Title
Elucidating Selective Gene Regulation by NF-B and Nuclear IκB in Macrophages

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Publication Date
2016

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Elucidating Selective Gene Regulations of NF-κB and Nuclear IκB in Macrophages

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Biology

by

George Yeh

2016

ABSTRACT OF THE DISSERTATION

Elucidating Selective Gene Regulations of NF-κB and Nuclear IκB in Macrophages

by

George Yeh

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2016

Professor Stephen T. Smale, Chair

The NF-κB family of transcription factors (RelA [RELA], c-Rel [REL], RelB [RELB], p50 [NFKB1], and p52 [NFKB2]) regulates many important biological processes including development, immunity, and inflammatory responses. It has a dominant role in regulating gene transcription in almost all mammalian cell types in response to a wide range of external, internal and environmental cues. Understandably, its mis-regulation results in the development of severe diseases, such as chronic inflammatory disorders, autoimmune diseases, and cancer. Although the NF-κB signaling cascade has been extensively studied and each individual NF-κB knockout strain (*Rela*$^{-/-}$ for RELA protein, *Rel*$^{-/-}$ for CREL protein, *Relb*$^{-/-}$ for RELB protein, *Nfkb2*$^{-/-}$ for NFKB2 protein, and *Nfkb1* $^{-/-}$ for NFKB1 protein) exhibited different phenotypes in mouse, whether there exists a distinctive subset of target gene for each member is poorly understood. Our lab previously reported that *Il12b* gene transcription is strongly dependent on c-Rel because of a unique 46-residue of it which results in strong binding to two non-consensus κB sequences in the *Il12b* promote. Here we further investigate the observed selectivity at the genomic scale

using chromatin RNA-seq and ChIP-seq technologies. Strikingly, genome-wise, c-Rel appears to be exclusively dedicated to the expression of *Il12b* in lipid A-stimulated mouse bone marrow-derived macrophages (BMDM), as no other genes demonstrated the same dependence and preference for c-Rel binding.

To carefully modulate NF-κB signaling, numerous regulatory layers are deployed by cells, one of which is mediated by the interaction with nuclear IκB proteins (BCL3 or Bcl-3, NFKBID or IκBNS, and NFKBIZ or IκBζ). Unlike prototypic IκBs (IκBα, IκBβ, and IκBε), which sequester NF-κB in the cytoplasm during homeostasis and release it upon stimulation, nuclear IκBs are located in the cell nucleus and are thought to serve as co-activators or co-repressors. Furthermore, nuclear IκB selectively interact with p50 (NFKB1) and p52 (NFKB2), two of the NF-κB proteins that do not contain transactivation domain (TAD). Both Bcl-3 and IκBζ, but not IκBNS, contain TAD and thus are able to alter the repressive nature of p50 or p52 via protein-protein interaction. To study the regulatory effects of nuclear IκBs in transcription, we perform chromatin RNA-seq and ChIP-seq with lipid A-stimulated BMDM. RNA-seq is performed with *Nfkb1*$^{-/-}$, *Nfkb2*$^{-/-}$, *Bcl3*$^{-/-}$, *Nfkbid*$^{-/-}$, and *Nfkbiz*$^{-/-}$ cells. Lipid A induces the expression of both primary and secondary response genes in BMDMs, including *Nfkb1*, *Nfkb2*, and all three nuclear IκBs. Our results indicate that while the absence of p52, Bcl-3, or IκBNS have only minor effects on TLR4-mediated gene transcription, a subset of inducible genes depends strongly on the expressions of p50 and/or IκBζ. Taking into account the corroborative data from RNA- and ChIP-seq experiments, we delineate and classify genes that are either directly or indirectly regulated by p50 and/or IκBζ.

The dissertation of George Yeh is approved.

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2016

In dedication to my wife and families

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Acknowledgements

I would like to thank my advisor, Stephen Smale, for his continuous support, guidance, and patience throughout my training as a graduate student. I would like to thank the members of my thesis committee Arnold J. Berk, Michael F. Carey, Albert J. Courey, and Reid C. Johnson for their kindness, guidance, and support. I also like to thank all past and present members of the lab who have been an integral part of my graduate journey, particularly Xin Liu, Prabhat Purbey, Peter Kim, Brandon Thomas, Hung-Hao Lo, Poonam Sachan, Cecilia Garcia, Richa Joti, Ann-Jay Tong, Miguel Edwards, Justin Langerman, and Abraham Chang. I am fortunate to have made lasting friendships with so many of you.

Lastly, I would like to thank my wife, Irene, for her forever love and support. My great appreciation goes to my families in Taiwan, especially my parents, my sister, our family dog Boa, who have always been supportive and giving me strengths.

Thank you, thank you, and thank you.

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George Yeh, Abraham Chang, Trevor Siggers, Kevin Williams, Shomyseh Sanjabi, and Stephen Smale. **2016**. Selective Regulation of *Il12b* Transcription by NF-κB c-Rel. Gene Expression & Signaling in the Immune System. Cold Spring Harbor Laboratory. (Poster)

Chapter 1

NF-κB, Nuclear IκB, and the Immune Response

NF-κB Proteins

Since its discovery 30 years ago, the NF-κB protein family (RelA [RELA], c-Rel [REL], RelB [RELB], p50/p105 [NFKB1], and p52/p100 [NFKB2]) has been shown as an integral part in many biological processes including cell survival, differentiation, proliferation, and immune responses. Particularly, due to its amenability to experimentation and importance in diseases, much of the studies focused on its functions in immune system, where NF-κB plays an important and evolutionarily conserved role (reviewed in Hayden and Ghosh, 2012). NF-κB proteins are transcription factors that bind to DNA, typically in the promoter or enhancer regions of genes, and regulate transcriptional activities.

