Mutations in multiple genes have been linked to PD, including alpha-synuclein (SNCA), leucine-rich repeat kinase 2 (LRRK2), glucosylceramidase beta (GBA), Parkin, and PTEN-induced kinase 1 (PINK1); several studies have suggested NF-κB as a therapeutic target for PD arising from multiple genetic etiologies. Consistent with the pro-inflammatory state, activation of NF-κB by nuclear translocation is observed in post-mortem brains of patients diagnosed with PD and in animal models of PD (111, 112). While nuclear p65 has been observed in neurons as well as astrocytes in tissue from PD patients, whether activated NF-κB occurs primarily in neurons or glia in mouse models of PD may depend upon the model under study. Toxicity induced by 1-methyl-4-phenyl−1,2,3,6-tetrahydropyridine (MPTP) has been employed to produce dopaminergic neuron death in mice for the study of PD. In the MPTP-toxicity model, the induction of pro-inflammatory astrocytes accompanied by NF-κB activation in astrocytes is reported (111). In this context, IKK inhibition by injection of a cell-permeant nemo-binding domain (NBD) peptide reduced pro-inflammatory astrocytes as well as substantia nigra dopaminergic neuron cell death in response to MPTP (111).

Several publications have supported a role for activation of c-Rel-containing dimers in conferring protection from neurodegenerative-associated stimuli through induction of anti-apoptotic genes (113–115). Mice globally deficient in the c-Rel subunit exhibit a significant late-onset loss of dopaminergic neurons and dopaminergic synaptic terminals in the substantia nigra of aged mice (18 months) as well as a deficiency in motor activity as compared to wild type mice (114), consistent with a PD phenotype. Lewy bodies and eosinophilic inclusions containing α-synuclein are characteristic findings in brains

TABLE 2 | A reference categorizing primary literature cited in this review according to the specificity of the brain cell type in which NF-κB is investigated: neurons, microglia, or astrocytes.

References	Title	First author	Year
NEURONS			
(7)	NF-kappaB p50-deficient mice show reduced anxiety-like behaviors in tests of exploratory drive and anxiety	Kassed	2004
(8)	Lack of NF-kappaB p50 exacerbates degeneration of hippocampal neurons after chemical exposure and impairs learning	Kassed	2002
(9)	NF-kappaB p50 subunit knockout impairs late LTP and alters long term memory in the mouse hippocampus	Oikawa	2012
(10)	c-Rel, an NF-kappaB family transcription factor, is required for hippocampal long-term synaptic plasticity and memory formation	Ahn	2008
(11)	A bioinformatics analysis of memory consolidation reveals involvement of the transcription factor c-rel	Levenson	2004
(12)	Regulation of nuclear factor kappaB in the hippocampus by group I metabotropic glutamate receptors	O'Riordan	2006
(13)	NF-kappa B functions in synaptic signaling and behavior	Meffert	2003
(14)	Late-Life Environmental Enrichment Induces Acetylation Events and Nuclear Factor kappaB-Dependent Regulations in the Hippocampus of Aged Rats Showing Improved Plasticity and Learning	Neidl	2016
(15)	The IkappaB kinase regulates chromatin structure during reconsolidation of conditioned fear memories	Lubin	2007
(16)	Forebrain-specific neuronal inhibition of nuclear factor-kappaB activity leads to loss of neuroprotection	Fridmacher	2003
(17)	NF-kappaB/Rel regulates inhibitory and excitatory neuronal function and synaptic plasticity	O'Mahony	2006
(18)	NF-kappaB transcription factor role in consolidation and reconsolidation of persistent memories	de la Fuente	2015
(22)	Differential contributions of microglial and neuronal IKKbeta to synaptic plasticity and associative learning in alert behaving mice	Kyrargyri	2015
(23)	A requirement for nuclear factor-kappaB in developmental and plasticity-associated synaptogenesis	Boersma	2011
(24)	Opposing action of nuclear factor kappaB and Polo-like kinases determines a homeostatic end point for excitatory synaptic adaptation	Mihalas	2013
(25)	Stimulation of ionotropic glutamate receptors activates transcription factor NF-kappa B in primary neurons	Kaltschmidt	1995
(26)	Synaptic activation of NF-kappa B by glutamate in cerebellar granule neurons *in vitro*	Guerrini	1995
(27)	Glutamate-dependent activation of NF-kappaB during mouse cerebellum development	Guerrini	1997
(28)	Glutamate promotes NF-kappaB pathway in primary astrocytes: protective effects of IRFI 016, a synthetic vitamin E analog	Caccamo	2005
(29)	Essential role for epidermal growth factor receptor in glutamate receptor signaling to NF-kappaB	Sitcheran	2008
(31)	From calcium to NF-kappa B signaling pathways in neurons	Lilienbaum	2003
(33)	Kainate receptors activate NF-kappaB via MAP kinase in striatal neurones	Cruise	2000
(37)	Glutamate-induced NFkappaB activation in the retina	Fan	2009
(38)	Stimulated nuclear translocation of NF-kappaB and shuttling differentially depend on dynein and the dynactin complex	Shrum	2009
(39)	Single-particle tracking uncovers dynamics of glutamate-induced retrograde transport of NF-kappaB p65 in living neurons	Widera	2016
(40)	Transcription factor NF-kappaB is transported to the nucleus via cytoplasmic dynein/dynactin motor complex in hippocampal neurons	Mikenberg	2007
(41)	Brain synapses contain inducible forms of the transcription factor NF- kappa B	Kaltschmidt	1993
(42)	Gene expression of the transcription factor NF-kappa B in hippocampus: regulation by synaptic activity	Meberg	1996
(43)	Hippocampal dynamics of synaptic NF-kappa B during inhibitory avoidance long-term memory consolidation in mice	Salles	2015
(44)	Targeting of NF-kappaB to Dendritic Spines Is Required for Synaptic Signaling and Spine Development	Dresselhaus	2018
(45)	The Huntington's disease mutation impairs Huntingtin's role in the transport of NF-kappaB from the synapse to the nucleus	Marcora	2010
(46)	NF-kappaB regulates neuronal ankyrin-G via a negative feedback loop	Konig	2017
(47)	IkappaB kinase/nuclear factor kappaB-dependent insulin-like growth factor 2 (Igf2) expression regulates synapse formation and spine maturation via Igf2 receptor signaling	Schmeisser	2012
(48)	NF-kappaB regulates spatial memory formation and synaptic plasticity through protein kinase A/CREB signaling	Kaltschmidt	2006
(49)	Role of p300 in regulating neuronal nitric oxide synthase gene expression through nuclear factor-kappaB-mediated way in neuronal cells	Li	2013
(50)	Constitutive nuclear factor-kappa B activity is required for central neuron survival	Bhakar	2002
(51)	NF-kappaB activity in transgenic mice: developmental regulation and tissue specificity	Schmidt-Ullrich	1996
(52)	Lymphotoxin beta receptor-mediated NFkappaB signaling promotes glial lineage differentiation and inhibits neuronal lineage differentiation in mouse brain neural stem/progenitor cells	Xiao	2018
(76)	NFkappaB activation is required for the neuroprotective effects of pigment epithelium-derived factor (PEDF) on cerebellar granule neurons	Yabe	2001
(77)	The canonical nuclear factor-kappaB pathway regulates cell survival in a developmental model of spinal cord motoneurons	Mincheva	2011

(Continued)

TABLE 2 | Continued

References	Title	First author	Year
(98)	The predominantly HEAT-like motif structure of huntingtin and its association and coincident nuclear entry with dorsal, an NF-kB/Rel/dorsal family transcription factor	Takano	2002
(104)	A SNP in the HTT promoter alters NF-kappaB binding and is a bidirectional genetic modifier of Huntington disease	Becanovic	2015
(105)	Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappaB	Rojo	2004
(106)	Deregulation of TDP-43 in amyotrophic lateral sclerosis triggers nuclear factor kappaB-mediated pathogenic pathways	Swarup	2011
(112)	Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease	Hunot	1997
(113)	NF-kappaB factor c-Rel mediates neuroprotection elicited by mGlu5 receptor agonists against amyloid beta-peptide toxicity	Pizzi	2005
(114)	Late-onset Parkinsonism in NFkappaB/c-Rel-deficient mice	Baiguera	2012
(119)	Parkin mediates neuroprotection through activation of IkappaB kinase/nuclear factor-kappaB signaling	Henn	2007
(120)	Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling	Sha	2010
(122)	TRIM9-Mediated Resolution of Neuroinflammation Confers Neuroprotection upon Ischemic Stroke in Mice	Zeng	2019
MICROGLIA			
(22)	Differential contributions of microglial and neuronal IKKbeta to synaptic plasticity and associative learning in alert behaving mice	Kyrargyri	2015
(55)	Regulation of inflammatory responses by neuregulin-1 in brain ischemia and microglial cells *in vitro* involves the NF-kappa B pathway	Simmons	2016
(56)	Nuclear factor-kappa B family member RelB inhibits human immunodeficiency virus-1 Tat-induced tumor necrosis factor-alpha production	Kiebala	2010
(58)	Role of microglial IKKbeta in kainic acid-induced hippocampal neuronal cell death	Cho	2008
(59)	A20 critically controls microglia activation and inhibits inflammasome-dependent neuroinflammation	Voet	2018
(60)	Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor	Parkhurst	2013
(61)	Microglia contact induces synapse formation in developing somatosensory cortex	Miyamoto	2016
(84)	Divergent Neuroinflammatory Regulation of Microglial TREM Expression and Involvement of NF-kappaB	Owens	2017
(87)	Genome-wide RNAseq study of the molecular mechanisms underlying microglia activation in response to pathological tau perturbation in the rTg4510 tau transgenic animal model	Wang	2018
(106)	Deregulation of TDP-43 in amyotrophic lateral sclerosis triggers nuclear factor kappaB-mediated pathogenic pathways	Swarup	2011
(108)	Microglia induce motor neuron death via the classical NF-kappaB pathway in amyotrophic lateral sclerosis	Frakes	2014
(109)	NF-kappaB activation in astrocytes drives a stage-specific beneficial neuroimmunological response in ALS	Ouali Alami	2018
ASTROCYTES			
(52)	Lymphotoxin beta receptor-mediated NFkappaB signaling promotes glial lineage differentiation and inhibits neuronal lineage differentiation in mouse brain neural stem/progenitor cells	Xiao	2018
(54)	RelB controls adaptive responses of astrocytes during sterile inflammation	Gupta	2019
(62)	Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury	Brambilla	2005
(63)	Transgenic inhibition of astroglial NF-kappaB protects from optic nerve damage and retinal ganglion cell loss in experimental optic neuritis	Brambilla	2012
(64)	Transgenic inhibition of astroglial NF-kappa B leads to increased axonal sparing and sprouting following spinal cord injury	Brambilla	2009
(65)	Nuclear factor-kappaB contributes to neuron-dependent induction of glutamate transporter-1 expression in astrocytes	Ghosh	2011
(66)	Astrocytic Process Plasticity and IKKbeta/NF-kappaB in Central Control of Blood Glucose, Blood Pressure, and Body Weight	Zhang	2017
(69)	Astroglial nuclear factor-kappaB regulates learning and memory and synaptic plasticity in female mice	Bracchi-Ricard	2008
(82)	NF-(kappa)B mediates amyloid beta peptide-stimulated activity of the human apolipoprotein E gene promoter in human astroglial cells	Du	2005
(102)	A critical role of astrocyte-mediated nuclear factor-kappaB-dependent inflammation in Huntington's disease	Hsiao	2013
(103)	Activation of the IkappaB kinase complex and nuclear factor-kappaB contributes to mutant huntingtin neurotoxicity	Khoshnan	2004
(106)	Deregulation of TDP-43 in amyotrophic lateral sclerosis triggers nuclear factor kappaB-mediated pathogenic pathways	Swarup	2011
(109)	NF-kappaB activation in astrocytes drives a stage-specific beneficial neuroimmunological response in ALS	Ouali Alami	2018
(111)	Selective inhibition of NF-kappaB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease	Ghosh	2007

Only references implicating or investigating specific cell types are included. Full reference information can be found in the bibliography of this article.

from individuals diagnosed with PD. Aged c-Rel$^{-/-}$ mice were observed to have elevated α-synuclein-positive inclusions that were selectively located within the dopaminergic neuron population, as opposed to either other neuronal types or glia. While no change in activated astrocytes was observed, an increase in numbers of activated microglia were observed by immunostaining in the brains of aged c-Rel-deficient compared to wildtype mice (114).

NF-κB has also been linked with proteins known to be involved in PD. Epistasis studies examining signaling of PD-associated genes in drosophila first suggested that PINK1 may act upstream of Parkin in a common pathway (116–118). Studies of the molecular mechanisms of neurodegeneration triggered by autosomal recessive mutations of either PINK1 or Parkin have demonstrated a role for NF-κB (119, 120). The expression of wildtype Parkin in a neuroblastoma cell line was shown to activate NF-κB through IKK, using luciferase reporter and gel shift assays, while expression of Parkin harboring pathogenic mutations associated with PD exhibited reduced capacity to activate NF-κB (119). Loss of Parkin function in either the neuroblastoma cell line, or in fibroblasts from PD patients with Parkin mutations, inhibited activation of the NF-κB pathway in response to cellular stressors (119). Mechanistically, parkin is reported to possess E3 ligase activity with Parkin activation mediating K63-linked polyubiquitination of IKKy and consequent NF-κB activation and protection against apoptosis (120). In addition, a brain-specific tripartite motif protein (TRIM9) with lowered levels in post-mortem brains of PD patients (121), was recently reported to function as an inhibitor of NF-κB activation by blocking IκBα degradation to restrict neuroinflammation (122). As TRIM9 manipulations have not been made in a cell-type selective manner, it is not yet clear whether the relevant TRIM9 functions in resolving NF-κB activation are occurring in neurons or glia (122). Collectively, research to date supports a role for deficiencies of NF-κB activation in reducing neuroprotection and neuron survival in association with PD. However, while NF-κB activation in neurons is clearly shown to be a protective feature disrupted by PD in multiple studies, consensus is lacking regarding the relative importance of NF-κB signaling in neurons compared to glial cells for pathology of PD.

CONCLUSION

Dysregulation of NF-κB activation and NF-κB-dependent gene expression have been implicated in a host of brain disorders. In this review, we have selected several as illustrative examples in considering cell and NF-κB subunit-type specific roles for brain NF-κB. In conclusion, NF-κB is widely expressed in all cell types in the brain but its activation or deficiency in different cell types reveals different functions and consequences both on cell-autonomous and non-cell autonomous levels (**Table 2** summarizes cell-type specific studies from this review). NF-κB functions in neurons under basal conditions to maintain neuronal health, synapse growth and plasticity-related functions, and under disease conditions, upregulation in neurons is associated with neuroprotective outcomes. While NF-κB is reported to have little basal activity in glia, under certain conditions NF-κB-dependent gene expression in glial subtypes can have beneficial outcomes in maintaining brain health through immune response and neuronal maintenance (such as in DAM), but chronic or excessive glial activation of NF-κB has been shown to be neurotoxic. The NF-κB subunits, RelA/p65, p50, and c-Rel have well-documented importance in healthy physiological responses in neurons and behavioral assays of cognition. The specific NF-κB subunits of functional importance in glial cells are for the most part less well-defined than in neurons, but RelA/p65 has been implicated in expression of inflammatory mediators in microglia. To date, roles for RelB and p52 reported in the literature appear mostly circumscribed to instances of pluripotent neural stem cells, transformed cells, such as glioma/glioblastoma, and several reports of expression in activated microglia. While initial characterization suggested that RelB expression was largely restricted to hematopoietic cells, examination of the nervous system was not specifically reported (123). However, the updated Human Protein Atlas (www.proteinatlas.org, based on commercially available antibodies) reports medium to high levels of RelB protein in human brain neurons in both the cortex and hippocampus. It remains unclear whether this potential discrepancy reflects as yet unknown functions for RelB and p52 in the healthy nervous system, or a difference between mouse and human nervous system expression.

Throughout this review, studies discussed as indicating cell-type specific roles for NF-κB are carried out generally by gene or pathway manipulation of NF-κB in a cell-type selective manner. In the setting of isolated cultured cell types, this type of manipulation can give rise to knowledge of cell-autonomous effects, such as RelA/p65-regulated growth of dendritic spines in hippocampal neurons (23). However, it should be noted that the outcomes (e.g., cognitive, behavioral, or neurodegenerative) observed from cell-type selective manipulations of NF-κB pathways can rarely be assigned as wholly cell-autonomous when assayed in the intact brain tissue or nervous system. Generating data to make this type of assignment would require extensive comparative NF-κB pathway manipulations in diverse cell-types, which has not been conducted in the nervous system to our knowledge. Given the current understanding of the complex interplay between the different cellular constituents, both in healthy cognitive processes and in disease, it is perhaps unlikely that a cell-type selective manipulation is capable of generating a purely cell-autonomous response in the intact nervous system. Nonetheless, cell-type selective initial manipulations of NF-κB can drive distinct outcomes in the intact nervous system and the current dearth of studies using spatially or temporally selective manipulations to target NF-κB subunits or NF-κB activation pathways represents a significant barrier to our understanding of NF-κB function in the nervous system. Elaborating our knowledge regarding specificity of NF-κB function in the CNS is an investment that can yield insights to the pleiotropic functions of NF-κB in healthy cognitive function and disease conditions.

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An NFκB Activity Calculator to Delineate Signaling Crosstalk: Type I and II Interferons Enhance NFκB via Distinct Mechanisms

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Nuclear factor kappa B (NFκB) is a transcription factor that controls inflammation and cell survival. In clinical histology, elevated NFκB activity is a hallmark of poor prognosis in inflammatory disease and cancer, and may be the result of a combination of diverse micro-environmental constituents. While previous quantitative studies of NFκB focused on its signaling dynamics in single cells, we address here how multiple stimuli may combine to control tissue level NFκB activity. We present a novel, simplified model of NFκB (SiMoN) that functions as an NFκB activity calculator. We demonstrate its utility by exploring how type I and type II interferons modulate NFκB activity in macrophages. Whereas, type I IFNs potentiate NFκB activity by inhibiting translation of IκBα and by elevating viral RNA sensor (RIG-I) expression, type II IFN amplifies NFκB activity by increasing the degradation of free IκB through transcriptional induction of proteasomal cap components (PA28). Both cross-regulatory mechanisms amplify NFκB activation in response to weaker (viral) inducers, while responses to stronger (bacterial or cytokine) inducers remain largely unaffected. Our work demonstrates how the NFκB calculator can reveal distinct mechanisms of crosstalk on NFκB activity in interferon-containing microenvironments.

Keywords: mathematical model, signaling crosstalk, interferon, NFκB, systems biology, translational inhibition, immunoproteasome, anti-viral response

INTRODUCTION

NFκB is the primary transcriptional regulator of inflammation (1), controlling the expression of inflammatory cytokines and chemokines that activate and coordinate both local and systemic immune responses, as well as tissue remodeling factors that facilitate immune cell invasion and tissue repair (2). Furthermore, NFκB controls cell survival genes and its activity is associated with chemoresistance in cancer cells (3). As a result, high NFκB activity in chronic disease is often associated with poor prognosis (4). Indeed, clinical histological screening to inform treatment strategies often involves assessment of NFκB expression or activity (5, 6).

The molecular mechanisms by which the primary NFκB protein RelA is activated in response to inflammatory cytokines or pathogen exposure have been elucidated. Inflammatory stimuli induce

phosphorylation by the IκB kinase (IKK) complex of IκBs, triggering their ubiquitin-dependent proteasomal degradation, and thus freeing NFκB to translocate to the nucleus to bind to DNA κB elements and induce transcription of target genes. NFκB target genes include several IκBs, which upon induction provide negative feedback on the system thus regulating the dynamics of NFκB activity (7–9).

Mathematical kinetic models of the IκB-NFκB signaling module have contributed to our understanding of the complex and often oscillatory dynamics of NFκB activity observed in single cells stimulated with a defined inflammatory agonist (10). However, due to cellular heterogeneity such oscillatory responses are rarely observed at the cell population level (11, 12). In primary or tissues cells such dynamic heterogeneity is likely to be even greater given their differential steady states (13). Indeed, in various clinical settings, overall NFκB activity in cell populations (average nuclear localization across many cells) examined in tissues has prognostic value. While recent studies have distributed the state of single-cell simulations to estimate cell population behavior (13, 14) such approaches are computationally challenging due to the need to numerically solve a large system of equations for each cell in the simulation. This may preclude comprehensive parameter scanning, preventing full characterization of possible responses. Only small models can be analytically solved to obtain concentrations without the need for relatively slow computational numerical solvers. In addition, due to the number of molecular species in larger models that cannot be experimentally measured, the iterative interpretation of experimental results with computational simulation can be challenging. For a given experimental observation, multiple reaction rates can often be perturbed to explain the result leading to challenges in targeting the next experiment. This calls for a simplified modeling framework that coarse-grains the known regulatory mechanisms when the data of interest do not demand detailed models. Simplified models of NFκB have previously been constructed and shown to be useful in elucidating the regulatory principles underlying its oscillatory control of single cells (15–18). However, no models have been reported that focus on the regulatory principles governing the quantitative average NFκB activity of many cells i.e., models that recapitulate the tissue scale NFκB activity. Though models representing the aggregate behavior of multiple cells or entire organs, i.e., tissue-scale models, are further abstractions of the regulatory mechanisms than models that recapitulate the intra-cellular regulatory dynamic, they have proven useful to investigate the dose-response and time-evolution of diverse biological phenomena, such as hormone control and the interplay between organ function, drug metabolism, and the responses to drugs (19–21).