In resting cells, basal levels of NF- κBs form as dimers and are sequestered in the cytoplasm by association with Inhibitor of κB (IκB) proteins (IκBα [NFKBIA], IκBβ [NFKBIB], and IκBε [NFKBIE]). Different members of the IκB family target different NF-κB dimers. For example, IκBα and IκBβ preferentially interact with RelA:p50 and c-Rel:p50 heterodimers, whereas IκBε binds only to RelA and c-Rel hetero- and homo-dimers (Thompson et al., 1995; Whiteside et al., 1997). This NF-κB-IκB interaction blocks the nuclear localization sequence (NLS) of NF-κB and prevents it from entering the nucleus. All IκB proteins carry a characteristic ankyrin-repeat domain (ARD) that can bind to the Rel-homology domain (RHD) of NF-κB, which is a relatively well-conserved 300-residue region found in the N-terminus of all five NF-κB

members. The RHD contains two immunoglobulin (Ig)-like domains linked by a non-structured linker; one Ig domain is responsible for sequence-specific DNA binding and the other for homo- or hetero-dimerization (see Figure 1 for an overview of NF-κB and IκB proteins).

RelA, c-Rel, and RelB all contain a well-characterized trans-activation domain (TAD) at the C-terminus that can recruit other transcriptional co-regulators to either initiate or repress gene transcription, depending on the context and cell type. A wide array of proteins has been identified to interact with NF-κB proteins and exert different effects on transcription. For example, p300, a histone acetyl transferase, interacts with c-Rel and RelA at gene promoters and activates gene transcription (Hariri et al., 2013). cAMP-response element binding protein (CREB), on the other hand, can interact with the same domain of RelA and blocks the activation mediated by p300 (Ollivier et al., 1996; Parry and Macman 1997). Furthermore, RelA can recruit histone deactylase 1 and 2 (HDAC1 and HDAC2) to modify histones and negatively regulate gene transcription (Ashburner et al, 2001).

p50 and p52, on the other hand, do not contain a TAD and their homodimers or p50:p52 heterodimers are traditionally considered as repressive transcription factors, unless other co-regulators (e.g. the nuclear IκBs described later) are recruited by them. They are translated initially as precursor proteins p105 and p100, respectively, which contain ankyrin repeats at the C-terminus and act like IκBs, sequestering NF-κB dimers in the cytoplasm. p105's C-terminal ankyrin-repeat domain is commonly referred as IκBγ and preferentially binds to

RelA, c-Rel, and p50 for cytoplasmic sequestration. In resting cells, p105 is constitutively processed co-translationally by a proteasome-mediated proteolytic process, generating NF-κB p50. Due to a glycine-rich STOP signal located between the RHD and ankyrin repeat in p105 and p100, both proteins undergo only partial degradation, generating p50 and p52, respectively. The proteolytic processing of p105 is greatly enhanced under classical NF-κB activation (discussed below), in which almost all p105 proteins are processed into p50. In contrast, only a fraction of the p100 protein undergoes a similar process at the resting state. The majority of p100-to-p52 proteolytic processing occurs under alternative NF-κB activation (discussed later).

The classical activation of NF-κB signaling can be triggered by many stimuli including oxidative stress (Morgan and Liu, 2011), cytokines (e.g. IL-1 and TNFα), and antigens recognized by pattern recognition receptors (PRRs) (e.g. LPS and CpG DNA) (see Figure 2). Once a stimulus initiates classical NF-κB activation, IKK complex, which consists of IKKα (CHUK), IKKβ (IKBKB), and IKKγ (IKBKG) protein, is activated to phosphorylate IκB proteins. IKKγ does not provide kinase activity; it primarily acts as a complex structural scaffold recruiting IKKα and IKKβ. In the classical NF-κB activation pathway, IKKβ is the main kinase at work and is responsible for the full activation of NF-κB signaling. This is evident by the fact that in IKKβ-deficient mice, NF-κB activation is severely defective, although IKKα could provide some residual activation functionality (Li et al., 1999). In contrast, IKKα-deletion in IKKβ-expressing cells has no effects on classical NF-κB activation pathway (Hu et al.,

1999). In classical NF-κB activation, once IκB proteins and p105 are phosphorylated by IKK complexes, they are subsequently recognized and ubiquitinated by SCF complex. The ubiquitinated IκBs are then recognized and degraded by 26S proteasome (Alkalay et al., 1995). This re-exposes NF-κB NLS and allow NF-κB dimers to translocate into the nucleus and bind to NF-κB binding motifs.

On the other hand, p100 processing requires activation of the alternative NF-κB activation pathway. In resting cells, p100 overwhelmingly prefers RelB as the dimerization partner, forming RelB:p100 dimers in the cytoplasm. Unlike the classical NF-κB activation pathway, the alternative activation pathway requires specific stimuli like CD154 (agonist of CD40 receptor ligand), lymphotoxin (LT, agonist of LT receptor), or BAFF (agonist of B-cell activating factor), which activates NF-κB via NF-κB inducing kinase (NIK). It is noteworthy that these stimuli not only activate the alternative pathway, but also concurrently the classical pathway that was mentioned before (Figure 3). At resting state, the NIK protein has a high turnover rate, being actively degraded by cIAP/TRAF2/TRAF3 complex. The activation of the alternative NF-κB signaling pathway results in the degradation of TRAF3, leading to the stabilization NIK, which can then phosphorylate and activate IKK complex. IKKα, but not IKKβ, is the major kinase that mediates p100 phosphorylation at the C-terminus. The phosphorylation is then again recognized and ubiquitinated by SCF complex, which marks p100 for proteasome-mediated partial degradation and forms p52.

The major dimer of this process, RelB:p52, is then able to translocate into the nucleus and mediates its gene regulations.

In summary, at resting state, NF-κB dimers are sequestered in the cytoplasm by cytoplasmic IκBs, p100, or p105. When activated by classical or alternative NF-κB activation pathway, the NF-κB dimer's NLS is re-exposed and translocates into the nucleus. Inside the nucleus, NF-κB binds to DNA by recognizing NF-κB binding motifs with plasticity (described later). Acting as a transcription factor, NF-κB dimers recruit other co-regulatory factors and either activate or repress gene transcriptions.

Complexity of NF-κB Proteins

Although a large body of literature has been focusing on the study of NF-κB, at the time of this report, one of the most critical questions is the how the selective regulation is mediated by different members of the NF-κB family. To bind DNA, two NF-κB proteins must form a dimer via the interaction of dimerization domains. Since there are five NF-κB proteins, there are potentially fifteen possible combinations of either homo- or hetero-dimers of NF-κB (Figure 4). So far, through *in vivo* and *in vitro* studies, twelve dimer species have been observed occurring naturally and can bind to DNA. RelB:RelB, c-Rel:p52, and RelB:c-Rel dimers have not been found to exist. RelB:RelB homodimer, through crystallography studies, forms an intertwined complex, cannot bind to DNA, and is rapidly degraded by the proteasome (Huang et al., 2005; Vu et al., 2013). It is mechanistically plausible to postulate that each dimer combination can regulate a

specific subset of genes based by its ability to recognize a specific DNA motif. Our lab has previously approached this question using unbiased protein binding microarray (uPBM) and surface plasmon resonance (SPR) to investigate the binding preferences among different types of dimers. Siggers et al. showed that NF-κB dimers can be categorized into three classes based on their preferred DNA sequences and lengths (Figure 5). Although considerable differences are observed between homodimers of RelA/c-Rel and p50/p52, the study also showed that considerable plasticity and overlap existing among all heterodimers. Interestingly, novel half sites were identified for p50 and cRel homodimers, broadening our understanding of NF-κB binding. However, this also raises the challenge of distinguishing NF-κB dimer specificity based on DNA sequences (Siggers et al., 2012).

Another approach to deciphering NF-κB functions has been to generate and analyze knockout mice (summarized in Table 1). In most cells types, the RelA:p50 heterodimer appears to be the most abundant dimer species, and it is considered the prototypical and dominant NF-κB dimer that drives the regulation of NF-κB-dependent genes. RelA homozygous knockout mice are known to be lethal on embryonic stage day 14-15 due to massive hepatic apoptosis induced by TNFα, which makes it difficult for a straightforward study of its overall functions in post-natal mice. To overcome this hindrance, *Rela*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and fetal liver cells have been isolated for extensive *in vitro* and *in vivo* experiments. Treating RelA-deficient fibroblast, macrophages, and hepatocytes with TNFα (TNF) induces excessive apoptosis but not in wild type

cells, indicating that RelA is required for protection from TNFα-mediated toxicity (Beg and Baltimore, 1996; Geisler et al., 2007). Chimeric mice, in which *Rela*$^{-/-}$ fetal liver cells were transplanted into wild type mice, were also studied *in vivo*. RelA-deficient lymphocytes are normal in function, as the antibody production, cytokine expression, and Th1-Th2 polarization are unaffected (Doi et al., 1997). On the other hand, RelA-deficient macrophages demonstrate considerable abnormalities, including reduced production of pro-inflammatory cytokines, high sensitivity to apoptosis, and reduced killing of intracellular parasites (Mise-Omata et al., 2009). The lethality of hepatic apoptosis in RelA-deficient mice can be overcome in *Rela*$^{-/-}$ *Tnf*$^{-/-}$ double knockout mice. This allows the investigation of RelA deficiency *in vivo*. However, given the considerable importance of TNF in mediating inflammatory response and the feed forward regulation of NF-κB signaling, it is difficult to make clear conclusions about the specific roles of RelA in this situation.

p50-deficient mice exhibited defects in immune responses involving B lymphocytes and nonspecific responses to infection. The B cells in p50-deficient mice did not proliferate in response to LPS, were defective in basal and specific antibody production, and had reduced cell populations in marginal zone and peritoneal B cells (Cariappa et al., 2000; Pohl et al., 2002). These mice were also defective in clearing L. monocytogenes and were susceptible to infection with S. penumoniae, partly due to B cells' defects in isotype switching (Sha et al., 1995, Snapper et al., 1996). In terms of gene regulation, p50-deficiency macrophages show reduced expression in IL-6 (*Il6)*, IL-10 (*Il10)*, and Cox-2 (*Ptgs2*), which are

important to mount proper innate and adaptive immune responses (Banerjee et al., 2006). Of note, when evaluating the effects of p50-deficiency, besides acting as a factor in NF-κB pathway, one has to keep in mind that p50 is also involved in the regulation of the ERK signaling pathway. Tpl-2 protein (encode by *Map3k8* gene, human analog: Cot gene), a mitogen-activated protein kinase kinase kinase, is required for the activation of ERK/MAPK. ERK/MAPK subsequently negatively regulates LPS-induced IFN-β (IFNB1) production. In resting cells, the Tpl-2 protein interacts with p105, which inhibits Tpl-2 kinase activity but also maintains its stability in cells. Upon LPS stimulation, p105 is proteolytically processed into p50, releasing Tpl-2, which subsequently phosphorylates and activates MAPK kinase 1 (MEK1), an upstream kinase of ERK1/2. Concurrently, both p50/p105-deficient and Tpl-2-deficient mouse macrophages exhibit higher IFN-β expression with LPS stimulation and fail to activate MEK/ERK signaling pathway, with the former due to the lack of stable Tpl-2 (Waterfield et al., 2003; Yang et al., 2011). Thus, besides acting as a transcription factor and interacting with NF-κB/IκB/DNA, p50/p105 can also function as a regulator of upstream signaling event other than NF-κB's.