One diverse cytokine family that defines tissue microenvironments are the interferons (22); the most prominent family members, IFNβ and IFNγ, exemplify type I and type II interferons, respectively. Interferons are typically coordinately activated with NFκB in sites of infection and play roles in inflammatory disease even if their primary physiological function is anti-viral gene expression. Indeed, both clinical and experimental studies point to crosstalk by interferons on NFκB-driven inflammatory signaling (23–27). For example, inflammatory symptoms and cytokine secretion during an infection with *streptococcus pneumoniae* are exacerbated by infection with influenza. Similar clinical symptoms during leishmaniasis are observed when the parasites harbor the Leishmania RNA Virus (LRV) (28, 29).

Laboratory studies have proposed two broad classes of cross-regulatory mechanisms: one mediated by chromatin, altering how induced NFκB controls gene expression, and the other mediated by the signaling networks, affecting the level of NFκB activity. In line with the former, IFN-mediated RNA pol II recruitment or IFN-mediated chromatin remodeling of NFκB-inducible genes have been identified as mechanisms potentiating inflammatory gene expression (30–34). In regards to the latter, IFNs have been reported to affect NFκB activity by altering signal transduction between TLRs and NFκB via expression of receptors, co-receptors and adapter proteins (35–41), or by altering translation control through phosphorylating eukaryotic initiation factors (eIF)2α and eIF4E, which may also diminish translation of IκBα (40, 42–45). However, these mechanisms must allow for a level of stimulus-specificity, as TLR4-mediated NFκB activation was, for example, found to be unaffected by IFNγ (34).

Here we construct a simple model of NFκB control, termed SiMoN, to capture the activity of populations of cells and employ it in an iterative and quantitative systems biology study to investigate how signaling crosstalk by micro-environmental type I and II IFNs influences NFκB signaling. We identify distinct, IFN type-specific mechanisms that amplify NFκB activation in a stimulus-specific manner.

RESULTS

A Simplified Model of NFκB Activity for Studying Cross-Regulation

Previously published mathematical models accurately recapitulate transient NFκB activities and oscillations caused by stimuli such as TNF or LPS (11, 12, 46–48) in fibroblasts and a macrophage cell line (49); these studies focused on a single enzymatic reaction that controls NFκB-activation: the IKK-mediated degradation of NFκB-bound IκB. To investigate the tissue scale control of NFκB and assist our intuitive understanding, a new mathematical model was constructed. To develop this simple quantitative tool we carefully considered the enzymatic reactions that control NFκB activity. Conceptualizing an abstracted model, we find that the amount of NFκB that is capable of binding DNA in the nucleus is determined by the abundance of the inhibitory IκB proteins, which in turn is a function of the biochemical reactions governing IκB synthesis and degradation (50). NFκB-bound IκBα is degraded through an IKK-mediated pathway, but free IκBα, that is IκBα not bound to NFκB, has a short half-life (51) determined by an IKK- and ubiquitination-independent pathway (**Figure 1A**). Thus, in principle, IKK-mediated NFκB activity (reaction K, **Figure 1A**)

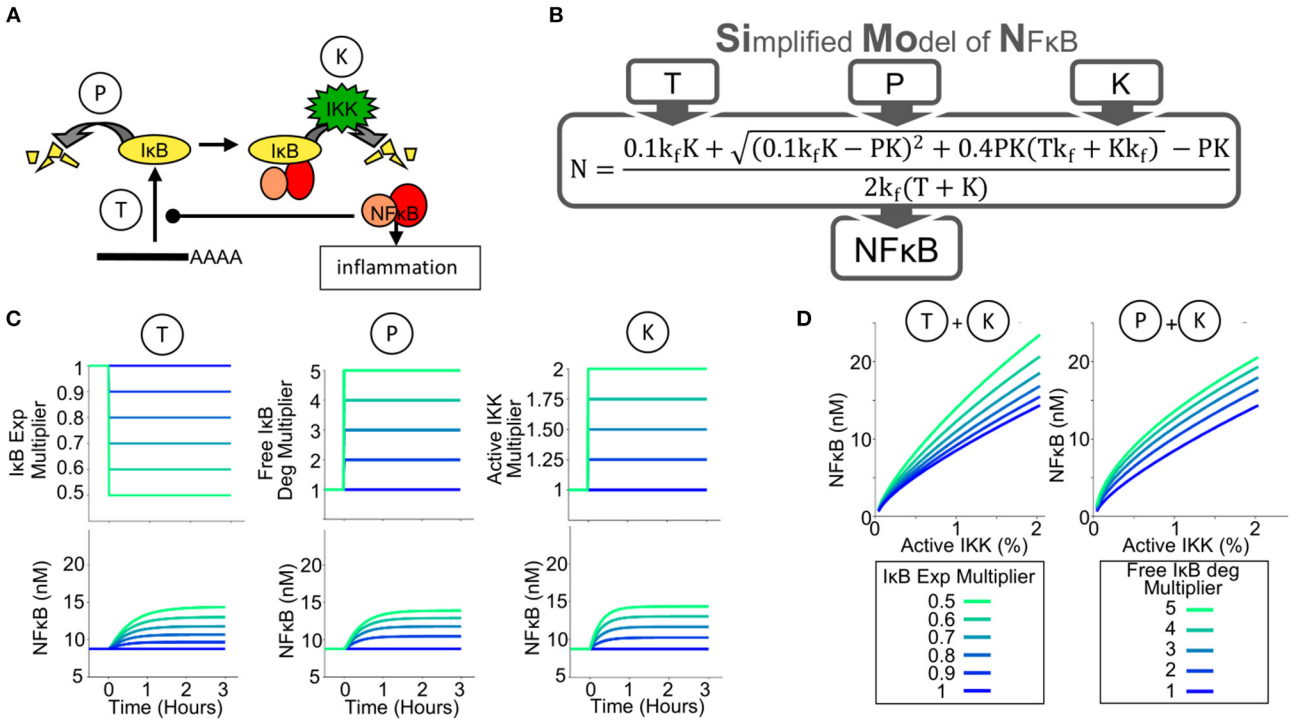

FIGURE 1 | A Simplified Model of NFκB Activity (SiMoN) can predict NFκB activity from 3 parameters. **(A)** Schematic of the key reactions controlling NFκB activity through IκB metabolism. The amount of free, transcriptionally active, NFκB (NFκB activity) is tightly controlled by the amount of IκB; therefore IκB synthesis (reaction T) and free IκB degradation (reaction P) may potentially offer alternative points of control. The primary, canonical activation pathway is through IKK (reaction K), however, interferons do not directly activate IKK. **(B)** Schematic of the Simplified Model of NFκB (SiMoN), which analytically calculates NFκB as a result of parameters T,P and K. **(C)** Modeled time-course concentrations of free NFκB (lower), in response to perturbed reaction rates obtained by multiplying the WT parameter value by the multiplier indicated (upper) utilizing the simplified model. **(D)** Steady-state free NFκB concentrations in response to: increased IKK activity and IκB translation inhibition (left) and increased IKK activity and free IκB degradation (right).

may be enhanced by reductions in IκB protein synthesis (reaction T, **Figure 1A**) or in the free IκB half-life (reaction P, **Figure 1A**).

The schema of the Simplified Model of NFκB (SiMoN) is given in **Supplementary Figure 1** in Systems Biology Graphical Notation (52) and consists of three ordinary differential equations (ODEs) representing the rate of change of free (active) NFκB, free IκB, and the NFκB-IκB complex. The concentration of each constituent is a function of IκB synthesis, free IκB degradation (an IKK-independent process) and degradation of IκB from the IκB-NFκB complex (an IKK-dependent process) (**Figure 1B**, parameters T, P, and K, respectively). SiMoN approximates the average of multiple single cell simulations of TLR NFκB responses (**Supplementary Figure 2**). Although this model lacks the complexity of other NFκB signaling models that describe the highly dynamic and variable NFκB responses at single cell resolution (10), it provides for a simplified, intuitive understanding of the reactions that may be perturbed by signaling crosstalk and carry physiological relevance within populations of cells. In addition to these benefits of interpretation, SiMoN provides analytical benefits over single-cell models. Indeed, by assuming that the network reaches a steady-state quickly when reaction rates change (the quasi-steady-state assumption), SiMoN can avoid the need for simulation with numerical differential equation solvers. An analytical solution for the quasi-steady-state concentration of NFκB as a function of the kinase activity of IKK (K), free IκB protein degradation (P), and IκB synthesis via translation (T) was found (**Figure 1B**). NFκB activity can thus be directly calculated when the values of these parameters are known, and experimentally-measured changes in these parameters can be directly interpreted.

We used SiMoN to examine how NFκB activity is a function, not only of IKK activity, but also of translation inhibition and IKK-independent free IκB degradation. Steady-state concentrations of free NFκB were calculated to be increased by either increasing active IKK, inhibiting IκB translation or increasing free IκB degradation (**Figure 1C**). Dose response analyses suggest that both inhibition of IκB synthesis and free IκB degradation substantially amplify the response of free NFκB to increasing IKK activity (**Figure 1D**). This means that environmental conditions that do not activate IKK or alter its activity may nevertheless potentiate or modulate NFκB activity. To establish whether analytically investigating NFκB with SiMoN could elucidate mechanisms of cross-regulation we turned to the biologically important scenario of interferon modulation of NFκB-driven inflammatory responses.

Type I and II IFNs Enhance NFκB Responsiveness to dsRNA

Exposure of naïve macrophages to Type I (IFNβ) or Type II (IFNγ) interferons alters their physiological functions and gene expression responses to pathogen-associated molecular patterns (PAMPs) or inflammatory cytokines [reviewed by Glass and Natoli (53); Ivashkiv and Donlin (54); Lawrence and Natoli (55)]. The underlying molecular mechanisms may involve changes to state of the chromatin or epigenome (34), or alterations to the signaling network state. We established two experimental systems to examine whether and how interferon signaling affected the control of NFκB signaling. To determine whether NFκB activity is modulated by Type I Interferon, bone marrow-derived macrophages (BMDMs) from either wild-type or type I interferon receptor-deficient (*ifnar*$^{-/-}$) mice were treated with LPS (sensed by TLR4) or the dsRNA mimetic poly(I:C) (sensed by TLR3, RIG-I, and MDA-5). *Ifnar*$^{-/-}$ macrophages do not sense the tonic or PAMP-responsive production of IFNβ that may be referred to as "IFNβ feedback" (56). Nuclear extracts analyzed by electrophoretic mobility shift assay (EMSA) revealed that in response to LPS NFκB induction was similar between the WT and *ifnar*$^{-/-}$ BMDMs (**Figure 2A**, lower panel), but in response to poly(I:C) it was similar only at the 1 h timepoint and significantly reduced at later time points in the knockout (0.2 and 0.1 vs. 1.0 and 0.6 relative DNA binding activity, **Figure 2A** upper panel).

As type II interferon is produced by T-cells and known to polarize naive macrophages to a more activated state, we addressed the role of type II interferon (IFNγ) on NFκB signaling in primary peritoneal macrophages elicited by thioglycollate (TEPMs). Cells were cultured with or without IFNγ for 24 h prior to exposure to poly(I:C) or LPS before we examined the effect of IFNγ priming on NFκB signaling by EMSA. Whereas, IFNγ did not affect LPS-induced NFκB activation, it strongly enhanced the NFκB responsiveness to poly(I:C) at 2 and 4 h (2.2 and 3.1 vs. 1 and 0.6 relative NFκB DNA binding activity, **Figure 2B**).

Recent single-cell imaging studies have revealed that NFκB nuclear localization dynamics can show diverse single-cell dynamics which can be obscured in bulk assays (49, 57, 58). To quantitatively measure the effects of type I and type II IFN pretreatment on NFκB dynamics BMDMs derived from a RelA-mVenus reporter mouse were stimulated with poly(I:C) and nuclear NFκB translocation was tracked in single cells. Plotting the nuclear NFκB trajectory for 577 cells in each condition, revealed that even in the context of cellular heterogeneity, either interferon (Type I or II) increased nuclear NFκB activation at late timepoints in response to poly(I:C) (**Figure 2C**). Indeed, the average of these single-cells trajectories confirmed this also (**Figure 2D**). Total NFκB abundance in response to poly(I:C) did not increase with either IFNβ or IFNγ co-stimulation, indicating increased nuclear NFκB was not due to increase abundance of NFκB protein (**Supplementary Figure 3**). Given that neither IFNβ nor IFNγ lead to IKK activation (as long as the preparations are endotoxin-free), these results suggest that late NFκB activity in WT macrophages responding to poly(I:C) may be enhanced by conditioning macrophages with type I or II interferon. We hypothesized that IFN-mediated regulation of IκB synthesis and/or free IκB degradation might underlie the observed cross-regulation, and we utilized SiMoN to dissect the mechanism.

Type I IFN Feedback Amplifies dsRNA-Induced NFκB Activity by Inhibiting IκBα Synthesis

Type I interferon signaling is known to result in inhibition of the translation of select mRNAs (59). To investigate whether type I interferon feedback alters IκBα translation, we measured IκBα protein synthesis in response to poly(I:C) directly in WT and *ifnar*$^{-/-}$ BMDMs. Following stimulation with poly(I:C) for 8 h, we pulsed with ^{35}S-labeled Methionine, and IκBα was immunoprecipitated to examine newly synthesized IκBα levels. Despite significantly lower concentrations of IκBα mRNA template (9.7 vs. 3.3 fold induction, 1.2 $\pm$0.6 $\log_2$ fold difference based on triplicates), the amounts of ^{35}S-Met IκBα levels were similar in WT and *ifnar*$^{-/-}$ BMDMs in response to poly(I:C) (**Figure 3A**, 3.7 vs. 3.2 fold induction, -0.1 ± 0.4 $\log_2$ fold change, based on triplicates), indicating that an IFNAR-dependent process inhibits translation during BMDM response to poly(I:C). Indeed, quantitation of the fold induction of synthesis (^{35}S-labeled IκBα) over the fold induction of the mRNA level shows that there is a 2-fold higher degree of IκBα translation in the *ifnar*$^{-/-}$ BMDMs than wild-type counterparts (**Figure 3A**, 1.1 $\pm$ 0.72 $\log_2$ fold change, based on summing the standard deviations in the quadrature). While there is substantial uncertainty in the quantitation of type I IFN-dependent translation inhibition the above-described measurements place the true value between 1 and 4-fold with 2-fold being the geometric mean.

During the early phase of the poly(I:C) timecourse, prior to any potential IFNβ feedback, NFκB activation is equivalent in wild-type and *ifnar*$^{-/-}$ macrophages. However, at later time points that may involve type I IFN feedback signaling, NFκB activation is significantly lower in *ifnar*$^{-/-}$ BMDMs (**Figure 2A**). To determine whether type I IFN-dependent translation inhibition may account for the defects in NFκB activation in *ifnar*$^{-/-}$ BMDMs, we used SiMoN to quantify the effect of translational inhibition and IKK activity on NFκB activation (**Figure 3B**). In both WT and *ifnar*$^{-/-}$ BMDMs, TLR3/TRIF signaling triggers IKK and NFκB activity during the early phase. By comparing NFκB activity using SiMoN with and without the addition of a 2-fold increase IκBα translation as identified experimentally in *ifnar*$^{-/-}$ BMDMs we found a qualitative agreement in decreased late-phase NFκB activity (**Figure 3B**). However, as the simplified model could only explain a 3-fold difference in late-phase NFκB activity, rather than the 6-fold difference observed experimentally, as such our analysis using SiMoN says that for NFκB to remain fully elevated in wild-type cells in response to poly(I:C), translation inhibition alone is not sufficient and an additional mechanism of cross regulation is required. We wondered whether IFNβ may also modulate IKK activity itself in response to poly(I:C)-induced NFκB activity.

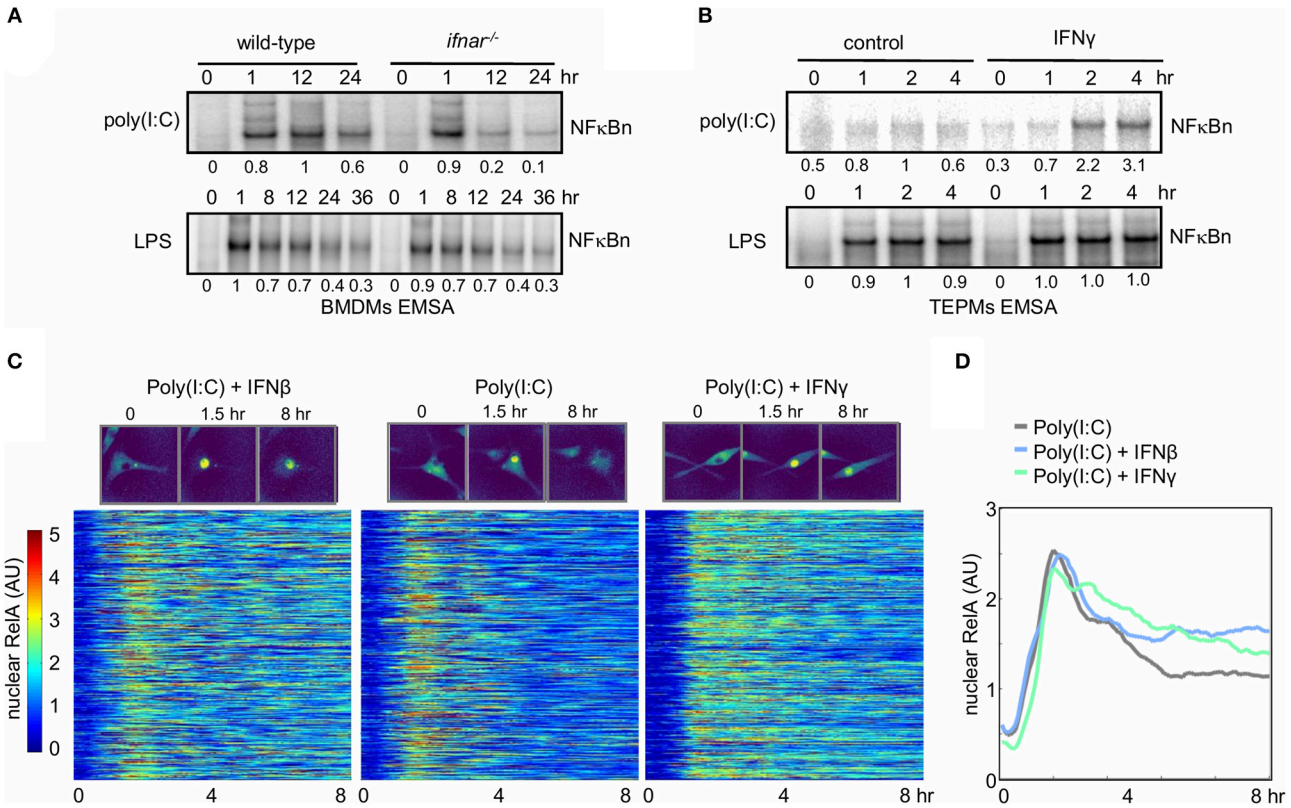

FIGURE 2 | Interferons potentiate NFκB activation in response to the viral PAMP poly(I:C). **(A)** Electrophoretic mobility shift assay (EMSA) of nuclear NFκB activity in wild-type and *ifnar*$^{-/-}$ BMDMs stimulated with LPS and poly(I:C). Quantitated activity is indicated below each band. **(B)** EMSA of nuclear NFκB activity in TEPMs cultured with or without IFNγ for 24 h prior to exposure to poly(I:C) or LPS. **(A,B)** show data representative of three biological replicates. Quantitations of phosphorimager data are relative to peak activity in controls which is set to 1. **(C)** Single-cell tracking of RelA-mVenus localization in 577 Poly(I:C) stimulated BMDMs cultured in the absence or presence (24 h) of IFNβ and IFNγ. Nuclear NFκB activity is indicated as nuclear:cytoplasmic ratio. The time-course response of each tracked cell is displayed as a row in the heatmap with brighter colors corresponding with increasing nuclear localization of NFκB. **(D)** The average nuclear NFκB activity of 577 tracked cells is shown for naïve and IFNβ- and IFNγ-primed conditions. **(C,D)** show data representative of two biological replicates.

The Type I IFN-Induction of RIG-I Enhances dsRNA-Responsive IKK Activation

To test the model-generated prediction of an additional molecular mechanism by which type I IFN regulates NFκB activity in response to poly(I:C), IKK activity was examined. In response to poly(I:C), the initial 1 h peak of IKK activity was similar between WT and IFNAR-deficient BMDMs (4.1. vs. 3.7 fold i.e., ≤10% different), yet IKK activity was lower at 8 and 12 h in *ifnar*$^{-/-}$ macrophages (2.1 vs. 1.5 fold at 8 h and 1.5 vs. 0.9 fold at 12 h, i.e., ≥30% different, **Figure 4A**). In contrast, the IKK activity profiles in response to LPS between WT and *ifnar*$^{-/-}$ BMDMs were similar (1.7 vs. 2.0 fold at 8 h and 1.9 vs. 1.9 fold at 12 h).

Whereas, type I IFN feedback is important for inhibition of IκBα synthesis, the IFN-dependent late-phase IKK activity enhances IκBα degradation in response to poly(I:C). Both LPS and poly(I:C) involve TRIF signaling to IKK and resultant induction of IFNβ; however, the fact that we only observed IFN feedback for potentiated NFκB activation in response to poly(I:C) but not LPS led us to investigate whether a TLR3/TRIF-independent mechanism for IKK activation may be boosted by type I IFN signaling. To determine whether a TLR3/TRIF-independent pathway contributes to late IFN-dependent IKK and NFκB activity, BMDMs from wild-type and *trif*$^{-/-}$ mice were treated with poly(I:C). As expected, we found that in the absence of TRIF signaling, NFκB and IRF/ISGF3 activation by poly(I:C) is severely diminished (**Supplementary Figure 4A**). However, while the early NFκB activity at 1 h was completely lost, a small amount of late 8–12 h NFκB activity was still observed in *trif*$^{-/-}$ BMDMs, pointing to a TRIF-independent mechanism to activate NFκB, one that may be boosted by type I interferon signaling.

We considered that the poly(I:C) added to the extracellular medium may be taken up by macrophages to activate intracellular cytoplasmic dsRNA receptors. The cytoplasmic dsRNA receptors MDA5 (melanoma-differentiation-associated gene 5) and RIG-I (retinoic-acid-inducible protein I) are known to activate the IRF3 pathway, as well as the IKK complex (60–62). We observed that RIG-I is inducibly expressed (> 5

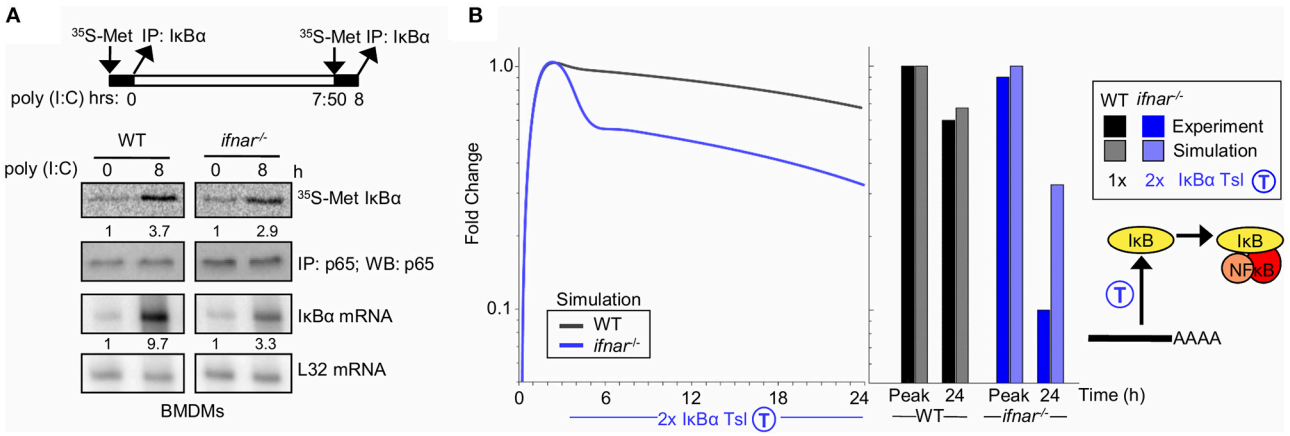

FIGURE 3 | Type I interferon signaling potentiates late NFκB activity by translational inhibition of IκBα **(A)** Experiments to determine IκBα translation rate in BMDMs. Top, schematic of the experimental design: ^{35}S-labeled Methionine pulsed at 0 h and following 8 h of Poly(I:C) stimulus. Middle, immunoprecipitates of IκBα following a ^{35}S-methionine pulse at either indicated timepoint. NFkB p65 immunoprecipitates are shown as normalization controls. Bottom, IκBα mRNA analysis using RNA protection assay. Ribosomal protein gene L32 is provided as a control. These data are representative of three biological replicates. Quantitations are relative to basal conditions which is set to 1. **(B)** Using SiMoN to determine whether the measured changes in the translation rate are sufficient to account for the NFkB activation defect in *ifnar*$^{-/-}$ BMDMs. Left, timecourse simulation of NFκB activity in response to IKK activation following poly(I:C) stimulation with and without a 2-fold increase in IκBα translation measured in *ifnar*$^{-/-}$ BMDMs **(A)**. Right, bar graph of NFκB activity at the peak and 24 h time point as quantified from the simulation and experiment (**Figure 2A**). This indicates that the increase in translation rate measured in **(A)** is not sufficient to account for the decrease in NFkB activity observed in **Figure 2A**.

fold) after 8 h of poly(I:C) or LPS treatment in an IFNAR-dependent manner (**Figure 4B**). In *trif*$^{-/-}$ BMDMs, which are deficient in autocrine IFNβ signaling, co-treatment with IFNβ was required to up-regulate RIG-I expression (6.2 vs. 1.3 fold). In addition, quantifying recent results from Cheng et al. (63) revealed transcriptional upregulation of RIG-I mRNA (Ddx58) in response to IFNβ conditioning (**Supplementary Figure 4B**). Thus, we hypothesized that complementing *trif*$^{-/-}$ BMDMs with exogenous IFNβ would enhance NFκB activation by poly(I:C). Indeed, IFNβ co-stimulation of *trif*$^{-/-}$ BMDMs enhanced induction of NFκB activity in response to poly(I:C) (8.8 vs. 3.2) but not LPS (**Figure 4C**). Furthermore, poly(I:C)-induced, TRIF-independent IKK activity was enhanced by co-treatment with IFNβ (**Figure 4D**). Together, these results suggest a model in which type I interferon amplifies poly(I:C)-induced NFκB activation through the expression of the intracellular dsRNA sensor RIG-I or MDA5 (64), which activates the canonical NFκB pathways through IKK.

To test whether poly(I:C) responsive NFκB activation is enhanced by RIG-I in this manner, we examined if IKK and NFκB activation in BMDMs is dependent on the RIG-I/MDA5 signaling adaptor IPS-1 (also known as mitochondrial anti-viral signaling protein, MAVS), which signals to IKK and IRF3 (64). Similar to what we observed in the *ifnar*$^{-/-}$ BMDMs, IKK activation by poly(I:C) in *ips1*$^{-/-}$ BMDMs is dampened at late time points (**Figure 4E**), suggesting that late poly(I:C) IKK activation is mediated by RIG-I/MDA5. Furthermore, unlike our results from *trif*$^{-/-}$ BMDMs (**Figure 4D**), IKK activation cannot be enhanced by co-treatment with IFNβ in the *ips1*$^{-/-}$ macrophages (**Figure 4E**). Indeed, poly(I:C)-induced NFκB activation in *ips1*$^{-/-}$ BMDMs was lower at 12 h than in wild-type counterparts (**Figure 4F**) (0.4 vs. 0.9), though not as low as observed in *ifnar*$^{-/-}$ BMDMs (**Figure 2A**) (0.2 vs. 1).

Our studies revealed two mechanisms by which type I interferon signaling may modulate NFκB activation (**Supplementary Figure 4C**). We first showed that interferon signaling inhibits translation of IκBα mRNAs (**Figure 3A**); we then, upon calculating with SiMoN that this alone was not sufficient (**Figure 3B**), found that type I interferon induces expression of the cytoplasmic receptor RIG-I which signals to canonical IKK (**Figure 4B**). Inclusion of both translation inhibition (quantified in **Figure 3**) and interferon-dependent IKK activity (quantified in **Figure 4A**) into calculations of NFκB activity with SiMoN fully explained the reduced late-phase NFκB activity in *ifnar*$^{-/-}$ cells (**Figure 4G**), and delineates how these two mechanisms combine to potentiate NFκB activation by poly(I:C) (**Figure 4H**). Examining the two mechanisms individually, we find that translational inhibition only partially accounts for the increase in NFκB activation and that the experimentally measured reduction in late-phase NFκB activity in *ifnar*$^{-/-}$ can only be explained when the measured translation inhibition is combined with a reduction in IKK activity (**Figure 4H**).

Interestingly, both mechanisms of crosstalk between type I interferon and NFκB signaling are specific for dsRNA, rather than LPS-triggered NFκB activation, albeit for different reasons (**Supplementary Figure 4C**). The RIG-I/MDA5-mediated cross-regulation mechanism is specific because these receptors sense dsRNA and not LPS. In contrast, the fact that the translational inhibition mechanism shows specificity for dsRNA-triggered NFκB activation may be explained by a kinetic argument: translational inhibition has a diminished effect on NFκB

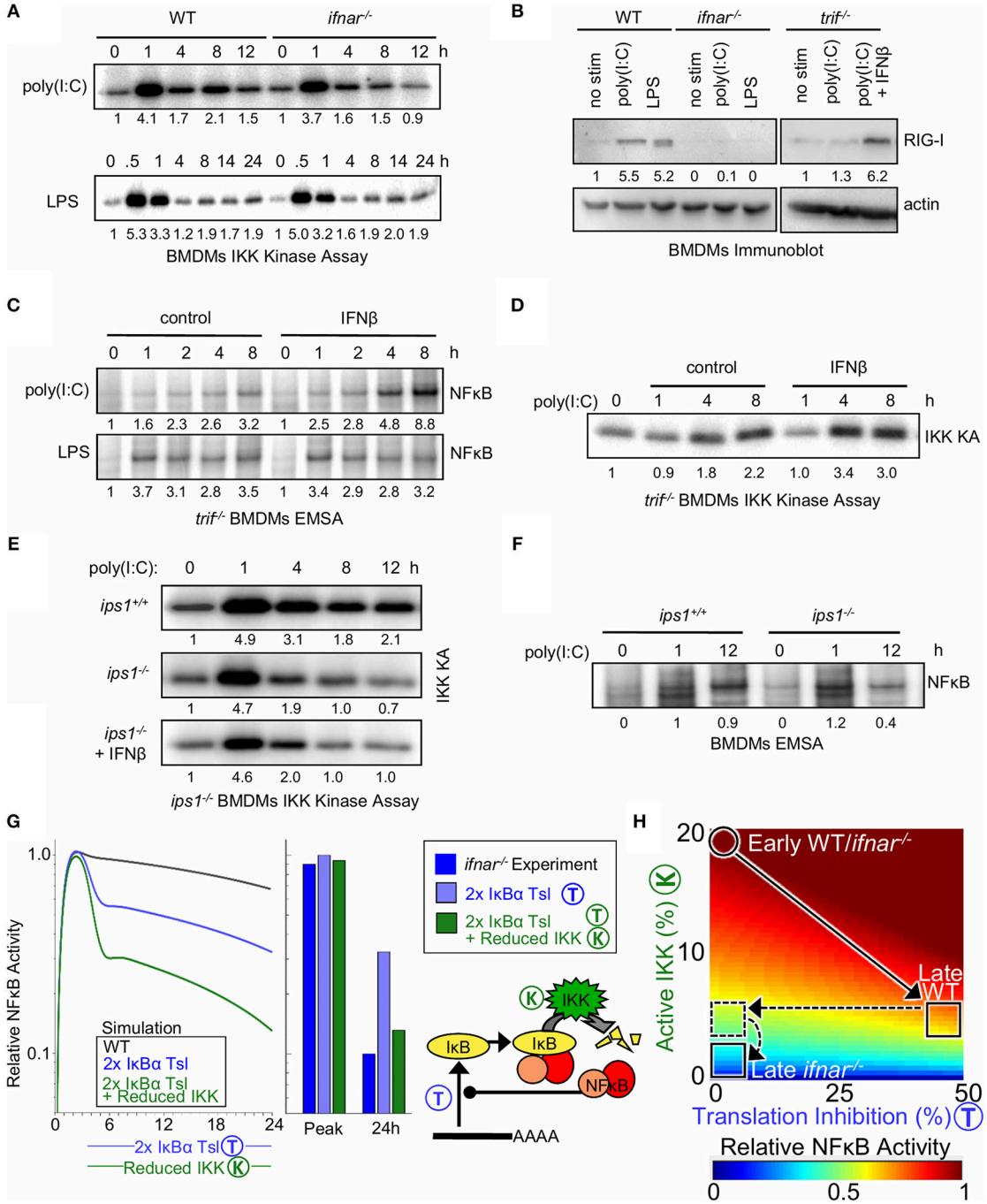

FIGURE 4 | Type I interferon potentiates late NFκB activation by poly(I:C) by decreasing IκB translation and increasing bound IκB degradation via elevated RigI expression. **(A)** Immunoprecipitation kinase assay (kinase A) of IKK activity in WT and *ifnar*$^{-/-}$ BMDMs in response to poly(I:C) and LPS. **(B)** Immunoblot of RIG-I expression after 8 h of poly(I:C) or LPS treatment in WT, *ifnar*$^{-/-}$ and *trif*$^{-/-}$ BMDMs; and rescue of *trif*$^{-/-}$ cells with IFNβ. **(C)** EMSAs of NFκB activation by poly(I:C) and LPS in *trif*$^{-/-}$ BMDMs with and without IFNβ co-treatment. **(D)** IKK activity in WT and *ips*$^{-/-}$ BMDMs exposed to poly(I:C) and in *ips*$^{-/-}$ cells with co-treatment with IFNβ. **(E)** IKK activity in *trif*$^{-/-}$ BMDMs with and without IFNβ co-treatment. **(F)** EMSAs of NFκB activation by poly(I:C) in ips1$^{+/+}$ and *ips1*$^{-/-}$ BMDMs. **(A–D)** show a dataset representative of at least three biological replicates, and **(E,F)** show a representative of two biological replicates (we gratefully acknowledge Zhijian James Chen for *ips1*$^{-/-}$ bone marrow). Quantitations are relative to basal or peak activity, which is set to 1. **(G)** (Left) Simulated NFκB timecourse in response to IKK activation representative of poly(I:C) stimulation, with a 2-fold increase in IκBα translation (blue) or with both IκBα translation inhibition and 50% IKK activity reduction as seen in *ifnar*$^{-/-}$ (green). (Right) Bar graph of NFκB activity at the peak and 24 h time point as quantified from simulations and experiments (**Figure 2A**). **(H)** Heatmap of NFκB activity calculated using SiMoN for 50 increasing IKK activity values and 50 increasing degrees of translation inhibition (2,500 total points). In both WT and *ifnar*$^{-/-}$ poly(I:C) stimulation results in increased IKK activity during the early phase. Following this WT cells undergo 50% translation inhibition and IKK activity decreases. *ifnar*$^{-/-}$ cells lack translation inhibition (horizontal dashed line, **Figure 3**), and have decreased late-phase IKK activity [vertical dashed line, this **(A–F)**].

activation when IKK-mediated IκB degradation is high. Thus, high IKK activity induced by LPS is sufficient to produce substantial NFκB activity and is only marginally enhanced by interferon-mediated IκBα translational inhibition.

IFNγ Potentiates NFκB Activation by Enhancing Free IκBα Degradation

Akin to type I interferon signaling in BMDMs, paracrine type II interferon used for priming TEPMs enhances nuclear NFκB DNA binding activity in response to poly(I:C) stimulation more than 2-fold, whereas it had little effect on LPS-induced NFκB activation (**Figure 2B**). To investigate the mechanism by which IFNγ potentiates NFκB responsiveness to poly(I:C) we again quantitatively examined the three tunable reactions controlling IκB metabolism using SiMoN (**Figure 1**). Specifically, we wondered whether IκB translation is inhibited in a Type II IFN-dependent manner in addition to the Type I-dependent inhibition we identified. However, we found no evidence that IFNγ treatment affects mRNA translation rates when translation rates were measured using the ^{35}S-Met pulse experiment (**Figure 5A**). Next, we tested whether IFNγ alters the IKK activity profile induced by poly(I:C) or LPS. To our surprise, IFNγ pre-treatment did not alter LPS- or poly(I:C)-induced IKK activity (**Figure 5B**).

As two out of the three reactions represented in SiMoN were found unaffected by IFNγ we tested the third, the degradation rate of unbound IκBα. Whereas, NFκB-bound IκBα is degraded through IKK-mediated phosphorylation and the ubiquitin-proteasome system, free IκBα is degraded independently of IKK activity through a ubiquitin-independent, but 20S proteasome-dependent mechanism (65, 66). To determine whether IFNγ affects the stability of free IκBα, we employed MEFs deficient in the NFκB proteins RelA, cRel, and p50 (termed "*nfkb*$^{-/-}$") in which all IκBα is in fact free, a previously established assay system for free IκBα turnover (51): *nfkb*$^{-/-}$ cells were treated with IFNγ, and IκBα levels were measured by Western blotting. IFNγ treatment of *nfkb*$^{-/-}$ cells resulted in a reduction of cellular IκBα (**Figure 5C**). We next sought to confirm that IFNγ-mediated reduction of free IκBα was due to enhanced degradation rather than reduced synthesis. We found that addition of the proteasome inhibitor MG132 after 24 h of IFNγ rescued the IκBα level, whereas addition of MG132 to cells treated for 4 h with the ribosomal inhibitor CHX did not (**Figure 5D**). Together, these data suggest that IFNγ enhances the proteasomal degradation of free IκBα.

We employed SiMoN to determine whether enhanced degradation of free IκB protein may account for the experimentally observed IFNγ-potentiated NFκB activity in response to poly(I:C). Our Western blot analysis is consistent with 10-fold higher degradation in IFNγ-primed cells; using this number in simulations along with low and high IKK activity curves representative of poly(I:C) and LPS, respectively, resulted in more than 2-fold amplification of NFκB activation in response to weak IKK activator poly(I:C) (**Figure 5E**). SiMoN predicted that increased free IκBα degradation affected the NFκB response speed, but did not substantially change late (>1 h) NFκB activity to strong IKK activating signals such as LPS but greatly increased the NFκB activity to weak activating signals such as poly(I:C) (**Figure 5E**). Strikingly, these predictions were validated by experimental quantitation of NFκB fold induction, which demonstrated similarly selective amplification of poly(I:C) but not LPS (**Figure 5F**). To understand this selective amplification we used SiMoN to quantify the relationship between IKK activity and NFκB and how this dose-response relationship is altered by free IκBα degradation. We observed a shift in the dose-response relationship between NFκB and IKK activities with increasing free IκBα degradation (**Figure 5G**). This shift selectively amplifies the NFκB response to weaker IKK-activating stimuli without substantially affecting strong IKK activators. Thus, the specificity of IFNγ-mediated potentiation of NFκB activation for poly(I:C), but not LPS, may be sufficiently explained by a kinetic argument: namely, weak signals are subject to modulation by crosstalk mechanisms, whereas strong signals are less sensitive to such modulation.

The IFNγ-Induced PA28 Proteasome Activators Accelerate Free IκBα Degradation

As IFNγ-stimulated degradation of free IκBα may tune NFκB responsiveness to poly(I:C) in tissue resident macrophages, we considered the potential molecular mechanisms. SiMoN predicts the molecular mechanism need not be poly(I:C) specific as selective amplification of weak NFκB activators can emerge through the kinetics of non-specific increased degradation of free IκBα. Whereas, ubiquitinated proteins are recognized and degraded by the 26S proteasome, which consists of the 20S barrel-shaped core and a 19S regulatory cap, free IκBα was shown to be degraded in a ubiquitin-independent manner (65). An alternative 11S regulatory cap, consisting of oligomers of the PA28α and PA28β proteins allows for ubiquitin-independent entry into the proteasome and has been implicated in antigen processing in antigen-presenting cells (66, 67).

Western-blotting revealed that IFNγ treatment increased PA28α and PA28β expression in both TEPMs (**Figure 5H**) and MEFs (**Figure 5I**). Using *nfkb*$^{-/-}$ MEFs allowed us to assay expression of free IκB protein, and examine whether PA28-mediated proteasomal degradation controls free IκB abundance. Knockdown of PA28α and PA28β by siRNA in *nfkb*$^{-/-}$ MEFs resulted in increased IκBα levels in cells, particularly in cells exposed to IFNγ (**Figure 5I**). Conversely, stable retroviral overexpression of PA28α and PA28β in *nfkb*$^{-/-}$ MEFs led to decreased levels of free IκBα (**Figure 5J**), demonstrating that increased expression of PA28α and PA28β are sufficient to increase degradation of free IκBα. Taken together, these data suggest that the 11S proteasomal cap components PA28α and PA28β are necessary and sufficient to increase free IκBα degradation in IFNγ-primed cells.