Unlike RelA and p50, NF-κB c-Rel is predominantly expressed in hematopoietic cells like lymphocytes, monocytes, and erythroid cells. However, in c-Rel-deficient mice, the development of cells from all hematopoietic lineage appears normal. On the other hand, c-Rel-deficient mice showed defects in humoral immunity by B and T cells as they were unresponsive to most mitogenic stimuli (Köntgen et al., 1995). c-Rel also regulates T helper cell 1 (Th1)

development during CD4+ T-cell responses. These phenotypes result from the inefficient production of important cytokines such as IL-2 (IL2), GM-CSF (CSF2), and IL-12 (IL12A+IL12B) (Plevy et al., 1997; Sanjabi et al., 2000; Sanjabi et al., 2005).

RelB expression is mainly restricted to thymus, spleen, lymph nodes, and intestine of adult mice (Carrasco et al., 1993). RelB-deficiency in mice results in the absence of dendritic cells (Burkly et al., 1995), defects in secondary lymphoid structures (Weih et al., 2001; Yilmaz et al., 2003) and multiorgan inflammation (Weih et al., 1995). RelB-deficient mice also lack Peyer's patches, properly formed splenic germinal centers, and subsets of thymic and DC populations (Wu et al., 1998).

Lastly, p52-deficient mice exhibited defects in B cell-mediated immune responses, splenic microarchitecture development, and secondary lymphoid structures (Caamaño et al., 1998; Franzoso et al., 1998; Beinke and Ley, 2004). Notably, the p52- and RelB-deficient mice exhibited striking overlap in phenotype studies, which strongly support their crucial roles, potentially via alternative NF-κB activation pathway, in adaptive immunity.

In summary, except *Rela*$^{-/-}$ mice, all other transgenic knockout mice can grow into adulthood and yet display distinct phenotypes. This strongly suggests that each NF-κB member is specifically regulating a subset of unique genes.

TLR4-mediated Immune Responses

As mentioned earlier, many stimuli induce the activation of NF-κB. The TLR family has been extensively studied and characterized due to its significance in mounting proper immune responses by detecting various pathogens, including viruses, bacteria, mycobacteria, fungi, and parasites. To date, there are 10 and 12 functional TLRs that have been identified in human and mouse, respectively. Different TLRs localize in difference compartments of cells and specialize in recognizing different PAMPs (see Table 2). TLR4 is one of the most extensively studied and specializes in recognizing Gram-negative bacterial lipopolysaccharides (LPS) or synthetic monophosphoryl lipid A. It is expressed primarily in monocytes, macrophages, dendritic cells, mast cells, and intestinal epithelium. TLR4 contains N-terminal leucine-rich repeats ectodomain, and a Toll/IL-1 receptor (TIR) endodomain, which recruits adaptor proteins MyD88 and TRIF. After the recruitment of MyD88 or TRIF to the TLR4 TIR, they subsequently recruit IRAKs and TRAF6 proteins, which then subsequently lead to the recruitment and activation of TAK1. TAK1 protein is essential for activating IKK complex and MAPKs, which subsequently induce classical NF-κB and AP-1 activation, respectively (see Figure 6). Notably, the recruitment of TRIF to TLR4 TIR happens after the internalization of ligand-bound TLR4 receptor into endosomes/phagosomes. Therefore, it is proposed that the cellular membrane-bound TLR4 (via MyD88) provides immediate NF-κB response while endosome-bound TLR4 (via TRIF) provides late NF-κB activation. Another key difference between MyD88 and TRIF is that the latter can also recruit TRAF3, which recruits TBK1/IKKi complex. The complex then activates IRF3 transcription

factor, an important protein to activate antiviral type I IFN response. TLR4-mediated classical NF-κB and type I IFN activation induce the secretion of important inflammatory cytokines, type I IFN, chemokines, transcription factors, co-factors, and antimicrobial peptides. Downstream of TLR4 activation, NF-κB induces the expressions of important genes including cytokines (e.g. *Il1b, Il6, Il12b, and Tnf)*, chemokines (e.g. *Il8*, *Ccl2*, and *Ccl3*), enzymes (e.g. *Nos2* and *Cox2)*, and adhesion molecules (*VAM1*, *ICAM1*, and *SELE*). These responses cause recruitment of neutrophils, activation of macrophages, and induction of IFN-stimulated genes, resulting in direct killing of the infected pathogens.

IL-12B Biology

IL-12B (IL12B) is a pro-inflammatory cytokine that is highly produced during the innate and adaptive immune responses. The main producers of it during immune responses are phagocytes like neutrophils, macrophages, and dendritic cells. IL-12B exerts multiple functions, partly due to its roles in forming two different cytokines, IL-12 and IL-23. IL-12B and IL-12A (IL12A) dimerize via disulfide linker to form IL-12, which is crucial in inducing IFN-γ production, cell proliferation, and cytotoxicity mediated by natural killer cells and T cells. Moreover, IL-12 is essential for the differentiation of naïve CD4+ T cells into Th1 cells, as well as their subsequent proliferation and maintenance. Th1 cells strongly secrete IFN-γ and IL-2, which in turn promote more T cell responses and macrophage activation, respectively.