To further demonstrate a direct role for the IFNγ-inducible PA28 proteins in free IκBα degradation, purified IκBα was subjected to an *in vitro* degradation assay with purified 20S proteasome. The presence of PA28 proteins accelerated the

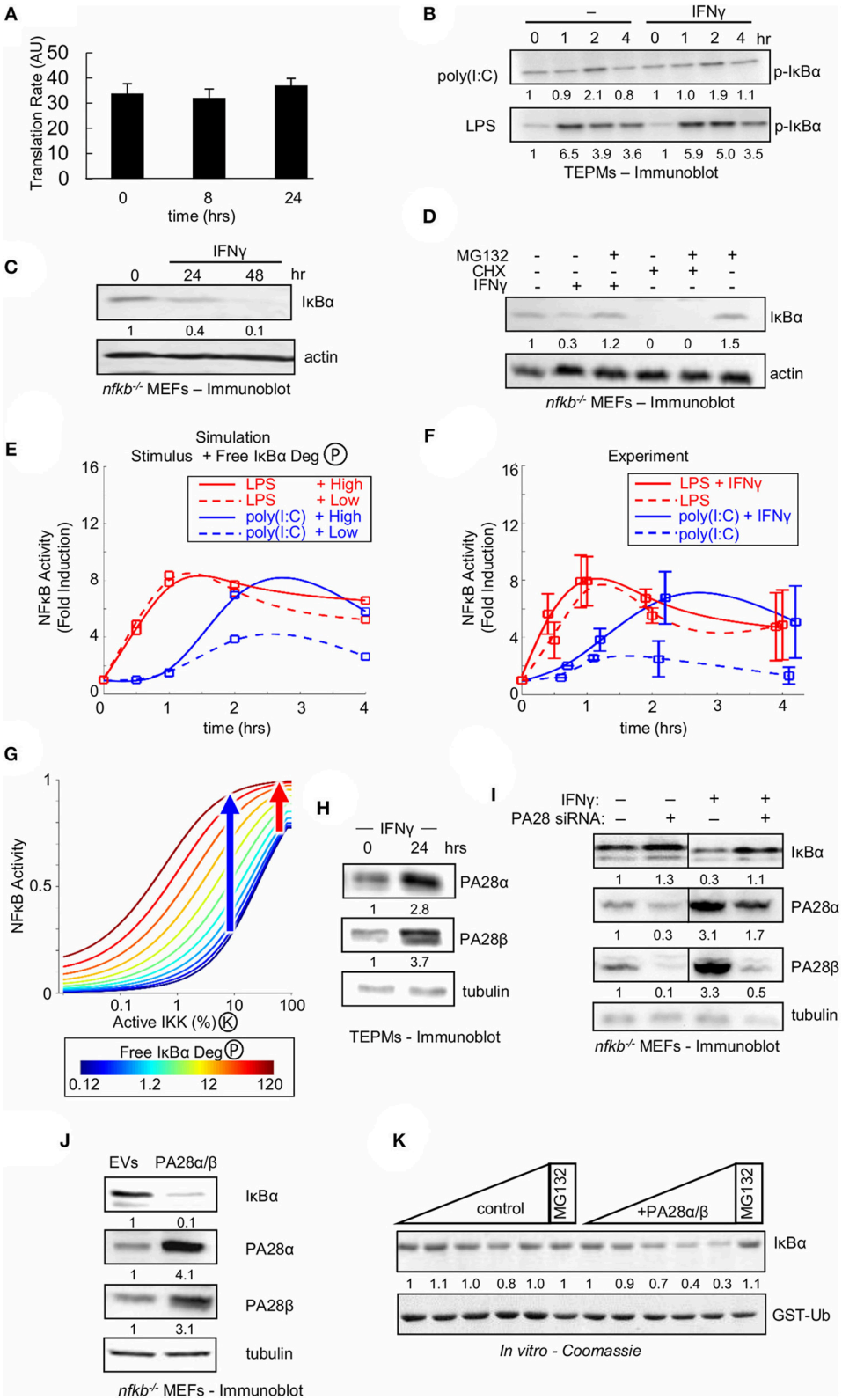

FIGURE 5 | Type II interferon amplifies weak NFκB activating stimuli by enhancing free IκBα degradation. **(A)** IκBα translational synthesis rates in naïve and IFNγ-conditioned TEPMs as revealed by ^{35}S-Met pulse assay. Average and standard deviation of three biological replicates are shown. **(B)** Immunoblot for p-IκBα in

(Continued)

FIGURE 5 | TEPMs exposed to either LPS or poly(I:C) with or without IFNγ priming. **(C)** Immunoblot of "free" IκBα compared to an actin control in MEFs deficient in canonical NFκB proteins RelA, cRel and p50 (termed "*nfkb*$^{-/-}$") exposed to IFNγ. **(D)** Free IκBα levels in *nfkb*$^{-/-}$ MEFs compared to an actin control. Immunoblot of lysates produced from MEFs exposed to 24 h priming with IFNγ or 4 h treatment with ribosomal inhibitor CHX, and followed by addition of proteasome inhibitor MG132. **(E)** Predictions from the Simplified Model of NFκB (SiMoN) with low (10% at peak) IKK activity, representative of poly(I:C) (blue), and high (40% at peak) IKK activity, representative of LPS. Values were calculated at 0, 0.5, 1, 2, and 4 h and fit with a smoothing spline for consistency with experimental time points. Free IκBα degradation was modulated from the default value (dashed lines) to 10-fold higher (solid lines) based on quantification of immunoblot in 5B. **(F)** Time course of NFκB induction (quantitated from EMSAs) in naïve or IFNγ-conditioned TEPMs stimulated with poly(I:C) and LPS. **(G)** Nuclear NFκB activity calculated using SiMoN as a function of bound IκBα degradation (IKK-activity, x-axis) and free IκBα degradation (colored lines). The blue and red arrows indicates the free IκBα degradation-dependent increase in NFκB activity for low and high IKK activities indicative of poly(I:C) and LPS, respectively. **(H)** Immunoblots of proteasome activator 28 (PA28) levels in TEPMs following exposure to IFNγ. **(I)** Immunoblots for IκBα and proteasome activator 28 (PA28) in *nfkb*$^{-/-}$ MEFs. Both conditions were repeated following PA28 siRNA-mediated knockdown. **(J)** Immunoblot of IκBα and PA28 levels in *nfkb*$^{-/-}$ MEFs transduced with retroviral transgenes. **(K)** Coomassie-stained SDS-PAGE showing free IκBα and PA28α/β levels following incubation with increasing amounts of purified 20S proteasome (upper panel) contrasted with GST-ubiquitin levels (lower panel), which serves as a negative control. **(B–D)** show a dataset representative of at least three biological replicates **(H–K)** show a dataset representative of two biological replicates. Quantitations are relative to basal or $t = 0$ activity, which is set to 1.

degradation of IκBα in this cell-free system (**Figure 5K**, upper), and this finding was specific to IκBα as the use of ubiquitin as the substrate in the same assay showed no change upon addition of PA28 proteins (**Figure 5K**, lower).

IFNγ-Mediated Degradation of Free IκBα Sensitizes NFκB to Weak Activating Signals

Our studies revealed that type II interferon signaling amplifies NFκB activation through increasing free IκBα degradation (**Supplementary Figure 5A**). SiMoN predicts that the amplifying effect of increasing free IκBα degradation is not specific to poly(I:C), but general to other weak NFκB inducing signals (**Figure 5G**). To further validate this prediction we utilized UV radiation, a known weak activator of NFκB, causing translation inhibition that allows for depletion of IκBα through its constitutive turnover (68). Consistent with the model predictions, pretreatment with IFNγ increased the NFκB response to UV in wild-type immortalized MEFs (**Supplementary Figure 5B**).

SiMoN was used to simulate the unfolded protein response (UPR) (69) which increases free IκBα degradation rates (simulating the presence of IFNγ). Whereas, increasing the free IκBα degradation rate had little effect on the response to large IKK activity changes such as for LPS (**Figure 5G**), it is predicted to result in a significant increase in the peak of NFκB activity in response to UPR (**Supplementary Figure 5C**).

To test this prediction and establish whether increased expression of PA28α and PA28β is sufficient to alter NFκB responsiveness to UPR, wild-type MEFs were retrovirally transduced with PA28α and PA28β. Overexpression of PA28α and PA28β increased the NFκB response to UPR induced by thapsigargin (**Supplementary Figure 5D**). The NFκB response to the strong IKK activator, TNF, however, was unaffected by the overexpression of PA28α and PA28β, consistent with the computational prediction that stronger inducers of IKK activity are not sensitive to increased free IκBα degradation (**Figure 5G** and **Supplementary Figure 5D**). In addition, pa28-deficient MEFs showed reduced response to thapsigargin (**Supplementary Figure 5E**). In addition, quantifying recent results from Cheng et al. (63) revealed transcriptional upregulation of PA28α/β (Psme1/2) in response to IFNγ conditioning (**Supplementary Figure 5F**). Together, these data support a model in which IFNγ enhances NFκB responses to weak stimuli by increasing the IKK-independent degradation of free IκBα via enhancement of the 11S proteasomal degradation pathway.

DISCUSSION

Here we presented a new simplified mathematical model of NFκB activity (SiMoN) and applied it to studying how interferons modulate NFκB activity. Although this model lacks the some of the molecular network detail of other NFκB signaling models that describe the highly dynamic and variable NFκB responses at single cell resolution (10), it provides for an intuitive understanding of how NFκB is controlled at the tissue scale. Specifically, the abstraction revealed that NFκB activity is governed fundamentally by three reactions that may be modulated by signaling crosstalk. This is an important modification of the prevailing research focus on just one of these: the IKK-controlled degradation of NFκB-bound IκB. Our work demonstrates that a focus on IKK alone has substantially limited previous studies into mechanisms of signaling crosstalk by cytokines that themselves do not activate NFκB. in this manner it is important to point out that other mechanisms that do not affect IκB metabolism may also control NFκB activity (e.g., the nuclear import/export machinery, post-translational modifications of NFκB, and expression of NFκB protein family members) and could be included in further studies.

In response to infection, innate immune responses must be delicately coordinated to ensure that it is sufficient to mount an effective defense, but not excessive so as to avoid the potentially harmful effects of inflammation. A central regulator of this response is NFκB, which can be activated by a variety of pathogen sensors, such as RIG-I/TLR3 and TLR4 in response to viral RNA and bacterial LPS, respectively. Infections also trigger an upregulation of type I interferon expression and expression of type II IFNγ by T and NK cells, thus providing a variety of cytokine milieus that potentially affect the NFκB-driven immune response. We have shown here how both type I and type II interferons engage in signaling

crosstalk with the core of IκB metabolism, effecting a stimulus-specific potentiation of NFκB activation, yet do so via different molecular mechanisms.

Through quantitative analysis of experimental data using SiMoN we identified two reactions in the core NFκB signaling module that are modulated by type I interferon feedback. Reduction in IκBα translation inhibition combined with modulation of IKK activity through RIG-I/MDA5 and IPS-1 results in increased late stage NFκB activation in response to poly(I:C) (**Figure 6A**). Type II interferon priming was found to modulate a third reaction, that we had not tested in response to type I interferon. Namely, type II interferon increased free IκBα degradation via the induction of immunoproteasomal cap proteins, thereby amplifying NFκB activation in response to weakly activating stimuli such as poly(I:C) (**Figure 6A**). IFNγ exposure also amplified the NFκB in response to ribotoxic stimuli, such as UPR, which induces NFκB signaling without inducing IKK (**Supplementary Figure 5C**), but showed less effect on LPS which activates NFκB by strongly inducing IKK.

Interestingly, the selective amplification of low IKK activating signals by IFNγ can be intuitively seen by studying the analytical solution to SiMoN. By first investigating a scenario without free IκB degradation such that the term P tends toward 0 we obtain:

$$\lim_{p\to 0} \frac{0.1k_fK + \sqrt{\left(0.1k_fK - PK\right)^2 + 0.4PK\left(Tk_f + Kk_f\right)} - PK}{2k_f\left(T + K\right)} = \frac{0.1k_fK + 0.1k_fK}{2k_f\left(T + K\right)} = \frac{0.1K}{\left(T + K\right)}$$

For a weak IKK activating stimulus ($K = 6\% \cdot k_{ikk}$) SiMoN gives ∼0.028 μM of NFκB activity and for strong IKK activators ($K = 60\% \cdot k_{ikk}$) SiMoN gives ∼0.078 μM of NFκB activity ($T = 0.055\ \text{min}^{-1}$ throughout). In contrast if we investigate the effect of enhancing free IκB degradation such that P is high we see that the limit does not depend on IKK activity:

$$\lim_{p\to \infty} \frac{0.1k_fK + \sqrt{\left(0.1k_fK - PK\right)^2 + 0.4PK\left(Tk_f + Kk_f\right)} - PK}{2k_f\left(T + K\right)} = 0.1\ \mu M$$

Therefore, the analytical solution reveals that free IκBα degradation can amplify NFκB activity in response to weak IKK activating over 3.5-fold (0.028 to 0.1 μM), but for strong IKK activating stimuli the amplification is far less substantial, with only around a 28% increase (from 0.078 to 0.1 μM).

Whether a stimulus is weak or strong depends on both dose and the pathways dose response. As LPS activation of NFκB is largely governed by the ultra-sensitive MyD88 pathway (49), LPS typically activates IKK strongly (or not at all). PolyIC on the other hand relies on the TRIF pathway, which, in macrophages, activates IKK more weakly. Thus, the crosstalk mechanisms identified here allow type I and type II interferons to potentiate NFκB activity in cells exposed to viral RNA, and less so when exposed bacterial LPS. Given the importance of coordinating innate immune defenses of localized macrophages, and system-wide adaptive immune responses during to viral infection, we suggest that the molecular mechanisms of interferon-NFκB crosstalk described here have pathophysiological relevance particularly where interferon signaling and inflammation are linked such as chronic inflammatory diseases and cancer (70, 71). By rigorously quantifying NFκB activation and IFN in physiological conditions, SiMoN may be used to explain seemingly conflicting physiological observations. For example, while greater inflammation is seen in leishmaniasis when the host IFN response is induced by parasites harboring Leishmania RNA virus (LRV) (28, 29) others have found TLR4 mediated NFκB activation to be unaffected by IFNγ (34). The selective IFN-dependent amplification of NFκB activity discovered here may reveal why some inflammatory conditions are susceptible to IFN-mediated crosstalk while others are not. Further work is required to quantify the degree of NFκB activation in diverse physiological conditions.

Further work is required to quantify the impact of selective amplification of NFκB activity on NFκB-target gene expression. A number of factors make such a task difficult, including gene-specific combinatorial control of NFκB-target genes in combination with other transcription factors interferon-regulatory factors [IRFs and STATs, Cheng et al. (72)]. Recent work has also identified highly gene-specific effects of interferons on chromatin accessibility and as such even genes lacking interferon responsive elements (IREs) may be subject to complex crosstalk (63). Similar signaling crosstalk may affect transcriptional elongation, mRNA processing and turnover. Disentangling these effects will require careful quantitative consideration, perhaps with the aid of a quantitative model of the mechanism controlling mature mRNA abundance.

The simplified model presented here enabled an analytical solution for the quasi-steady-state concentration of NFκB as a function of bound IKK activity, free IκB degradation, and IκB translation affinity (**Figures 1B, 6B**). NFκB activity can thus be calculated when the values of these parameters are known, without the need for timecourse simulations. This has enabled us to make SiMoN available through a web interface (signalingsystems.ucla.edu/tools/SiMoN.html) to allow others to interpret the impact of perturbations in these core processes on NFκB activity. Indeed, NFκB activity may be visualized in a four-dimensional plot (color cube) as a function of the three reactions (**Figure 6C**). Slices of the color cube in any of the three dimensions reveal NFκB activity as a function of two of the reactions at specific values of the third reaction. Thus, within a single image NFκB activity can be related to the activity of three interferon-tunable reactions that control IκB synthesis and degradation.

MATERIALS AND METHODS

Mathematical Modeling

A new mathematical model was constructed that consists of three ordinary differential equations (ODEs) to describe NFκB activation in response to TLRs and enable studies

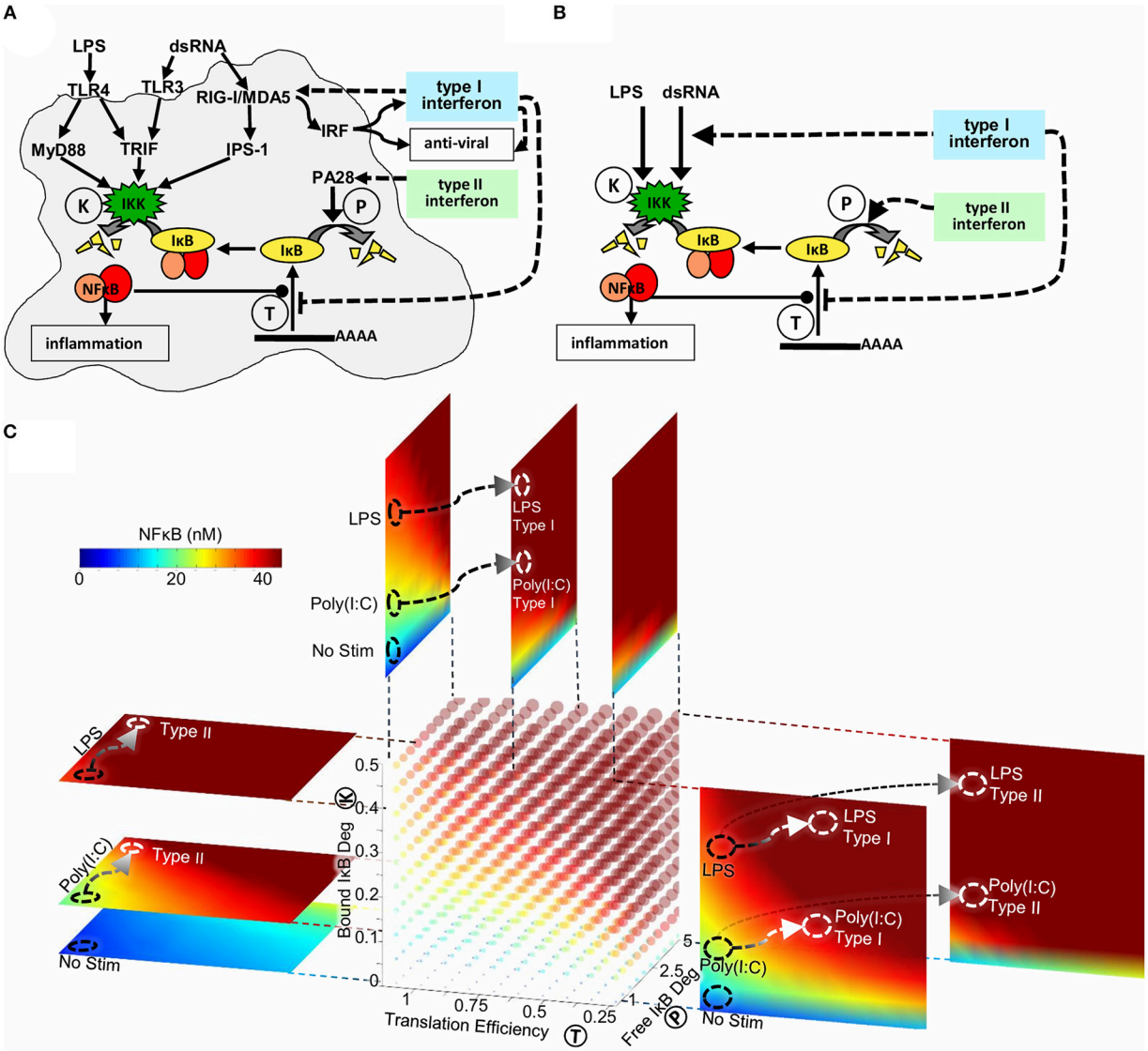

FIGURE 6 | The mechanisms underlying interferon signaling crosstalk on NFκB. **(A)** Type I interferons reduce IκB expression and increase IKK activity through RIG-I and IPS-1. Type II interferons increase free IκB degradation through a PA28-dependent process. **(B)** Type I interferons reduce translation of IκBα and increase the expression of the cytosolic viral sensor to allow for enhanced IKK mediated degradation of NFκB-bound IκBα. Type II interferon increases the degradation rate of free IκBα. All mechanisms potentiate the NFκB response to weak signals emanating from viral PAMP sensors, but have little effect on bacterial-MyD88-mediated responses. **(C)** Three-dimensional heatmap of nuclear NFκB concentrations as a function of three biochemical reactions: IKK activity (reaction K), IκB translation efficiency (reaction T) and free IκB degradation (reaction P). The point in this parameter space reached following Poly(I:C) and LPS stimulus is marked with black circles. Signaling crosstalk by Type I and Type II interferons produce distinct trajectories through this three-dimensional parameter space (marked with white arrows to white circles).

of signaling crosstalk in cell populations. NFκB activity is a function of its interaction with IκB, whose abundance is controlled via NFκB-dependent synthesis and two degradation reactions (51).

$$\frac{dNF\kappa B}{dt} = -k_f \cdot [NF\kappa B] \cdot [I\kappa B] + k_{ikk} \cdot ikkActivity \cdot [NF\kappa B - I\kappa B] \quad (1)$$

$$\frac{dI\kappa B}{dt} = -k_f \cdot [NF\kappa B] \cdot [I\kappa B] + k_{I\kappa BExp} \cdot [NF\kappa B] - k_{I\kappa BDeg} \cdot [I\kappa B] \quad (2)$$

$$\frac{dNF\kappa B - I\kappa B}{dt} = +k_f \cdot [NF\kappa B] \cdot [I\kappa B] - k_{ikk} \cdot [NF\kappa B - I\kappa B] \quad (3)$$

All parameters were derived from the existing cellular model of NFκB regulation (12) as follows:

Name	Value	Units	Description
$k_{I\kappa BDeg}$	0.12	min^{-1}	Rate of free IκBα degradation - (Werner et al, 2005)
k_f	30	$\mu M^{-1} min^{-1}$	Rate of NFκB IκBα binding - (Werner et al, 2005)
k_{ikk}	0.36	min^{-1}	Rate of IKK-mediated NFκB-bound IκBα degradation - (Werner et al, 2005)
$ikkActivity$	0.01-1	Multiplier	Scales k_{ikk} from 1-100% activity. Equivalent to "ikk_multiplier" scaling parameter used in Werner et al (2005). This parameter Used to scale k_{ikk} from its maximum rate ($ikkActivity = 1$) to 1% activity ($ikkActivity = 0.1$) such that the rate of bound IκBα degradation is a combination of both parameters: $k_{ikk} \cdot ikkActivity \cdot [NF\kappa B - I\kappa B]$
$k_{I\kappa BExp}$	0.055	min^{-1}	IκBα protein production in the previous model (Werner et al, 2005) was dependent on IκBα mRNA, which was included as a molecular species. mRNA is not included here. As a result the translation from Werner et al (2005) is not applicable here. Around 94nM of IκBα (bound and unbound) is present at steady-state in Werner *et al.* 2005. This $k_{I\kappa BExp}$ value was chosen to obtain approximately the same IκB mRNA concentration (92nM) in the reduced model presented here.

Model construction and analysis was performed in COPASI:Biochemical System Simulator (73). When compared to the model of Werner et al. (12) from which it was derived, SiMoN reduces complexity by assuming all reactions are in a single cellular compartment with all unbound (12) NFκB assumed to be transcriptionally active. Indeed, the majority of inhibited NFκB is found in the cytoplasm with free NFκB quickly translocating to the nucleus in both experimental and model systems (10). In addition, only the predominant NFκB-inhibitor (IκBα) is considered, and IκBε and IκBβ, which are bind a relatively minor portion of NFκB are ignored (74). To further simplify the model, the two reactions of NFκB-dependent IκBα mRNA expression and subsequent protein synthesis are reduced to a single NFκB-dependent IκBα protein production reaction in SiMoN, similar to other reduced models (15–18).

For the exploratory analysis in **Figure 1**, a steady-state phase was run with default parameters and then initial conditions were updated to the final concentrations from the steady-state phase. The indicated parameters were then scanned using the "Parameter Scan" task in COPASI with a 3-h time course. $k_{I\kappa BExp}$ was scanned from 0.5 to 1x the default parameter value with samples every 0.1 (**Figure 1A**), $k_{I\kappa BDeg}$ was scanned from 1 to 5x the default parameter value with samples every 1 (**Figure 1B**) and the *ikkActivity* multiplier was scanned from 1 to 2x the default parameters with samples every 0.25 (**Figure 1C**). Two dimensional parameter scans were performed using nested parameter scan tasks in COPASI to repeatedly perform a steady-state analysis at each parameter value as indicated (**Figure 1D**). In order to quantify the effect of IκBα translation on Poly(I:C) responses (**Figure 3B**) the model was modified to add an additional modifier to the rate of IκBα expression (Iκ Bα expression $= k_{I\kappa}BExp \cdot$ tslModifier, tslModifier $= 1$). A Copasi event was added to trigger at 200 min updating the translation rate modifier parameter, and a parameter scan task in Copasi was used to scan this modifier at 1 (no change) and 2 (double IκBα expression). IKK activity dynamics were simulated by modulating the multiplier of NFκB-bound Iκ B degradation reaction (parameter *ikkActivity*). Input curves for and poly(I:C)-induced IKK activity (**Figure 3A**) were quantified using ImageJ software (75). A piecewise function, which interpolated between the time points in the figure, was created to represent IKK activity through modulating the multiplier of NFκB-bound Iκ B degradation reaction (parameter *ikkActivity*).

In order to simulate the modulation of IKK activity $ifnar^{-/-}$ (**Figure 4G**) two additional multipliers were added scaling early IKK activity (0–200 min) and late IKK activity (>200 min) and these were set to 0.9 and 0.6, respectively to represent the fold change in IKK activity measured in $ifnar^{-/-}$ BMDMs by IKK kinase assay (**Figure 4A**). Simulations of the effect of free IκBα degradation on Poly(I:C) and LPS responses (**Figures 5E,F**) were obtained by multiplying the *ikkActivity* by 0.5 for Poly(I:C) and 2 for LPS to give peak IKK activity at ∼7% for Poly(I:C) and ∼30% for LPS, and a parameters scan task was use to adjust $k_{I\kappa BDeg}$ to 12 min^{-1} for both input curves. Simulations of the unfolded protein response (**Supplementary Figure 5C**) involved applying, at time $t = 0$, a 50% reduction on the IκB translation rate, while keeping the NFκB-bound IκB degradation reaction rate (dependent on IKK activity) constant at its basal level. In the analytical analysis and figures parameters are abbreviated: $ikkActivity \cdot k_{ikk} =$ K, $k_{I\kappa BDeg} = P$, $k_{I\kappa BExp} = T$.

Mouse Strains and Cell Culture

Bone Marrow-Derived Macrophages (BMDMs) were generated from C57BL/6, $trif^{-/-}$, $ips1^{-/-}$, and $ifnar^{-/-}$ mice with L929 cell–conditioned medium for 8 days. Thioglycollate Elicited Peritoneal Macrophages (TEPMs) were isolated from the peritoneal cavity 4 days after injection of thioglycollate. Mouse Embryonic Fibroblasts (MEFs) of indicated genotype

(wild-type or *nfkb1*$^{-/-}$*crel*$^{-/-}$*rela*$^{-/-}$) were prepared from embryonic day 12 to 14 embryos and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine calf serum for up to six passages. Cells were stimulated with LPS (0.1 μg/ml; Sigma, B5:055), poly(I:C) (50 μg/ml: Amersham Biosciences), IFNβ (250 U/ml: PBL Biomedical Laboratories), IFNγ (eBioscience: 10 U/ml), or thapsigargin (Sigma-Aldrich). For siRNA, the target sequences for PA28α and PA28β were AAGCCAAGGTGGATGTGTT and AGCGAGCAAGGCCAGAAGC, respectively. Oligonucleotides were transfected into *nfkb1*$^{-/-}$*crel*$^{-/-}$*rela*$^{-/-}$ MEFs with lipofectamine. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) which accredits UCLA's animal care program. UCLA's Animal Welfare Assurance number with the Department of Health and Human Services Office of Laboratory Animal Welfare is A3196-01. The protocol was approved by the UCLA Institutional Animal Care and Use Committee, known as the Chancellor's Animal Research Committee (ARC).

Live Cell Imaging of NFκB Localization

BMDMs derived from a RelA-mVenus reporter mouse (to be described) were plated on eight-well μ-slides (ibidi) and stimulated with poly(I:C) without or with IFNβ or IFNγ. Conditions were maintained at 5% CO_2 and 37°c throughout imaging with a Zeiss AxioObserver using a 40x oil immersion objective, LED (light-emitting diode) fluorescence excitation, and CoolSnap HQ2 camera. RelA-YFP and H2B-mCherry images were collected every 5 min over 12 h and exported into MATLAB where analysis was performed as previously described (76).

Biochemical Assays With Cell Extracts

Nuclear extracts from BMDMs were prepared by high salt extraction. Western blotting analysis and Electrophoretic Mobility Shift Assays (EMSAs) were conducted with standard methods as described previously (12, 46, 68). The κB EMSA probe was: GCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGG. For Western blotting analysis and supershift assays we used antibodies against p65 (Santa Cruz Biotechnology, sc-372), p50 (sc-114), α-tubulin (sc-5286), p50 (N. Rice, NC-1263), lamin A/C (Cell Signaling, #2032), PA28α/β (Cell Signaling, #2408/2409), and IRF3 (Cell Signaling, #4962); Guinea pig anti-RIG-I was used as described previously (77). IKK activity assays were previously described (68). *In vivo* pulse labeling of BMDMs was done with 100 μCi/ml trans ^{35}S-Met label (MP Biomedicals, Inc.) using the indicated time courses. IκBα was immunoprecipitated (sc-371) and proteins were resolved on 8% SDS-PAGE, visualized by autoradiography, and quantified with Imagequant software. Ribosomal inhibitor cyclohexamide (CHX) and proteasomal inhibitor MG132 were used to block protein synthesis and degradation, respectively, and as described previously (69). Gene expression studies employed quantitative RNAse protection or qPCR assays, as described (78, 79). Quantitative data of biological replicates was analyzed with indicated statistical tests and visualized in R, Prism, or Excel software.

Proteasomal *in vitro* Degradation Assay

As previously described (80), 20S proteasome particles were purified from bovine blood using four chromatographic steps (Q-sepharose, Sephacryl S-300, Phenyl sepharose and Mono-Q). PA28α and β subunits were expressed *in E. coli* and purified separately followed by hetero complex formation by refolding following the method described by Song et al. (81). PA28αβ was mixed in 4-fold molar excess with 20S at 25°C, and the resulting proteasome complex was incubated with recombinant IκBα immediately following its elution from a Superdex 200 column. Recombinant IκBα was mixed in varying molar ratios with purified proteasome in a reaction buffer containing 200 mM NaCl, 20 mM Tris HCl, pH 7.1, 10 mM MgCl2, and 1 mM DTT and incubated at 25°C. The reaction was quenched by the addition of 4X SDS dye and boiling for 1 min at 95°C. The products were then separated by SDS-PAGE and visualized by Coomassie staining. To ensure the specificity of the degradative activity of the proteasome the degradation assay was also performed using stably folded GST tagged di-ubiquitin (GST-diUb).

AUTHOR CONTRIBUTIONS

Computational modeling was performed by SM. Experiments were performed by EM, JH, and AA. AH conceived the project and AH, SM, and GG designed and coordinated the study. The manuscript was prepared by SM, EM, and AH with contributions from AA, QC, and GG. All authors interpreted the results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01425/full#supplementary-material

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REVIEW
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Signaling Crosstalk Mechanisms That May Fine-Tune Pathogen-Responsive NFκB

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Precise control of inflammatory gene expression is critical for effective host defense without excessive tissue damage. The principal regulator of inflammatory gene expression is nuclear factor kappa B (NFκB), a transcription factor. Nuclear NFκB activity is controlled by IκB proteins, whose stimulus-responsive degradation and re-synthesis provide for transient or dynamic regulation. The IκB-NFκB signaling module receives input signals from a variety of pathogen sensors, such as toll-like receptors (TLRs). The molecular components and mechanisms of NFκB signaling are well-understood and have been reviewed elsewhere in detail. Here we review the molecular mechanisms that mediate cross-regulation of TLR-IκB-NFκB signal transduction by signaling pathways that do not activate NFκB themselves, such as interferon signaling pathways. We distinguish between potential regulatory crosstalk mechanisms that (i) occur proximal to TLRs and thus may have stimulus-specific effects, (ii) affect the core IκB-NFκB signaling module to modulate NFκB activation in response to several stimuli. We review some well-documented examples of molecular crosstalk mechanisms and indicate other potential mechanisms whose physiological roles require further study.

Keywords: NFκB, PAMPs (pathogen-associated molecular patterns), interferon-beta (IFNβ), signaling crosstalk, immunoproteasome, TRIF, A20 (TNFAIP3), IκBs

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INTRODUCTION

NFκB signaling mediates inflammatory and innate immune responses; the signaling components that comprise the core signaling pathway are well-understood and have been amply reviewed, for example by Mitchell et al. (1), Leifer and Medvedev (2), Pandey et al. (3), and Hayden and Ghosh (4). Here, therefore, is only a brief summary. Of 15 possible NFκB dimers, the predominant mediator of NFκB inflammatory gene expression is the ubiquitous RelA:p50 heterodimer (1). At rest, inhibitors of κB (IκB)s sequester RelA:p50 in the cytoplasm by masking its DNA binding region and nuclear localization signal (5–7). In response to stimuli, IκBs are phosphorylated by IκB kinase (IKK), which triggers their ubiquitination and proteolysis (8, 9). Then, RelA:p50 translocates from the cytoplasm to the nucleus, where it binds and activates promoters and enhancers of target genes, such as *nfkbia,* which codes for IκBα (10, 11). Since IκBα synthesis is induced by RelA:p50, a tightly coupled negative feedback loop emerges that regulates NFκB activity in a highly dynamic and stimulus-specific fashion (11–13). To tune NFκB signaling, crosstalk mechanisms regulate

signal transduction from TLRs to IκBs to NFκB (**Figure 1**). We describe crosstalk mechanism at four levels: receptors, adaptors, enzymatic complexes, and the IκB-NFκB signaling module (**Figure 2**). Here, we focus on a few well-established crosstalk mechanisms, and mention others that deserve further study.

To ensure effective host defense against pathogens and to maintain tissue integrity, immune cells must integrate multiple signals to produce appropriate responses (14). Cells of the innate immune system are equipped with pattern recognition-receptors (PRRs) that detect pathogen-derived molecules, such as lipopolysaccharides and dsRNA (3). Once activated, PRRs initiate series of intracellular biochemical events that converge on transcription factors that regulate powerful inflammatory gene expression programs (15). To tune inflammatory responses, pathways that do not trigger inflammatory responses themselves may modulate signal transduction from PRRs to transcription factors through crosstalk mechanisms (**Figure 1**). Crosstalk allows cells to shape the inflammatory response to the context of their microenvironment and history (16). Crosstalk between two signaling pathways may emerge due shared signaling components, direct interactions between pathway-specific components, and regulation of the expression level of a pathway-specific component by the other pathway (1, 17). Since toll-like receptors (TLRs) are the best characterized PRRs, they provide the most salient examples of crosstalk at the receptor module. Key determinants of tissue microenvironments are type I and II interferons (IFNs), which do not activate NFκB, but regulate NFκB-dependent gene expression (18–21). As such, this review focuses on the cross-regulation of the TLR-NFκB signaling axis by type I and II IFNs.

Whereas, IFNγ is the only type II IFN, the type I IFN family consists of multiple forms of IFNα and IFNβ (22, 23). Type I IFNs ligate interferon-α receptors (IFNAR), which leads to the activation of Janus-activated kinase-1 (JAK1), tyrosine kinase 2 (Tyk2), and IFN-stimulated gene factor 3 (ISGF3) complex, which consists of signal transducer and activator of transcription 1 (STAT1), STAT2, and IFN-regulatory factor (IRF)-9 (23). IFNγ ligates IFNγ-receptor (IFNGR), which leads to the activation of JAK1 and JAK2 and the subsequent STAT1 phosphorylation and homodimerization (22).

RECEPTOR MODULES

Receptor Abundance and Localization

IFNγ is a well-described crosstalk mediator that enhances NFκB signaling (**Figure 3**) (20). By upregulating the expression of TLRs, IFNγ enhances the detection of pathogen-associated molecular patterns (PAMPs) by TLRs in different cellular compartments. At the plasma membrane, TLR2 and TLR4 recognize microbial cell wall components, such as lipopolysaccharides and lipoproteins (24). Similarly, endosomal TLRs, such as TLR3 and TLR9, recognize double stranded RNA and CpG oligonucleotides (24). IFNγ upregulates TLR2, TLR3, TLR4, and TLR9 at the mRNA and protein levels (25–30). Similarly, the inflammatory cytokine, tumor necrosis factor (TNF) upregulates the mRNA expression of TLR2 (31). The significance of TNF-induced and IFNγ-induced upregulation of TLR abundance on NFκB signaling dynamics is unknown. In addition to recognizing PAMPs, TLRs recognize host-derived molecules, such as extracellular matrix proteins, heat-shock proteins, nucleic acids, and high mobility group box 1 (32–37). Whereas, high TLR abundance facilitates detection of pathogens and mobilization host defenses, it may also increase susceptibility to autoimmune diseases and sepsis (24).

Accessory Protein Abundance

In addition to upregulating TLR expression, IFNγ also upregulates expression of TLR accessory proteins (**Figure 3**), such as myeloid differentiation factor 2 (MD2) and CD14 (29, 38, 39). Both accessory proteins facilitate the binding of lipopolysaccharide (LPS) to TLR4, in part by regulating localization of TLR4 (40–42). In fact, MD2 is necessary for localization of TLR4 to the plasma membrane, where it can bind LPS and transduce signals to downstream components (41, 43). After activation, TLR4 undergoes dynamin-mediated endocytosis into endosomes, where it continues transmitting signals (44). In the absence of CD14, endocytosis of TLR4 and subsequent signal transmission are attenuated. Further, CD14 and MD2 promote the association of endosomal TLR4 to downstream adaptors, which are critical for signal transduction (41, 42). Although CD14 is primarily associated with TLR4-mediated signaling, it also facilitates TLR2, TLR3, and TLR9 signaling (45–47). Interestingly, accessory proteins may contribute to inflammation in Alzheimer's disease (AD) and atherosclerosis (48). CD36, a scavenger receptor, recognizes amyloid β and oxidized LDL, which contribute to pathogenesis of AD and atherosclerosis, respectively (48). CD36 forms a heterotrimeric complex with TLR4 and TLR6 to induce production of inflammatory mediators (48). Further, IFNγ-activated macrophages significantly upregulate the expression CD36 in disease models of atherosclerosis (49).

Signaling Adapters

While IFNγ upregulates the expression of TLRs and accessory proteins that promote inflammatory responses, it also upregulates negative feedback regulators to maintain homeostasis (**Figure 3**). To enable negative feedback, IFNγ, TNF, and type I IFNs induce the expression of a family of E3 ubiquitin ligases, aptly named suppressors of cytokine signaling (SOCS) (18, 25, 50). SOCS1 was reported as a negative regulator of TLR4 signaling that is essential for the formation of endotoxin tolerance (51). The putative mechanism by which SOCS1 inhibits TLR signaling is through ubiquitin-mediated degradation of TIR domain containing adaptor (TIRAP), which recruits myeloid differentiation primary response gene 88 (MyD88) to TLR2 and TLR4 by mitigating the effects of electrostatic repulsion (52). The significance of SOCS1 is evident from the fact that SOCS1 deficiency causes neonatal lethality in mice due to overwhelming inflammation (53). However, loss of IFNγ rescues *socs1*$^{-/-}$ mice, which suggests that the primary role of SOCS1 is to restrain IFNγ-dependent inflammation and pathology.

Since TLRs do not possess the catalytic activity to activate NFκB directly, they engage adaptors such as MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF)

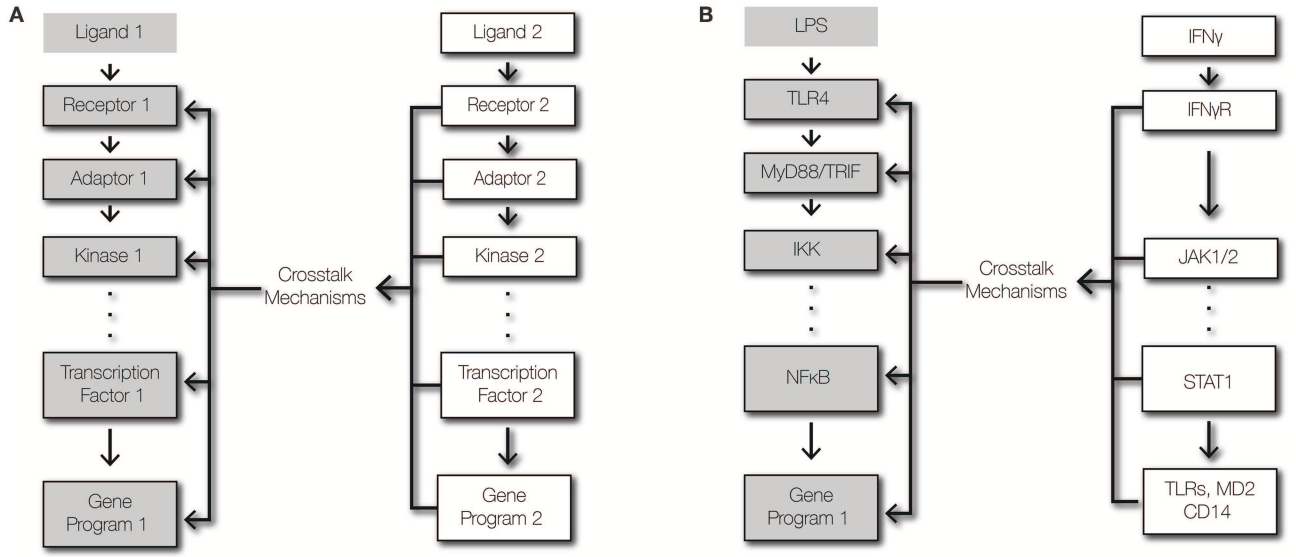

FIGURE 1 | Signaling and crosstalk. **(A)** Regulatory crosstalk is defined here as the signal transduction within a pathway being altered by a second pathway that affects the abundances or functions of signaling components. **(B)** Schematic of signaling crosstalk from IFNγ signaling to TLR4-NFκB signaling.

to propagate signals downstream (54, 55). The expression of MyD88 may be controlled by IFNγ, since *myd88* mRNA is IFNγ-inducible (25). Furthermore, MyD88 degradation may also be regulated by the anti-inflammatory cytokine, transforming growth factor (TGF)β, through Smad6-dependent recruitment of Smad ubiquitin regulatory factor (Smurf) 1/2 E3 ubiquitin ligases (56). However, the physiological significance of these crosstalk mechanisms remains to be fully elucidated.

ENZYMATIC COMPLEXES

Signal transduction from TLRs to NFκB involves recruitment of several enzymes to the TLR signaling complex (3). The recruited kinases and ubiquitin ligases allow for signal amplification while providing pathway specificity (13, 57). The enzymes upstream of the IKK signaling complex provide multiple avenues and nodes for signal integration and crosstalk (57–59). Both the catalytic activity and abundance of these enzymes can be subject to cross-regulation (**Figure 4**). After engaging TLRs, MyD88 forms an oligomeric complex with IL1R-associated kinases (IRAK) called the Myddosome (60). Formation of the Myddosome complex brings IRAK4 dimers and IRAK1/2 dimers into close proximity for efficient signal transduction (61). In response to IFNγ stimulation, immune cells upregulate the expression of IRAKs and MyD88 (25, 29, 62). In contrast, TNF stimulation upregulates the expression of negative regulators of TLR signaling, such as IRAK-M (63). The expression of IRAK-M in macrophages abrogates signaling downstream of IRAKs, inhibits TLR-induced NFκB activation, and mediates endotoxin tolerance (64). As limiting components in TLR signal transduction, MyD88, and IRAKs form critical junctures for regulatory control of inflammatory responses (60, 65). During endotoxin tolerance, the abundance of IRAKs and the association of TLRs with MyD88 are reduced (62). Therefore, crosstalk at this module can serve a dual purpose: priming and tolerance.

Similar to TNF receptor 1 (TNFR1), TRIF engages the adaptor protein tumor necrosis TNFR1-associated death domain protein (TRADD) and the kinase receptor-interacting protein (RIP)1 (66, 67). NFκB activation through TRIF-RIP1 signaling is dependent on Pellino-1, which is an E3 ubiquitin ligase that is essential for the formation of ubiquitin scaffold on RIP1 (68); however, the E3 ubiquitin ligase activity of Pellino-1 may be dispensable for TRIF-dependent activity (69). Whereas, loss of Pellino-1 expression abolishes TRIF-dependent RIP1 ubiquitination, loss of Pellino-1 E3 ubiquitin ligase activity does not affect RIP1 ubiquitination (68, 69). Although the inducible expression of Pellino-1 mRNA (*Peli1)* is dependent on IFN-regulatory factor 3 (IRF3), evidence suggests *Peli1* is also a target gene of ISGF3, which is induced by type I IFNs (70). Whether type I IFNs enhance TRIF-NFκB in a Pellino-1-dependent manner is unknown. Since the loss of Pellino-1 confers resistance to septic shock in response to TLR3 and TLR4 activation, it is possible that type I IFNs cross-regulate TRIF-NFκB through Pellino-1 to regulate septic shock (68). However, direct evidence is lacking.