The study of IL-12 intrigues great interests in therapeutics, as IL-12B-deficient mice are resistant to many experimentally induced autoimmune conditions, including brain paralysis (multiple sclerosis model), arthritis inflammation (rheumatoid arthritis model), and gut disease model (Crohn's disease model). However, the resistance is not seen in the knockout mice of the other subunit, IL-12A, suggesting that IL-12B potentially forms other factors with other proteins. Later, IL-12B was found to dimerize with IL-23A (IL23A) and form IL-23 cytokine, which is responsible for the aforementioned experimentally induced autoimmune conditions. Other studies relate the production of IL-23 to the maintenance of T helper cell 17 (Th17) (see Figure 7). Th17 cells play a role in adaptive immunity protecting the host against pathogens by secreting effector cytokines like IL-17A, IL-17F, IL-21, and IL-22. Concurrently, Th17 cells are strongly associated with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and psoriasis. To date, the pursuit of controlling IL-12 or IL-23 expression as a therapy for immune-mediated inflammatory diseases continues (Teng et al., 2015).

Nuclear IκBs

While the importance of IκB in sequestering and regulating NF-κB activation is well documented, three other ankyrin repeat-containing proteins have been identified. Bcl-3 (BCL3), IκBNS (NFKBID), and IκBζ (NFKBIZ) contain 7, 6, and 5 ankyrin repeats, respectively, and can all interact with NF-κB dimers (See Figure 9). Unlike the aforementioned IκBs (IκBα, IκBβ, and IκBε), which

primarily shuttle between cytoplasm and nucleus, and retain NF-κBs in the cytoplasm (termed "cytoplasmic IκBs" from now on), Bcl-3, IκBNS, and IκBζ all contain an NLS in their N-terminus and primarily reside in the nucleus (termed "nuclear IκBs" from now on). Due to their unique locations in cells, nuclear IκBs are considered to primarily interact with NF-κBs after their translocation into the nucleus.

In terms of expression, at resting state, while the cytoplasmic IκBs are already expressed at considerable levels and sequester NF-κBs in the cytoplasm, the nuclear IκBs are expressed at very low basal levels in most cell types. Nonetheless, both cytoplasmic and nuclear IκBs are NF-κB target genes and are strongly induced upon NF-κB activation. In terms of transcriptional control, while cytoplasmic IκBs all take part in inhibiting NF-κB activation with NF-κB NLS blocking and cytoplasmic retention, nuclear IκBs interact with NF-κB dimers that have entered the nucleus and can either suppress or activate gene transcriptions, depending on the context and cell type.

Another key difference is their targets of interaction; through extensive biochemical and crystallography studies, it has been well-documented that nuclear IκBs strongly prefer to interact with p50 and (or) p52 NF-κBs; Bcl-3 has been shown to interact with both p50 and p52 homodimers while IκBζ and IκBNS specifically bind to p50 homodimers. In addition, IκBζ has been reported to interact with RelA subunit as well. This is in contrast to the preferred targets of cytoplasmic IκBs, which primarily bind with RelA and c-Rel NF-κB subunits. This selectivity is of great interests with respect to NF-κB research because NF-

κB families are well known for their functional redundancies and complexity. The selective protein-protein interaction may indicate one of the key mechanisms in mediating specific transcriptional control.

Another key feature of the nuclear IκBs is the presence of TAD in Bcl-3, IκBζ, but not IκBNS. As mentioned earlier, p50 and p52 do not contain a TAD and their homodimers are considered as repressive transcription factors. The binding of Bcl-3 and IκBζ, but not IκBNS, therefore, has the potential to activate gene transcription by binding to p50 or p52 homodimers that are occupying the promoters or enhancers of genes.

Many studies of nuclear IκBs focus on the phenotypes of knockout mice and their regulatory effects on a handful of genes. Bcl-3 is the first member to be identified and thought to be a putative proto-oncogene due to its high expression level in B cell chronic lymphocytic leukemia (CLL) as well as a wide variety of cancers. In CLL, Bcl-3 is constitutively expressed due to a t(14;19) chromosome translocation (Ohno et al., 1990). Subsequently, Bcl-3 has been shown to be an important co-factor required for the expressions of cyclin D1 (CCND1) and MDM2, two important proteins that regulate cell cycle progression (Kashatus et al., 2006; Westerheide et al., 2001). However, Bcl-3 has also been shown to be a negative regulator of TNFα transcription as well by binding and stabilizing p50 homodimer at TNFα promoter, which prevents RelA- or c-Rel-containing NF-κB dimers from binding and perpetuating the expression of TNFα. Concurrently, wild type mice that receive Bcl-3-deficient bone marrow die of severe septic shock due to an unmitigated inflammatory response after repetitive intraperitoneal

LPS injection whereas control mice that receive wild type bone marrow do not (Carmody et al., 2007). The contradicting (activating vs. inhibiting) roles of Bcl-3 in gene regulation has also been observed in transient transfection assays (as an activator: Bours et al., 1993 and Fujita et al., 1993; as an inhibitor: Kerr et al., 1992 and Richard et al., 1999).