The primary E3 ubiquitin ligase that transduces signals from MyD88 to IKK is TRAF6 (71–73). Downstream of IRAKs, TRAF6 facilitates the formation of K63-linked ubiquitin scaffold and the recruitment of IKK to the TLR signaling complex (73). TLR-NFκB signaling is regulated by ubiquitin editing enzymes, such as A20 and cylindromatosis (CYLD) (74, 75). We will focus the next section on A20 though it is not IFN-controlled but provides important signaling crosstalk (**Figure 4**).

A20 is a highly inducible NFκB target gene that attenuates cytokine- and pathogen-mediated inflammatory signaling (76, 77). Loss of A20 is lethal, due to excessive inflammation, cachexia, and organ failure (78, 79). Furthermore, dysregulated

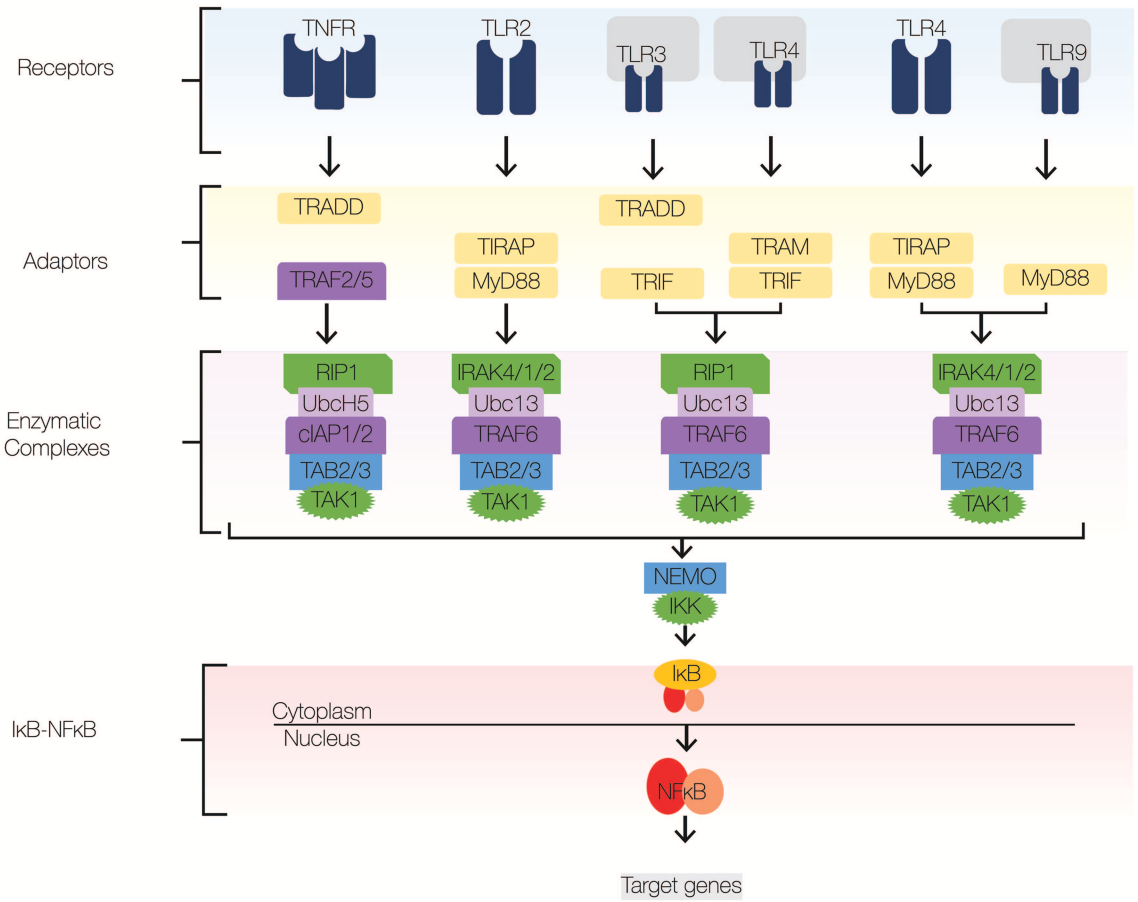

FIGURE 2 | NFκB signaling pathway. The major signaling components of the NFκB signaling pathway include receptors, adaptors, enzymatic complexes, and the IκB-NFκB complex. Upon ligand recognition, cognate receptors engage adaptor proteins that recruit kinases and ubiquitin ligases to the signaling complex. TLR signaling employs adaptor proteins MyD88 and TRIF; both of which contain TIR domains. Sorting adaptor proteins such as TIRAP and TRAM facilitate MyD88 and TRIF association with the signaling complex. MyD88 engages an enzymatic complex that includes IRAK4, IRAK1, IRAK2, TRAF6, Ubc13, TAB2/3, and TAK1. TRIF engages a similar enzymatic complex, which includes RIP1 instead IRAK4,1,2. The enzymatic complexes facilitate the recruitment and activation of IKKβ, which induces the degradation of IκBs and subsequent nuclear translocation of NFκB. Navy blue, TLRs; yellow, adaptors; green, kinases; dark purple, E3 ligases; light purple, E2 conjugases.

A20 signaling contributes to the pathogenesis of atherosclerosis and rheumatoid arthritis (80–82). A20 is an essential negative feedback regulator and terminator of TLR signaling (77). It edits ubiquitin tags on TRAF6 and RIP1 (75, 83). A20 removes K63-linked ubiquitin chains from RIP1 and may add K48-linked ubiquitin chains to target RIPK1 for proteasomal degradation (75). Additionally, A20 disrupts the interactions between TRAF6 and E2 ubiquitin conjugating enzymes, Ubc13 and UbcH5; A20 also enhances proteasomal degradation of Ubc13 and UbcH5c, by catalyzing the deposition of K48-linked ubiquitin chains (83). By mediating signaling crosstalk between TNFR and TLR/IL1R signaling pathways, A20 serves as a memory of recent inflammatory signaling (58, 63).

A20-binding inhibitor of NFκB activation 1(ABIN1; also known TNIP1) is a TNF-inducible binding partner of A20 (84–86). ABIN1 modulates A20-mediated inhibition of IKK-NFκB signaling by enhancing the de-ubiqutination of the IKK regulatory subunit, IKKγ/NEMO (84). The exact mechanism of ABIN1-mediated inhibition of IKK has yet to be elucidated. The observation that ABIN1 has a high affinity for polyubiquitin chains has informed some candidate mechanisms (87). One potential mechanism involves ABIN1 serving as an adaptor that brings A20 and its targets into close proximity (88). Another potential mechanism involves competition with the regulatory subunit of IKK, IKKγ/NEMO for polyubiquitin binding (88). Similar to the loss of A20, the loss of ABIN1 ($tnip1^{-/-}$) may lead to embryonic lethality (89). $Tnip1^{-/-}$ mice that reach adulthood develop autoimmune disorders spontaneously (87, 90). ABIN3 is another TNF-inducible binding partner of A20 (18, 91). The significance of ABIN3-mediated negative regulation of TLR-NFκB signaling has yet to be established and the mechanism has yet to be elucidated.

Monocyte chemotactic protein [MCP]-induced protein 1 (MCPIP1; also known as Regnase-1a or ZC3H12A) is a TNF-, IL1β-, and IL4-inducible deubiquitinase that negatively regulates NFκB activity (92–94). In the absence of MCPIP1, TLR-induced

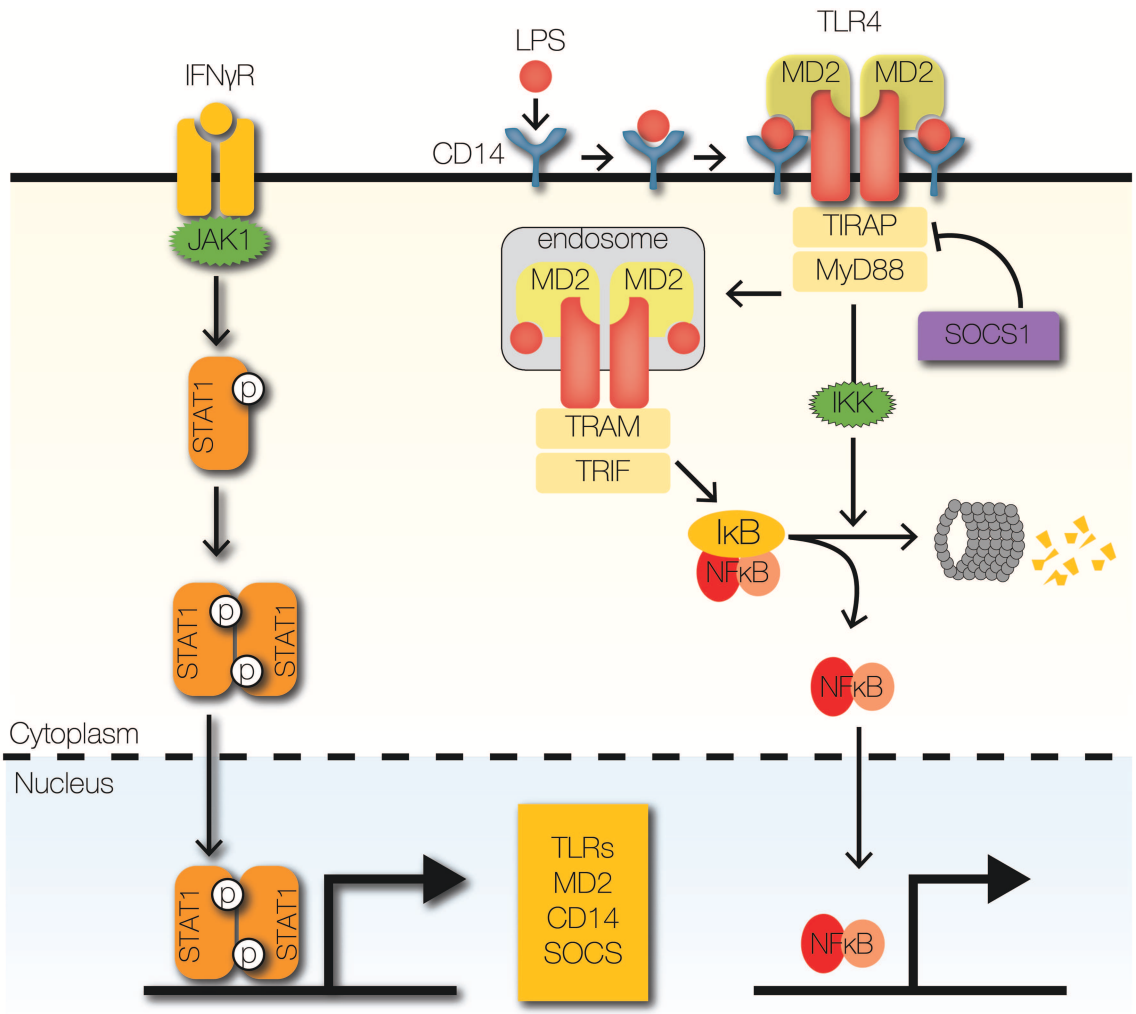

FIGURE 3 | Signaling crosstalk at receptors and adaptors. IFNγ receptor activation leads to the phosphorylation and nuclear translocation of STAT1 homodimers. STAT1 upregulates the expression of several signaling components of the TLR signaling pathway, such asTLRs and co-receptors MD2 and CD14. SOCS1, a STAT1-inducible negative regulator of STAT1 signaling, promotes the degradation of TIRAP by facilitating K48-ubiquitin-mediated proteolysis.

IKK phosphorylation, and NFκB nuclear translocation are enhanced as a result of elevated TRAF6 ubiquitination (93). The biological importance of MCPIP1 is highlighted by the fact that *Zc3h12a*$^{-/-}$ mice develop lymphadenopathy, splenomegaly, growth retardation, and chronic autoimmunity and die prematurely (92, 93).

NFκB-IκB MODULE

IκB Synthesis

Regulation of IκBα synthesis via translational control of *nfkbia* mRNA, which encodes IκBα, can mediate cross-regulation of NFκB activity (**Figure 5B**). Type I IFNs, such as IFNβ, enhance TLR-NFκB signaling by repressing the translation of *nfkbia* (19). Further, stress responses to ultraviolet radiation (UV) and unfolded proteins (UPR) enhance NFκB activity through translation repression of *nfkbia* (95, 96). Translation of *nfkbia* is controlled by eukaryotic initiation factor (eIF)2α and eIF4E [J. (97, 98)]. Translational repression of *nfkbia* by eIF2α depends on its phosphorylation by eIF2α kinases, such as PKR (interferon-induced, double-stranded RNA-activated protein kinase), PERK (pancreatic eIF2α kinase/RNA-dependent-protein-kinase-like endoplasmic-reticulum kinase), and GCN2 (general control non-derepressible-2) (96, 97, 99, 101). Whereas, PKR is activated by type I IFNs, GCN2, and PERK are activated by UV and UPR, respectively (100, 101).

IFNγ may also inhibit *nfkbia* translation and enhance NFκB activity by inhibiting the phosphorylation and activation of eIF4E (102). eIF4E-dependent inhibition of IκBα is controlled by MAPK and mammalian target of rapamycin (mTOR) pathways (98, 102). Interestingly, translation inhibition of IκBα significantly upregulates IFNβ production in response to double-stranded RNA stimulation (98). This observation hints at the possibility of positive feedback regulation of NFκB activity

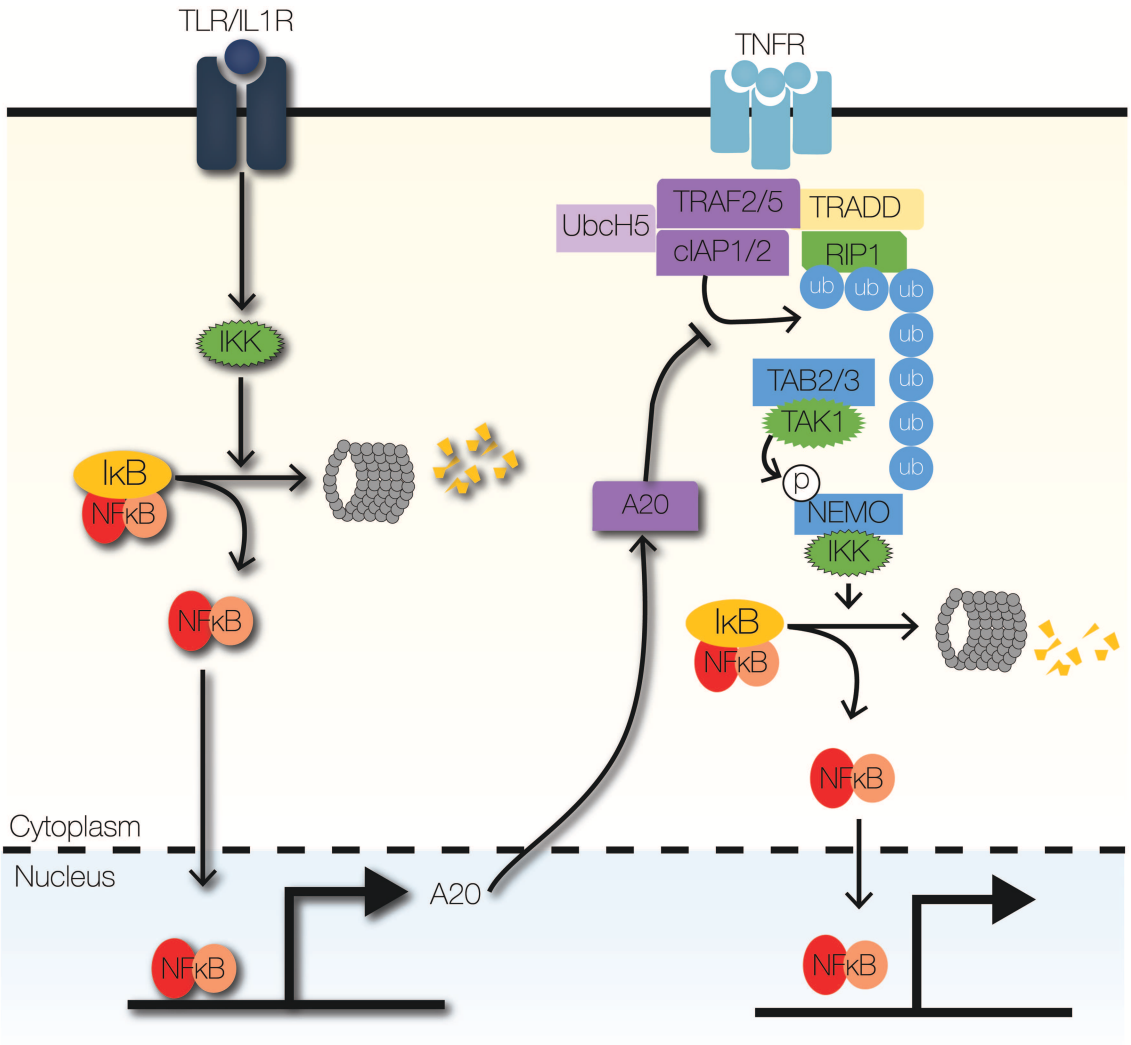

FIGURE 4 | Signaling crosstalk at enzymatic complexes. TLR signaling can modulate TNF signaling through the actions of A20, a ubiquitin-editing enzyme. A20 inhibits the recruitment of IKK to the TNFR signaling complex by inhibiting K63-linked ubiquitination of RIP1. Further, A20 increases the degradation of RIP1 by facilitating K48-linked ubiquitination of RIP1.

by type I IFNs. Currently, detailed investigations to examine this positive feedback regulation are lacking.

IκB Degradation

Control of IκB degradation can mediate signaling crosstalk to NFκB (**Figure 5B**). IFNγ enhances NFκB activity by enhancing the degradation of free IκBα, which are unbound to NFκB dimers (19). Free IκBs have short half-lives (<10 min) and can be degraded independently of IKK activity and ubiquitination (99, 103); however, proteolysis of free IκBs is dependent on proteasomal degradation (99, 103). IFNγ enhances proteolysis of free IκBα by the immunoproteasome, which shares the 20S core of the 26S proteasome, but utilizes an 11S cap rather than a 19S cap (19, 104). IFNγ upregulates key components of the IκBα-associated 11S cap: PA28α and PA28β (19). Furthermore, pathological TNF signaling enhances NFκB activity by upregulating the degradation of IκBε by the immunoproteasome in a murine model of inflammatory bowel disease (105). TNF induces the expression PA28γ component of the immunoproteasome cap in colonic epithelial cells, which leads to severe colonic inflammation due to elevated NFκB activity (105).

NFκB Trapping

Cytoplasmic trapping of RelA:p50 dimers by high-molecular weight IκB complexes (IκBsomes) permits multiple layers of inflammatory regulation (106, 107). It provides a gateway for crosstalk through developmental signals and provides a history of recent inflammatory signaling (**Figure 5A**). Members of the TNF receptor superfamily that transduce developmental signals, such as B-cell activator factor and lymphotoxin-β (LTβ), induce degradation of IκBδ, which is induced in

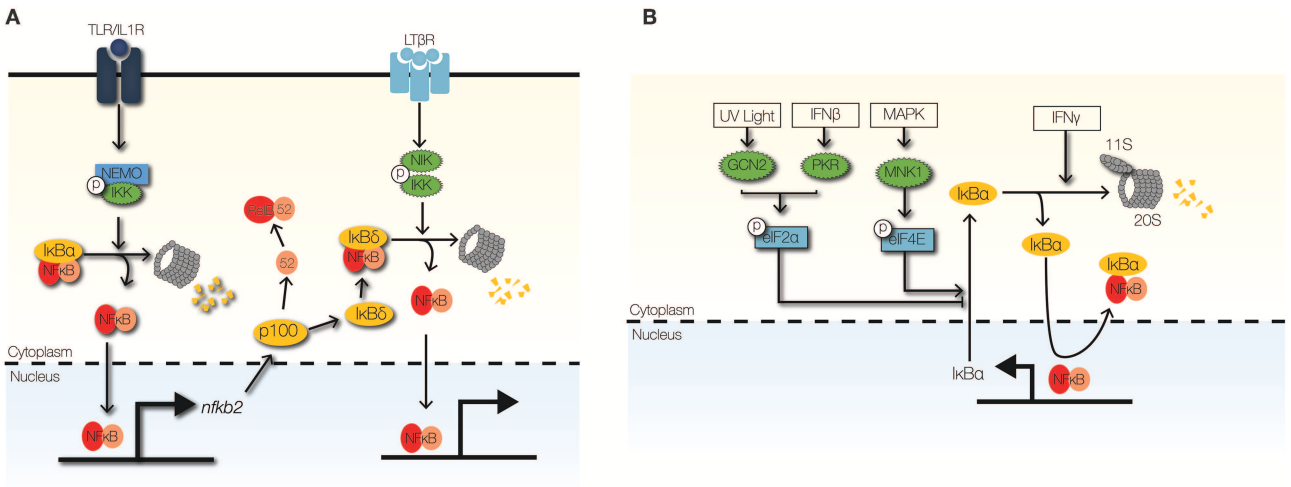

FIGURE 5 | Signaling crosstalk at the IκB module. **(A)** The non-canonical NFκB signaling pathway can cross-regulate the canonical NFκB through NIK-IKK1-mediated degradation of IκBδ. High-molecular weight complexes of IκBδ trap RelA:p50 dimers in the cytoplasm to limit inflammatory NFκB activity. **(B)** Stimulus-responsive transcription initiation factors regulate the synthesis of IκBα. GCN2 and PKR phosphorylate eIF2α to inhibit IκBα synthesis in response to UV light and IFNβ, respectively. In contrast, phosphorylation of eIF4E by MNK1 stabilizes the IκBα mRNA. IFNγ promotes the proteolysis of IκBα/ε by upregulating the 11s cap of the immunoproteasome.

response to inflammatory stimuli such as TLR ligands (108, 109). Although it is induced less rapidly than IκBα, IκBδ possesses a longer half-life and may function as a late brake on NFκB activity (110). Since IκBδ levels are invariant to canonical IKK-degradation, IκBδ functions as regulator of available NFκB dimers that can be activated by inflammatory stimuli (108). Finally, in the absence of IκBδ, priming with TNF or IL1β enhances NFκB signaling rather than inhibiting NFκB signaling (110).

CONCLUDING REMARKS

Maintaining a delicate balance between effective host defense and deleterious inflammatory responses requires precise control of NFκB signaling (111). Multiple regulatory circuits have evolved to fine-tune NFκB-mediated inflammation through context-specific crosstalk (112). In this work, we have highlighted specific components of the NFκB signaling pathway for which crosstalk regulation is well-established. Despite decades of research, our current understanding of NFκB signaling remains insufficient to yield effective pharmacological targets (111, 113). Effective and specific pharmacological modulation of NFκB activity requires detailed, quantitative understanding of NFκB signaling dynamics (57). Furthermore, achieving cell-type and context-specific modulation of NFκB would be a panacea for many autoimmune and infectious diseases, as well as malignancies (112–114).

To dissect the dynamic regulation of NFκB signaling, quantitative approaches with single-cell resolution are required (115). By measuring the full distribution of signaling dynamics and gene expression in single cells, rather than simple averages, one can decipher cell-intrinsic properties from tissue-intrinsic properties (116–118). Such single-cell analyses may reveal strategies for targeting pathological cell populations with high specificity, which can mitigate adverse effects of pharmacological therapy (57, 113). Furthermore, with the aid of mathematical and computational modeling, one can conduct experiments *in silico* that may be prohibitive *in vitro* or *ex vivo* (57, 119, 120).

Finally, cross-regulatory pathways may fine-tune NFκB activity in a gene-specific manner. Many studies have identified the molecular components of gene-regulatory networks (GRNs) that control NFκB-dependent gene expression (15, 121). The regulatory mechanisms that define the topology of these GRNs include chromatin remodeling, transcription initiation and elongation, and post-transcriptional processing (15). They allow for combinatorial control by multiple factors and pathways, as well as cross-regulation (15). Further work will be required to delineate them in various physiological contexts.

AUTHOR CONTRIBUTIONS

AA conducted the literature review, prepared figures, and wrote the manuscript. AH provided supervision, outlined the scope, and edited the manuscript.

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Assaying Homodimers of NF-κB in Live Single Cells

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NF-κB is a family of heterodimers and homodimers which are generated from subunits encoded by five genes. The predominant classical dimer RelA:p50 is presumed to operate as "NF-κB" in many contexts. However, there are several other dimer species which exist and may even be more functionally relevant in specific cell types. Accurate characterization of stimulus-specific and tissue-specific dimer repertoires is fundamentally important for understanding the downstream gene regulation by NF-κB proteins. *In vitro* assays such as immunoprecipitation have been widely used to analyze subunit composition, but these methods do not provide information about dimerization status within the natural intracellular environment of intact live cells. Here we apply a live single cell microscopy technique termed Number and Brightness to examine dimers translocating to the nucleus in fibroblasts after pro-inflammatory stimulation. This quantitative assay suggests that RelA:RelA homodimers are more prevalent than might be expected. We also found that the relative proportion of RelA:RelA homodimers can be perturbed by small molecule inhibitors known to disrupt the NF-κB pathway. Our findings show that Number and Brightness is a useful method for investigating NF-κB dimer species in live cells. This approach may help identify the relevant targets in pathophysiological contexts where the dimer specificity of NF-κB intervention is desired.

Keywords: RelA, NF-κB, transcription factor, number and brightness, microscopy, oligomerization, dimerization

INTRODUCTION

Nuclear Factor-kappaB (NF-κB) is arguably the most important signaling pathway involved in immune responses (1). The specificity of NF-κB action as a transcription factor (TF) is partly mediated by the particular dimers that translocate into the nucleus in response to extracellular stimuli or stress (**Figure 1A**). In the nucleus, NF-κB homo- and hetero-dimers (**Figure 1B**) reversibly interact with specific DNA sequence motifs to activate the transcription of hundreds of target genes (2, 3). Depending on which of the 5 different NF-κB TF proteins comprise the dimers that translocate to the nucleus (4), different gene expression profiles can be induced (5, 6). Yet, because the NF-κB TF family is comprised of up to 15 different dimer species (1) (**Figure 1B**), developing a thorough understanding of how individual NF-κB dimers regulate transcription has proved to be an exceedingly difficult task.

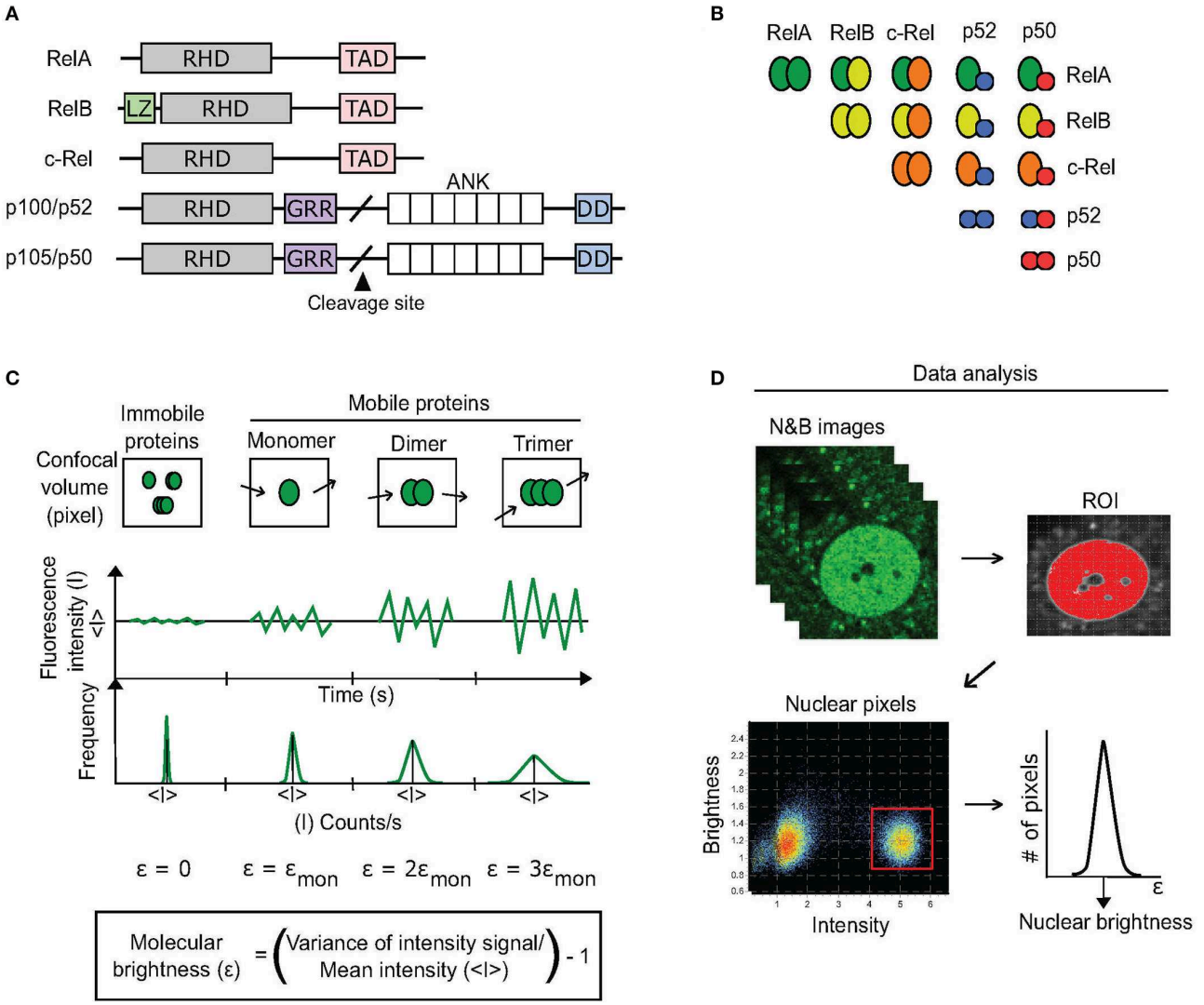

FIGURE 1 | N&B approach to discern dimerization status of RelA. **(A)** There are five homologous proteins of the NF-κB TF family. All contain a Rel homology domain (RHD) that promotes dimerization with other RHD-containing proteins as well as DNA binding. RelA, RelB, and c-Rel each also contain a transcription-activation domain (TAD) that enables them to activate transcription. Cleavage of p105 and p100 produces p50 and p52, respectively. Additional domains include: LZ, leucine zipper; GRR, glycine-rich region; ANK, ankyrin-repeat domain; DD, death domain. **(B)** 15 potential NF-κB dimers exist based on RHD interactions. **(C)** The N&B assay measures the oligomer state of a protein by determining its molecular brightness (ε) within a region of interest (ROI) in a cell. A fluorescent protein's molecular brightness is determined by calculating the fluctuations (variance) in mean fluorescence intensity (<I>) that are caused by the movement of protein oligomers (monomers, dimers, trimers, k-mers) within every pixel (confocal volume) of the ROI over time (confocal imaging acquisition). The ratio of the variance to the mean fluorescence intensity of the pixels is equal to the protein's brightness (ε) + 1. Because immobile proteins do not produce such movement-based fluctuations, their molecular brightness is equal to 0. **(D)** For quantifying a protein's brightness within an ROI, in our case the nucleus, the brightness values of each pixel comprising the ROI are extracted from a stack of N&B images (see Methods). The brightness values are then fitted to a Gaussian distribution to determine the protein's overall brightness within the ROI.

Molecular biology approaches such as immunoprecipitation, immunoblotting, and EMSAs are not particularly well-suited for investigating more than a few dimer species simultaneously. Importantly, they cannot be used to analyze dimers in live intact single cells and do not distinguish between different oligomer species. Advances in imaging technologies have revolutionized biological research by superseding many of the constraints inherent to classical molecular biology techniques. In addition to enabling the visualization of molecular biological processes in real-time, microscopy techniques enable high throughput time-course measurements from the same individual cell in a relatively direct and non-invasive manner. Despite the importance of NF-κB, to our knowledge only a few imaging studies have investigated the dimerization status of this TF in living cells, using fluorescence cross-correlation spectroscopy (FCCS) or Förster-resonance energy transfer (FRET) (7–9).

The Number and Brightness (N&B) assay is a live-cell imaging technique that measures the aggregation or oligomerization state of proteins of interest in a specific area of a cell, such as the nucleus (10). Its use has revealed insights into the oligomerization status of crucial TFs, including the hormone receptor transcription factors: glucocorticoid receptor (GR) (11–13), androgen receptor (AR) (12), and progesterone receptor (PR) (12); as well as CCAAT/enhancer-binding protein alpha (C/EBPα) (12). It has also been used to quantify the aggregation of DNA (14), Huntingtin (15), and amyloid peptides (16), as well as other proteins (17–26). Besides a brief inclusion in a report (13), the N&B assay has not been used to study NF-κB TF dimers.

We conducted a series of experiments to explore the technological feasibility of using the N&B assay to measure the homodimer status of the NF-κB TF RelA in single living cells. We discovered that the N&B assay detects the presence of a mixed RelA dimer status in the nuclei of stimulated immortalized and primary fibroblasts, with RelA homodimers seemingly comprising a substantial proportion of the overall RelA dimer species. Moreover, we obtained evidence suggesting that the N&B assay can be used to quantify pharmacological perturbations of NF-κB dimers.

METHODS

Materials

The mEGFP-N1 (54767) plasmid from Michael Davidson, and the RelA cFlag pcDNA3 (20012) (27) from Stephen Smale, were purchased from Addgene. The pSF-EF1α-Ub-Neo vector (OG606) was purchased from Oxford Genetics. The mEGFP-mutGR (monomeric glucocorticoid receptor (GR) mutant; monomer control) (GFP-GRN525), mEGFP-GR (wild-type GR), mEGFP-AR (androgen receptor), and mEGFP-PR (progesterone receptor) plasmids, as well as the dihydrotestosterone (DHT) and progesterone (PR), were previously described (12) and kindly provided by the Hager lab (NIH, Bethesda, MD). Additional reagents included lipopolysaccharide (LPS) (Enzo Life Sciences; ALX-581-008); TNFα (R&D; 410-MT-010); dexamethasone (Dex) (Sigma; D4902); withaferin A (WFA) (681535; EMD Millipore); and trichostatin A (TSA) (Sigma; T8552). Primary antibodies included polyclonal rabbit anti-RelA (Santa Cruz; SC-372), monoclonal rabbit anti-p50 (Santa Cruz; sc-114) (which also detects p105), polyclonal rabbit anti-RhoGDI (Sigma; R3025), and monoclonal rabbit anti-GAPDH (Cell Signaling Technology; 14C10). Secondary antibody consisted of polyclonal anti-rabbit IgG (Jackson Immunoresearch; 211-035-109).

Cloning

To generate the mEGFP-RelA plasmid encoding an N-terminal fusion protein, cDNA was amplified by PCR using Phusion polymerase mix (New England BioLabs (NEB); M0532S) and the indicated primers (**Table S1**). Constructs were digested with restriction enzymes from NEB (EcoR1-HF, R3101S; EcoRV-HF, R3195S; BsrGI-HF, R3575S) and ligated into the pSF-EF1α-Ub-Neo vector using Promega LigaFast Rapid DNA Ligation kit (Fisher Scientific; PR-M8221). The resulting plasmid was used to transform DH5α competent cells (ThermoFisher; 18265017). Plasmid derived and expanded from a single antibiotic resistant clone was purified using an EndoFree Plasmid Maxi kit (Qiagen; 12362). Plasmid construction was verified by DNA sequencing.

Cell Culture and Transfections

NIH3T3 cells (CRL-1658) were purchased from ATCC. Cells were cultured and maintained in a 37°C humidified environment of 5% CO_2/95% air in growth media composed of phenol-red-free Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; 21063-029) supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts; 100-500) and 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (1% p/s/g) (Gibco; 15140-122). Passaging of cells was performed using brief exposure to 0.25% trypsin-EDTA (Gibco; 25200-056). Cells in DMEM containing only 10% FBS (lacking p/s/g) were transiently- or stably-transfected with the respective expression vectors using Fugene HD (Promega; E2311) according to the manufacturer's protocol. Pools of cells with stable-integration of EF1α-promoter driven mEGFP-RelA were obtained via selection in G418 sulfate solution (500 μg/mL) (Mirus; MIR5920). Expression and function of the fluorescent fusion proteins was tested through a combination of immunoblotting and confocal microscopy (**Figure S1**). Primary mouse adult fibroblasts (MAFs) for the N&B assay were obtained from ear pinna minced and digested in 200 μg/mL Liberase TM (Sigma; 5401119001) in a 37°C water bath for 1 h. Digested pinna were then diluted 5-fold in growth media and centrifuged at 1,000 rpm for 5 min. Liberase-containing media was aspirated, and cells were resuspended in growth media and cultured for ∼1 week. Upon reaching greater than ∼50%, but less than ∼90% confluence, primary fibroblasts were passaged using 0.25% trypsin-EDTA using routine cell culture procedures. Primary cells were passaged no more than twice before being used in the study.

Immunoblotting Lysates of Stably-Transfected Cells

Cells were lysed in ice-cold RIPA cell lysis buffer (Millipore; 20-188) containing Complete Ultra Mini protease inhibitors (Roche; 05892970001). Lysates were vortexed, centrifuged at 13,000 rpm for 15 min, and homogenized using insulin syringes. Concentrations of proteins were estimated using Protein Assay Dye Reagent Concentrate (Biorad; 5000006). Samples containing equal amounts of protein were heated at 95°C for 5 min in LDS sample buffer (ThermoFisher; NP0007) containing 10% 2-Mercaptoethanol (Gibco; 31350-010) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 4–12% NuPage Bis-Tris pre-cast gels (ThermoFisher; NP0322BOX) and MOPS buffer (ThermoFisher; NP0001). Proteins were transferred using transfer buffer (ThermoFisher; NP00061) and 0.45 μm PVDF membranes (Millipore; IPVH00010). Membranes were blocked for 30 min in 5% (w/v) non-fat milk and then sequentially incubated with primary and HRP-conjugated secondary antibodies. HRP activity was detected using SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher; 34580).

Microscopy for Generation of Stably-Transfected Cells

All imaging was performed using a Zeiss LSM880 AxioObserver confocal microscope with an associated environmental chamber for live-cell imaging. During imaging all cells were maintained in a 37°C humidified environment of 5% CO_2/95% air. Cells were seeded at medium confluence on 35 mm glass-bottomed dishes (MatTek; P35G-1.5-20-C) and cultured overnight before imaging in phenol-red-free growth media. Images were acquired using a 488 nm laser and a 40X/1.4 NA Plan-Apochromat oil-immersion objective with a fully open pinhole (600 μm).

Microscopy for the N & B Assay

All imaging was performed using the same hardware and cell culture conditions as described above, unless described otherwise. Before imaging, cells were treated with indicated concentrations of ligands, as described in the Results section and associated figure legends. Treatment was not required to induce nuclear localization of the monomeric control (mEGFP-mutGR) as it is constitutively nuclear. mEGFP-GR nuclear localization was induced by pre-incubating cells with Dex (100 nM) for at least 1 h prior to imaging. mEGFP-GR maintained nuclear localization throughout the course of the experiments which typically lasted no more than 1–2 h after beginning imaging (per 35 mm dish). When mEGFP-RelA-expressing cells were treated with TNFα (10 ng/mL) or LPS (100 ng/mL), subsequent nuclear translocation was evident within 15–45 min and persisted for no more than ∼1 h during which time N&B imaging was performed. For each nucleus analyzed, N&B microscopy was performed as previously described with minor adjustments (12). Briefly, a time-lapse stack of 150 images (256 × 256 pixels) was acquired using a 63X/1.4 NA Plan-Apochromat oil-immersion objective; a pinhole corresponding to 1 Airy unit; and a zoom of 6.6. Dwell-time per pixel was 8.24 μs with a subsequent frame scan time of 1.27 s. Laser power (488 nm) was set at 3% to detect near endogenous levels of mEGFP-RelA (**Figure S1**). Fluorescence signal was detected using a gallium arsenide phosphide (GaAsP) detector set to photon-counting mode. Additional imaging parameters include performing the 16-bit acquisition using a digital gain of 0.2. Images were acquired using one direction scanning, rather than bidirectional, to ensure a fixed re-sampling time of the mobile fluorescent proteins. No averaging was performed for line acquisitions. Nuclei were excluded from imaging if: they were not the only nuclei within a cell; they were radically misshapen or not intact; or if they had an approximate average fluorescence intensity under 4 or >17 (well-below pixel saturation values). Moreover, if nuclei exhibited significant rotational movement, or horizontal or lateral movement of greater than ∼1 μm, they were discarded from analysis. The first 10 frames of each image stack were discarded to remove the initial impact of photobleaching from measurements. Image stacks were analyzed using the N&B option of the "GLOBALS for Images" software developed by the Laboratory for Fluorescence Dynamics (University of California, Irvine, CA), with the divider set to 1.

Dimeric Population Estimation

Since N&B cannot separate mixtures of oligomeric states, the resulting brightness value represents a weighted-average combination of the species present in the illumination volume (10). In general, the dependence of the brightness value is given by a non-linear combination of the brightness and the occupation number of each species (28). Assuming RelA can only exist in monomeric or dimeric forms, then the expected brightness (ε_{exp}) obtained by the N&B assay is given by:

$$\varepsilon_{exp} = \frac{\varepsilon mon^2 * Nmon + \varepsilon dim^2 * Ndim}{\varepsilon mon * Nmon + \varepsilon dim * Ndim}$$

Where εmon and εdim represent the brightness of the monomeric and dimeric species (i.e., values of 1 and 2, respectively); and Nmon and Ndim are the molar fraction of monomers and dimers, respectively.

Mice

The *Nfκb1*$^{-/-}$*Rel*$^{-/-}$ mice (herein referred to as p50/c-Rel double-KO mice) were generated by intercrossing *Nfκb1*$^{-/-}$ mice (B6.Cg-*Nfκb1*^{tm1Bal}/J; Jackson Laboratory) and *Rel*$^{-/-}$ mice (29). The mEGFP-RelA knock-in (KI) mice were generated by inserting the mEGFP coding sequence (without stop codon) after the start codon (ATG) of RelA at the endogenous locus using CRISPR/Cas9 editing technology. Briefly, specific sgRNAs targeting the proximity region of RelA start codon were designed using the online tool MIT CRISPR Design (crispr.mit.edu) and generated *in vitro* using MEGAshortscript T7 transcription kit (Thermo Fisher Scientific; AM1354). sgRNAs were purified using MEGAclear kit (Thermo Fisher Scientific; AM1908). A double strand (ds) DNA donor template (∼7 kb) containing the mEGFP fusion sequence was obtained from Genewiz (genewiz.com). Cas9 mRNA (TriLink Biotechnologies; L-6125), sgRNAs and dsDNA donor template were microinjected into one-cell stage zygotes obtained from C57BL/6Ncr × B6D2F1/J mice to generate mEGFP-RelA KI animals (EM, FTA, LT, MHS, in preparation). *Nfκb1*$^{-/-}$*Rel*$^{-/-}$ and mEGFP-RelA KI MAFs were obtained as described above. Wild-type equivalent *Nfκb1*$^{+/+}$*Rel*$^{+/+}$ MAFs were obtained from B6.129-Il12b^{tm1Lky}/J mice (Jackson Laboratory), as the p50/c-Rel double-KO mice were bred using mice homozygous for both alleles. All MAFs were obtained from female mice aged 7–14 weeks. All mice were maintained under specific pathogen-free conditions at the animal facility of National Institute on Aging, and animal care was conducted in accordance with the guidelines of NIH.