Despite the convoluted regulatory functions of Bcl-3, its physiological functions are well-documented. Bcl3-deficient mice exhibit impaired formation of germinal centers, splenic structures, as well as reduced antigen-specific antibody IgG production when challenged by influenza virus (Schwarz et al., 1997; Franzoso et al., 1997). Interestingly, these phenotypic effects overlap with the observed phenotypes of p50-deficient mice (Sha et al., 1995), strengthening the concept that Bcl-3 coordinates closely with p50 and both are indispensable to exert specific functions in immune response and development. As with p50, Bcl-3 collaborates with p52 within stroma to generate medullary thymic epithelial cells (mTEC), which are essential for negative selections of autoreactive T cells. The loss of both Bcl-3 and p52, but not either one alone, leads to a profound breakdown in central tolerance, resulting in rapid and fatal multi-organ inflammation (Zhang et al., 2007).

IκBNS is originally identified as a protein that was expressed in T cells undergoing negative selection in thymus (Fiorini et al., 2002). However, IκBNS-deficient mice exhibit normal thymic as well as peripheral T cell development, albeit their thymocytes and T cells produce significantly less IL-2 (IL2) and IFN-γ (IFNG) (Touma et al., 2007). Later studies discovered that IκBNS has a more

potent role in B cell biology; B cells from IκBNS knockout mice had impaired proliferative response to LPS and anti-CD40. IgM and IgG expressions are drastically reduced in the serum of IκBNS-deficient mice when challenged with influenza virus. In agreement with this finding, IκBNS KO mice lack B1 B cells and exhibit a reduction in marginal zone B cells (Touma et al., 2011). This strongly suggests that IκBNS significantly impacts the development and functions of B cells and plasma cells. Interestingly, although flu-specific IgM antibodies are normal in Bcl-3 KO mice, the lack of flu-specific IgG antibodies in both Bcl-3 and IκBNS KO mice indicate Bcl-3 and IκBNS contain overlapping yet distinct functions in humoral responses. Also, as mentioned before, the impaired B cell proliferative response to stimuli is also observed in p50-deficient mice, suggesting the importance of p50 in associating and mediating the biological functions of IκBNS. In terms of gene identified to be regulated by IκBNS, IL-6 (IL6) and IL-12B (IL12B), two secondary inflammatory response genes, are shown to be upregulated in IκBNS-deficient mice (Hirotani et al., 2005; Kuwata et al., 2006). With IL-6, IκBNS binds and stabilize p50 homodimer at its promoter and prevent its transcriptional activation. Lastly, IκBNS is also expressed with IL-10 (IL10) treatment and is essential in preventing endotoxin shock. Together with the evidence that IκBNS is constitutively expressed in colonic macrophages, its role is apparent in deterring excessive inflammatory responses.

IκBζ (named MAIL or INAP initially) is first discovered as a LPS-induced, nuclear-resident protein that share high homology with Bcl-3 protein (Kitamura et al., 2000). IκBζ is quickly induced by IL-1 or TLR but not by TNF.

This discrepancy results from post-transcriptional regulation, as IκBζ mRNA is stable in IL-1 or TLR induction but not with TNF (Yamazaki et al., 2005). IL-17A (IL17a) was later shown to be essential for the stabilization of IκBζ mRNA, as stimulating cells concurrently with IL-17A and TNF, but not either one alone, induces the expression of IκBζ (Yamazaki et al., 2005). When expressed, two isoforms, IκBζ(L) (full length) and IκBζ(S) (missing amino acid 1-99) are generated by alternative splicing. However, since IκBζ(L) is the predominant isoform and both variants contain the essential functional domains including TAD, NLS, and ankyrin repeats, whether the two isoforms exert different functions remained to be investigated (Yamamoto et al., 2004). Besides the characteristic ankyrin repeats at C-terminus, IκBζ's N-terminus is unique as no significant homology to any other known proteins is identified. Sequential protein truncation study, however, identified a region of N-terminus as the TAD domain of IκBζ, which is relatively rich in glutamines and prolines, the features of transcription activation domains of several transcription factors, including Sp1 and c-Jun (Courey and Tjian, 1988; Struhl, 1988).

Interestingly, in opposition to IκBNS's functions in inhibiting the expressions of secondary response genes like IL-6 and IL-12B, IκBζ is found to be essential for both genes' proper expressions while dispensable for the induction of primary response gene (Yamamoto et al., 2004). The seemingly juxtaposed functions between IκBNS and IκBζ pose an attractive regulatory mechanism that seems to establish based on the competition and availability between the two nuclear IκBs, which remains to be examined closely with further

studies. The binding of IκBζ to gene promoters or enhancers has been shown crucial in inducing H3K4 trimethylation (H3K4me3) and subsequent RNA polymerase II (Pol II) and TATA-box binding protein (TBP) recruitment for secondary response genes but not for primary response genes (Kayama et al., 2008). It is not required, however, for chromatin remodeling of secondary gene promoters. Therefore, IκBζ exert its functions downstream of chromatin remodeling and upstream of H3K4me3 modification and preinitiation complex assembly. It should be noted that IκBζ has been suggested to cooperatively activate transcription with C/EBPs on selective genes, including *Lcn2* (for neutrophil gelatinase-associated lipocalin, or NGAL) and *Defb4a* (beta-defensin 2) (Matsuo et al., 2007). Despite its preference to interact with NF-κB p50, IκBζ had been reported to interact and inhibit NF-κB p65 DNA binding and repress NF-κB target gene expression, e.g. E-selectin (*Sele*) (Yamazaki et al., 2001; Totzke et al., 2006).

Distinct phenotypes have been observed in IκBζ-deficient mice. Albeit they grow normally after birth, they manifest atopic dermatitis-like lesions from 4-5 weeks of age and have higher concentrations of IgE (Shiina et al., 2004). The dermatitis lesions mostly likely arise from the elevated expression of TARC and eotaxin in skin, both of which are known chemoattractants for T helper 2 cells and eosinophils. IκBζ is essential to T helper cell subset Th17 development. IκBζ-deficient mice fail to develop Th17 cells and were resistant to experimental autoimmune encephalomyelitis (EAE), a mouse model of human multiple sclerosis and Th17 cell-mediated autoimmune diseases. This is further evident by

the fact that Rag2-deficient mice into which IκBζ-deficient CD4+ T cells are transferred do not develop EAE while wild type CD4+ T cells do (Okamoto et al., 2010). This suggests that IκBζ might also take part in other Th17-mediated immunopathologies, including rheumatoid arthritis and psoriasis. Of note, recently IκBNS has also been shown to be crucial for the development of Th17 (Annemann et al., 2015). The interrelationships between IκBζ and IκBNS in terms of Th17 development is of great interests and remained to be further studied.

In summary, since the last two decades, much progress has been made in understanding the structure, mechanism, and physiological functions of each nuclear IκB protein. Each nuclear IκB-deficient mouse exhibits distinct yet overlapping phenotypes, suggesting their partial redundancies as well as uniqueness in terms of functionality. These phenotypes, to some degree, overlap with those of p50- or p52-deficient mice, highlighting the importance of nuclear IκB-NF-κB interactions and how nuclear IκBs can regulate gene transcriptions and cell development, particularly in lymphocyte, in a mechanism that is distinctly different from that of cytoplasmic IκBs. For example, NF-κB can rapidly induce the transcription of primary response genes (gene with enriched CpG islands in their promoter regions and do not require nucleosome remodeling), whereas nucleosome remodeling is required for the expression of secondary response genes. Since all three nuclear IκBs are induced after initial NF-κB activation, it is possible they regulate the expression of NF-κB-dependent secondary genes after nucleosome remodeling. So far, most of the gene regulation

studies of nuclear IκBs focus on individual genes within specific relevant contexts. No systematic, parallel study of all nuclear IκBs at the same time have been performed.

Figure Legends

Figure 1-1: Schematic of NF-κB and IκB Proteins.

The diagrams here showcase the structure and organization of all five NF-κB subunits and three cytoplasmic IκBs. RHD: Rel Homology Domain. GGG: Glycine-Rich Domain. TA: Trans-Activation domain. Notice that RelA, c-Rel, and RelB, but not p50 or p52, contain TA and are considered trans-activating transcription factors. p50 and p52 are considered repressive transcription factors. Cytoplasmic IκBs, p100, and p105 utilize ankyrin (ANK) repeats to interact with NF-κB's RHD and sequester it in the cytoplasm by shielding its nuclear localization sequence (NLS).

Figure 1-2: Classical NF-κB Activation.

In resting state, NF-κB are sequestered in the cytoplasm by assembly with cytoplasmic IκBs, p100, or p105. This protein-protein interaction blocks the nuclear localization sequence (NLS) of NF-κB. The classical NF-κB activation can be triggered by various stimuli, including pathogen-associated molecular patterns (PAMPs, via different Toll-like receptors [TLRs], TNF (via TNFR1 and TNFR2 receptors), RANKL (RANK ligand, via RANK receptor), CD30L (CD30 ligand, via CD30 receptors on T and B cell membranes), CD40L (CD40 ligand, via CD40 receptors on antigen presenting cells), and antigen-MHC complex (via T cell and B cell receptors [TCR/BCR]). Some stimuli, for example CD40, can activate both classical and alternative NF-κB activation pathways. The stimulation

triggers the activation of IKK complex, primarily via the phosphorylation of IKKβ. Activated IKKβ phosphorylates cytoplasmic IκBs or the C-terminus of p105 protein, which marks them for subsequent poly-ubiquitination mediated by SCF complex and degradation mediated by 26S proteasome. This causes the re-exposure of NLS of NF-κB, allowing it to translocate into the nucleus and bind to NF-κB DNA motifs.

Figure 1-3: Alternative NF-κB Activation.

NF-κB, particularly RelB:p100 heterodimers, can be activated by specific stimuli like lymphotoxin β (via LTβR), B cell activating factor (BAFF, via BAFF-R) and previously mentioned CD40L (via CD40). At resting state, RelB preferentially dimerize with NF-κB p100. p100 contains ankyrin repeats at its C-terminus, which masks the NLS of RelB. NF-κB-inducing kinase (NIK) is subject to constant degradation, which is mediated by cIAP/TRAF2/TRAF3 complex. The activation of alternative NF-κB pathway induces the degradation of TRAF3, and NIK expression becomes stabilized. NIK subsequently activates IKKα of IKK complex by phosphorylation, and the activated IKKα preferentially targets and phosphorylates p100 C-termini, which lead to their poly-ubiquitination and eventual degradation. Again this re-exposes the NLS of RelB and p52, allowing RelB:p52 dimer to translocate into the nucleus.

Figure 1-4: NF-κB Dimers.

There are potentially fifteen NF-κB dimers. The top nine dimers all have at least one trans-activation domain (TAD) and could potentially activate gene transcription. The three dimers in row five are considered repressive transcription factors due to the lack of TAD. However, they can recruit TAD-containing co-factors like Bcl-3 and IκBζ, which can potentially active gene transcription. Out of the fifteen possible combinations, three of them haven't been identified *in vivo*: c-Rel:p52, RelB:RelB, and RelB:c-Rel. RelB:RelB can form a interwined dimer that cannot bind to DNA and are quickly degraded in cells.

Figure 1-5: NF-κB Dimers Motif Classes.