Co-immunoprecipitation of RelA Dimers

Whole-cell extracts from 10 × 10^6 cells per condition were prepared in 1 mL of cell lysis buffer containing 1% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and Complete Ultra Mini protease inhibitors (Roche; 05892970001). Lysates were centrifuged at 13,000 rpm for 10 min at 4°C. The cleared supernatants were collected and 50 μL of the supernatant was saved as the input sample. For immunoprecipitation, 1.5 μg of rabbit anti-RelA antibody (Santa Cruz; sc-372) was added to 80 μL of Dynabeads protein A (Invitrogen; 10001D) in 500 μL

of phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) for 20 min at room temperature to form bead-antibody complexes. The remaining cell lysate supernatants (~950 μL) were added to the bead-antibody complexes and incubated with rotation overnight at 4°C. The beads were washed three times with PBST and then the immunoprecipitated complexes were eluted in 25 μL of a 1:1 dilution of elution buffer (Invitrogen; 10006D) and 2xSDS sample buffer (Novex; LC2676) by heating at 70°C for 10 min. 25 μL of eluted protein complexes were resolved with a 8–16% Tris-Glycine gel (Invitrogen; XP08160) and visualized by immunoblotting using indicated antibodies.

Statistical Analysis

Quantitative data are represented as median values with their respective quartiles unless stated otherwise. Significance (relative to controls) was tested using unpaired two-tailed Student's *t* test. *p*-values ≤ 0.05 were deemed statistically significant.

RESULTS

RelA Homodimers Comprise a Substantial Proportion of Nuclear NF-κB in Live 3T3 Fibroblasts

Briefly, the N&B assay measures the oligomer state of a protein (monomer, dimer, multimer) within a cell by determining its molecular brightness (ε) via confocal microscopy (10) (**Figures 1C,D**). To do so, the approach relies on fluorescently-tagged proteins and specialized image analysis software (see Methods). In a model system, the molecular brightness of fluorescently-tagged proteins that exist as monomers is set to one. The fluorescent proteins that form homodimers have molecular brightness value of two, while k-mer proteins have brightness of k (**Figure 1C**). In cells, proteins can also exhibit intermediate values of molecular brightness (e.g., ε = 1.5-fold the brightness of monomers), which can be indicative of mixed populations of oligomeric species.

To investigate the state of RelA-containing dimers using the N&B assay, we transiently-transfected NIH3T3 (3T3) fibroblasts with a plasmid encoding the fluorescent fusion protein mEGFP-RelA. We also generated 3T3 cells stably-expressing mEGFP-RelA (**Figure S1**). The cells were then treated with one of two stimuli [tumor necrosis factor-alpha (TNFα), which produces a quick but oscillatory response (30), or lipopolysaccharide (LPS), which produces a delayed response (31, 32)]. Relative to a mEGFP-tagged monomer control with a brightness of ~1, we observed a median brightness (ε) value of 1.52 for RelA in the nucleus of transiently-transfected cells treated with TNFα (**Figures 2A–C**). Because of the numerous possible RelA-containing dimers among which only mEGFP-RelA-tagged homodimers are presumed to produce brightness values of ε = 2, while the others are to give ε = 1 (**Figure 2A**), the observed brightness value suggests that RelA homodimers form a considerable portion (roughly 35%, see Methods for details) of all potential RelA dimer species in individual fibroblasts (**Figure 2A**). When 3T3 fibroblasts stably-expressing mEGFP-RelA were treated with TNFα, a similar nuclear brightness value of 1.61 (~43% dimers) was detected (**Figures 2B,C**). Furthermore, when treated with LPS, a nuclear RelA brightness value of 1.75 (~60% dimers) was obtained (**Figures 2B,C**). As a comparison control, we transiently-transfected 3T3 fibroblasts with cDNA encoding a different TF, mEGFP-tagged glucocorticoid receptor (mEGFP-GR), which is known to form homodimers in the nucleus (13). Upon treating mEGFP-GR-expressing cells with the GR-ligand dexamethasone (Dex), we observed a nuclear brightness value of ε = 1.52 (**Figures 2B,C**), suggesting that a substantial portion of nuclear GR (including dimers containing endogenous untagged GR) was present as homodimers of mEGFP-GR, as expected.

To confirm whether the N&B assay, in our hands, could quantify the presence of TF oligomers with brightness values

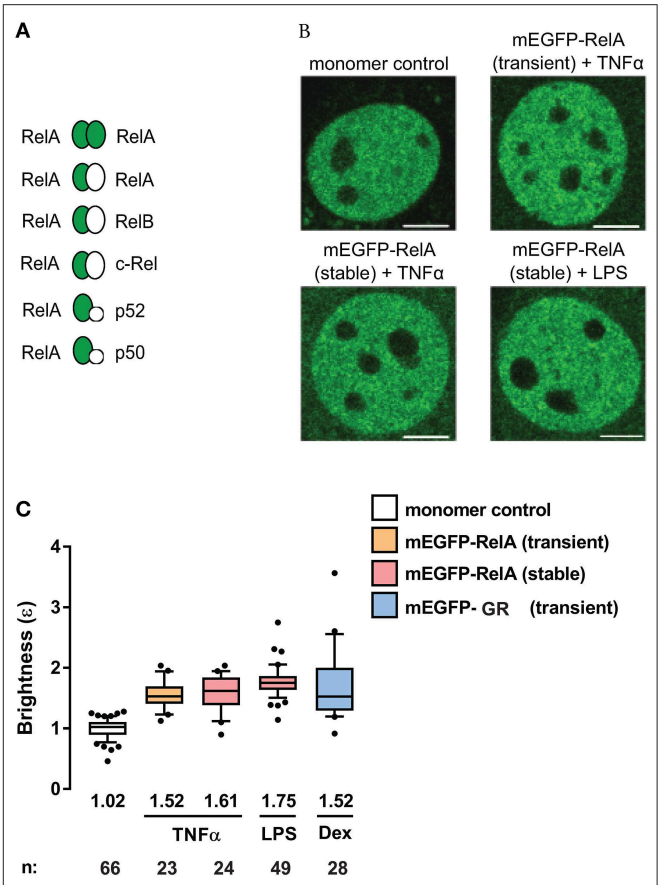

FIGURE 2 | RelA exhibits substantial homodimer levels in mouse fibroblasts. **(A)** Schematic of possible mEGFP-RelA (green ellipse) interactions with mEGFP-RelA or other non-mEGFP-tagged NF-κB proteins (white ellipse). **(B)** Representative confocal micrographs of nuclei in 3T3 fibroblasts transiently-expressing monomer control, transiently-expressing mEGFP-RelA, or stably-expressing mEGFP-RelA under different treatment conditions. Image intensity scale was adjusted for optimal viewing. Scale bar: 5 μm. **(C)** Quantification of nuclear mEGFP-RelA brightness (ε) values relative to the monomer control in transfected cells (B) treated with 10 ng/ml TNFα, 100 ng/ml LPS, or 100 nM Dex. Data was obtained from at least two independent experiments performed on different days. Whiskers are drawn down to the 10th percentile and up to the 90th percentile. Number of nuclei and median values of each sample are presented below each boxplot.

greater than those of RelA or GR, we assayed TFs known to form higher-order oligomers in the nucleus. Fibroblasts were transiently-transfected with mEGFP-AR (androgen receptor) or mEGFP-PR (progesterone receptor) and treated with their ligands dihydrotestosterone or progesterone, respectively (**Figure S2**). Upon activation, we observed a nuclear brightness value of $\varepsilon = 2.67$ for AR and a brightness of $\varepsilon = 2.07$ for PR, indicating that AR and PR form higher-order oligomers in immortalized fibroblasts (**Figure S2**) as previously shown (12). Moreover, detection of brightness values $\varepsilon > 2$ verified a sufficiently wide N&B dynamic range for studying various oligomers using this assay. As we did not perform the experiments in RelA, GR, AR, or PR knockout cells, we could not quantify the impact of untagged endogenous RelA, GR, AR, and PR proteins on the observed brightness data.

Primary Fibroblasts Maintain RelA Homodimers Independently of c-Rel and p50

Having observed RelA homodimers representing a substantial proportion of RelA-containing dimers in immortalized fibroblasts, we next sought to extend our analysis to primary cells. Therefore, we transiently-transfected wild-type (WT) mouse adult fibroblasts (MAFs) with plasmids encoding the monomer control or mEGFP-RelA, and treated the RelA-transfected cells with LPS. The N&B assay resulted in a relative RelA nuclear brightness value of $\varepsilon = 1.29$ in the primary fibroblasts (**Figures 3A,B**). While this value indicates a relatively lower proportion of RelA homodimers in comparison to immortalized 3T3 fibroblasts, it confirms that a substantial portion (roughly 20%) of RelA-containing dimers in the nucleus exist as homodimers in stimulated primary fibroblasts.

As previous studies have indicated the presence of RelA:p50 and RelA:c-Rel heterodimers (33), we next tested whether eliminating RelA binding partners (p105/p50 and c-Rel) would result in an increased relative abundance of RelA homodimers. To that end, we obtained MAFs from p50 (*Nfκb1*)/c-Rel (*Rel*) double-knock-out (KO) mice and performed the N&B assay. Surprisingly, we obtained a nuclear RelA brightness value of $\varepsilon = 1.31$ in the double-KO cells (**Figures 3A,B**) relative to the monomer control, suggesting roughly equivalent levels of RelA homodimers in the WT and double-KO fibroblasts. The result was likely due to tagged RelA monomers readily forming dimers with untagged RelA monomers made available due to the lack of c-Rel and p50 protein (**Figure 2A**), as well as interactions with other untagged NF-κB TF monomers (**Figure 2A**), rather than substantially increasing levels of tagged RelA homodimers.

Dimerization of NF-κB Subunits Can Be Perturbed by Small Molecules

A recent study by Dikstein and colleagues reported that Withaferin A (WFA), a naturally occurring anti-inflammatory and anti-cancer phytochemical, disrupts RelA dimerization by interacting with a conserved hydrophobic core domain and dimerization scaffold within RelA and other NF-κB subunits (34). To investigate whether small molecules such as WFA can disrupt RelA-containing dimers in living cells, we performed the N&B assay on the 3T3 fibroblasts stably-expressing mEGFP-RelA, pretreated with either 0.5 or 1 μM WFA for 1 h and stimulated with LPS (**Figure 4A**). Due to the pleiotropic effects of WFA, including IKKβ hyperphosphorylation (35), WFA concentrations >1 μM completely inhibited nuclear translocation of mEGFP-RelA. Therefore, we used lower concentrations (≤1 μM) for our assay. RelA nuclear brightness values decreased from $\varepsilon = 1.75$ for

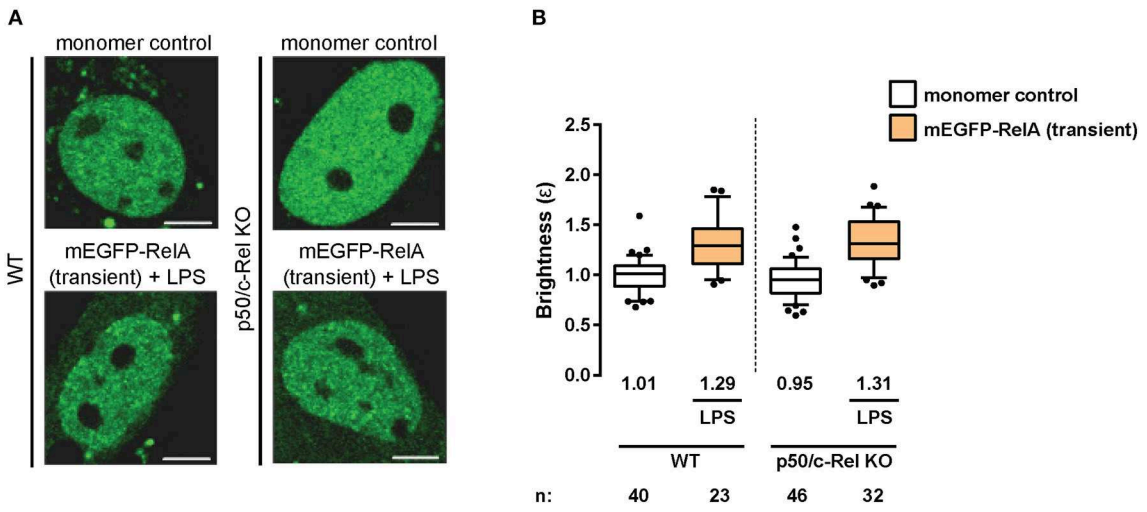

FIGURE 3 | RelA homodimers in primary fibroblasts. **(A)** Representative confocal micrographs of nuclei in wild-type (WT) and p50/c-Rel double-KO primary MAFs transiently-expressing monomer control or mEGFP-RelA. The latter were treated with LPS (100 ng/mL). Image intensity scale was adjusted for optimal viewing. Scale bar: 5 μm. **(B)** Nuclear RelA brightness (ε) values relative to the monomer control in transfected primary fibroblasts **(A)** treated with 100 ng/ml LPS. Data was obtained from at least two independent experiments performed on different days. Whiskers are drawn down to the 10th percentile and up to the 90th. Number of nuclei and median values of each sample are presented below each boxplot.

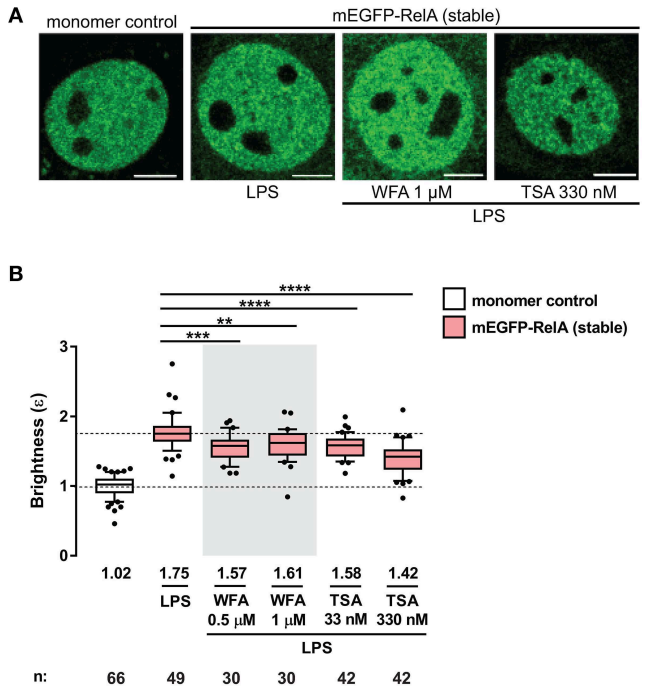

FIGURE 4 | Perturbation of RelA homodimers by pharmacological agents. **(A)** Representative confocal micrographs of nuclei in transiently- and stably-transfected 3T3 fibroblasts pre-incubated with WFA or TSA before LPS treatment (100 ng/mL). Control and LPS images are the same as those in **Figure 2B**. Image intensity scale was adjusted for optimal viewing. Scale bar: 5 μm. **(B)** Brightness (ε) values of nuclear RelA in fibroblasts under different conditions. Control and LPS samples are the same as those in **Figure 2C**. Data was obtained from at least two independent experiments performed on different days. Whiskers are drawn down to the 10th percentile and up to the 90th. Number of nuclei and median values of each sample are presented below each boxplot. Unpaired two-tailed Student's *t*-test (**$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$) was performed for statistical comparisons.

LPS-only treated cells (**Figure 4B**) to ε = 1.57 or ε = 1.61 for cells exposed to either 0.5 μM or 1 μM WFA prior to LPS treatment, respectively (**Figure 4B**). Although modest, the WFA-induced decrease in the brightness of RelA suggests that WFA perturbs the abundance of RelA homodimers and that such changes are quantifiable using the N&B assay.

We also examined the effects of another small molecule, trichostatin A (TSA), a class I and II histone deacetylase (HDAC) inhibitor which alters the global chromatin landscape by increasing the level of acetylated histones. To test whether RelA dimerization is affected by TSA, 3T3 fibroblasts stably-expressing mEGFP-RelA were pretreated with TSA for 2 h before LPS stimulation and N&B analysis (**Figure 4**). Interestingly, TSA-pretreatment significantly reduced brightness values of RelA. RelA brightness ε decreased from 1.75 in the nuclei of LPS-only treated cells (same control as for WFA) to 1.58 or 1.42 in the nuclei of TSA-pretreated cells, depending on the concentration of TSA (**Figure 4B**). Since the fraction of chromatin-bound TFs can range from 20% to 50% in live cells (36, 37), an intriguing possibility is that the global alteration in chromatin induced by TSA may influence the dimerization status of RelA. Such a reverse (gene to TF) action has been reported, where specific chromatin interfaces result in allosteric conformational changes of GR which impact gene-specific regulation (38). On the other hand, the HDAC inhibitor effect may be partly through non-histone targets, including acetylation of RelA (39–41) or RelA-regulating proteins (42, 43). The mechanisms of TSA action underlying the unexpected dimer perturbation are beyond the scope of this report and will require separation of effects on histones and non-histone targets.

Finally, we attempted to measure the perturbation of RelA-containing complexes (RelA:RelA, RelA:p50, and RelA:p105) by WFA and TSA using the conventional methods of immunoprecipitation and immunoblotting. While a trend toward a reduced abundance of RelA:p105 was observed for LPS-treated cells pre-incubated with WFA or TSA, the overall results were inconclusive (**Figure S3**). We suspect that the *in vitro* biochemical methods lack the sensitivity for detecting subtle changes in TF dimer composition that occur within intact live cells, highlighting the potential utility of the N&B assay.

DISCUSSION

The function of NF-κB TFs has been widely studied over the years in various cell-types and biological contexts. However, studies focusing on the dimerization states of NF-κB TFs have been relatively scarce due to the difficulties associated with obtaining and interpreting *in vitro* biochemical data. Nevertheless, using systems-based *in silico* modeling and experimental validations, a previous study indicated that RelA homodimers constitute ∼25% of total RelA-containing dimers in mouse embryonic fibroblasts (44). The remainder of RelA dimers were determined to be comprised of other dimers, mostly RelA:p50 (44) (**Figure 2A**). Our N&B data from primary transfected MAFs suggest ∼20% of RelA-containing dimers are RelA:RelA homodimers (**Figures 3A,B**), which is in accordance with the aforementioned study. However, whereas p105/p50 KO embryonic fibroblasts had increased abundance of endogenous RelA homodimers (to nearly 50% of the total RelA-dimer population) (44), our data suggest that ectopically expressed mEGFP-tagged RelA likely dimerizes with untagged RelA and other NF-κB subunits in the absence of p105/p50 and c-Rel in primary adult fibroblasts (**Figures 3A,B**).

A significant caveat of our study and many live-cell imaging approaches is the use of ectopically expressed fluorescent fusion proteins as well as the presence of untagged endogenous proteins. We mitigated the complications associated with ectopic expression by avoiding cells showing excessively high mEGFP signal; all our N&B data were from individual cells expressing low levels of the transgene. To obtain more definitive information about the composition of NF-κB dimers in living cells, it is imperative to study their biophysics in primary cells where the endogenous locus encoding the relevant subunit is replaced by its fluorescent fusion construct. Such a fluorescent knock-in (KI) reporter would retain the natural regulatory environment and would not harbor any untagged proteins

(45). N&B assay of such KI reporter systems would enable accurate quantification of relative compositions of different NF-κB homodimers and heterodimers in real-time in single living cells. Toward this end, we recently generated such a KI mouse strain. When the N&B assay was performed on TNFα or LPS-treated MAFs obtained from the mEGFP-RelA KI mouse (in which the entire population of RelA protein was tagged with mEGFP (**Figure S4**) (see Methods), the results suggested again that RelA homodimers constitute a significant portion of the overall RelA dimer species in primary fibroblasts (slightly <35%).

The potential utility of the N&B assay in finding drugs that target NF-κB dimerization is evidenced by our observation that pre-treatment with withaferin A (WFA) or trichostatin A (TSA) modestly but significantly reduces RelA homodimer levels upon activation by LPS. Based on the primary roles that RelA and other NF-κB TFs fulfill in various immunological and pathological contexts, drug-induced perturbation of NF-κB dimers may have potent and clinically desirable consequences. With improvements in automated microscopy, the N&B assay may be useful in a drug screening platform in future high-throughput studies of NF-κB dimerization status. We also expect that the N&B assay and other live-cell imaging approaches will continue to reveal valuable information about NF-κB and other TFs in their natural states, thereby shedding light on the functional role of their biophysical characteristics.

DATA AVAILABILITY STATEMENT

The materials and data that support the findings of this study are available upon reasonable request.

ETHICS STATEMENT

This study was carried out in accordance with the guidelines of the National Institute on Aging (NIA) and approved by the NIA Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

EM, SC, and M-HS designed the experiments and interpreted results. DP provided training, plasmids, and valuable assistance for the N&B assay. EM and SC performed the imaging experiments, data analysis, and drafted the manuscript. K-SO designed and performed the co-immunoprecipitation. MK crossbred the p105/p50 and c-Rel KO strains to produce the double KO strain. EM and FT generated the mEGFP-RelA knock-in mice. LT supervised the generation of the knock-in mice. M-HS revised the manuscript with input from all.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02609/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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