Using unbiased protein binding microarray (uPBM), the preferred DNA sequences and motif lengths are identified for 8 different NF-κB dimers. **(A)** Pair-wise comparison of ten NF-κB dimers, showing the Pearson correlation between each of the two dimer species. The hierarchical cluster is formed based on the comparison matrix. RelA:RelA homodimer shows the strongest correlation with c-Rel:c-Rel homodimer. p50:p50 homodimer shows the highest correlation with p52:p52. Heterodimers all highly correlate with each other. The most distinct difference is seen between p50/p52 homodimers and c-Rel/RelA homodimers. **(B)** Three NF-κB DNA motif classes are identified that correspond to three NF-κB dimer groups: 1) p50/p52 homodimers, 2) heterodimers, and 3) c-Rel/RelA homodimers. Each representative DNA motif site is determined by using the top twenty-five κB sites bound by each class member. p50/p52 homodimers prefer

longer (12 bp) motifs with multiple 5'-Gs. RelA/c-Rel homodimers prefers shorter motifs (9 bp) that are highly symmetrical, having 5'-GGAA on both ends. Heterodimer, on the other hand, prefers motifs that are in between.

Table 1-1: NF-κB Knockout and Transgenic Mice.

A compilation of studies based on NF-κB knockout or transgenic mice, with annotations in lethality, defects, and phenotypes.

Table 1-2: TLRs and Their Respective Agonists.

This table lists pathogen-associated molecular patters (PAMPs) and their corresponding detecting TLRs. For each PAMP, its pathogenic source (i.e. species) is also listed. Assisting pathogen recognition receptors (PRRs) are also listed here.

Figure 1-6: TLR4 Activation.

TLR4 signals through the MyD88-TIRAP adaptor pair to activate NF-κB and AP-1 signaling. Downstream, via TRAF6, TAK1 activates NF-κB and AP-1 transcriptions factors by activating IKK complex and MAPKs, respectively. LPS triggers endocytosis of TLR4 via dynamin-dependent pathway, leading to the termination of initial MyD88 signaling. In endosomes (or phagosomes), a second phase of TRIF-TRAM adaptor pair-mediated signaling is initiated via TRAF6 and TRAF3 for NF-κB/MAPKs and IRF3 activation, respectively. IRF3 triggers the

production of IFN-β, an important anti-viral response. NF-κB activation occur in two phases: the early phase is mediated by MyD88, and the late phase by TRIF.

Figure 1-7: Schematic of IL-23, IL-12, Their Receptors, and Downstream Signaling Pathways.

IL-23 comprises of IL-23A and IL-12B subunits. Its respective receptors are IL-12Rβ1 and IL-23R, which activate JAK-STAT pathway but mainly on STAT3. This induces the secretion of IL-17A, IL-17F, IL-22, and stabilizes T helper cell *in vivo*. IL-12 is a heterodimer consists of IL-12A and IL-12B subunits. IL-12 signals through IL-12Rβ1 and IL-12Rβ2 receptors, which stimulate JAK2 and TYK2 activity, leading to the phosphorylation of primarily STAT4 and other STAT molecules. STAT4 induces the production of IFN-γ, which is required for the development of T helper cell 1 immune responses.

Figure 1-8: Schematic of Nuclear IκBs.

The schematic shows the overall structures of the three nuclear IκBs. Bcl-3, IκBζ, and IκBNS contain 7, 5, and 6 ankyrin repeats (ANK), respectively. Notice that all three nuclear IκBs contain nuclear localization sequence (NLS) and reside within nucleus as their typical residing cellular compartment. Only Bcl-3 and IκBζ compose well-characterized TAD domain. Since Bcl-3 and IκBζ specifically prefer interactions with p50 and/or p52 homodimers, which lack TAD and are considered repressive transcription factors, Bcl-3 and IκBζ can potentially

reverse the inhibitive transcriptional control by binding to NF-κBs p50 and p52. However, as noted in Chapter 1 discussion, while IκBζ's role in gene trans-activation is obvious and strong, Bcl-3 exhibits mixed, and sometimes contradicting, regulatory controls on gene transcriptions.

Table 1-3: Nuclear IκB Knockout Mice and Phenotypes.

Distinct phenotypes can been seen in different nuclear IκB knockout mice. Bcl-3 specifically interacts with p50 or p52 homodimers, and can inhibit excessive TNF expression and endotoxin shock. Bcl-3 collaborates with p52 in spleen stroma to generate medullary thymic epithelial cells (mTEC), which are essential for the negative selection of autoreactive T cells. In addition, Bcl-3-deficient mice also lack splenic architecture and germinal center formation, which is crucial for follicular dendritic cells to present antigen-antibody immune complexes to facilitate clonal selection and differentiation of antigen-activated B cells, leading to the generation of plasma cells and memory B cells. IκBζ primarily acts as a pro-inflammatory trans-activator as it is indispensable for the induction of many important cytokines like IL-6, IL-12B, and IL-17A. It is also important for the development of T help cell 17 (Th17), as IκBζ-deficient mice lack Th17 cells and are resistant to experimental autoimmune encephalomyelitis (EAE), a disease mediated by Th17. IκBNS seems to primarily inhibit excessive NF-κB activation, as IκBNS-deficient mice are sensitive to induced colitis and endotoxin shock. Interestingly, in contradiction to IκBζ, IκBNS inhibits the expression of IL-6 and

IL-12B. Like Bcl-3, IκBNS$^{-/-}$ mice also exhibits defects in developments of B1, germinal centers, and marginal zone B cells.

Figure 1-1: NF-κB and IκB Proteins.

(From Bonizzi and Karin, 2004)

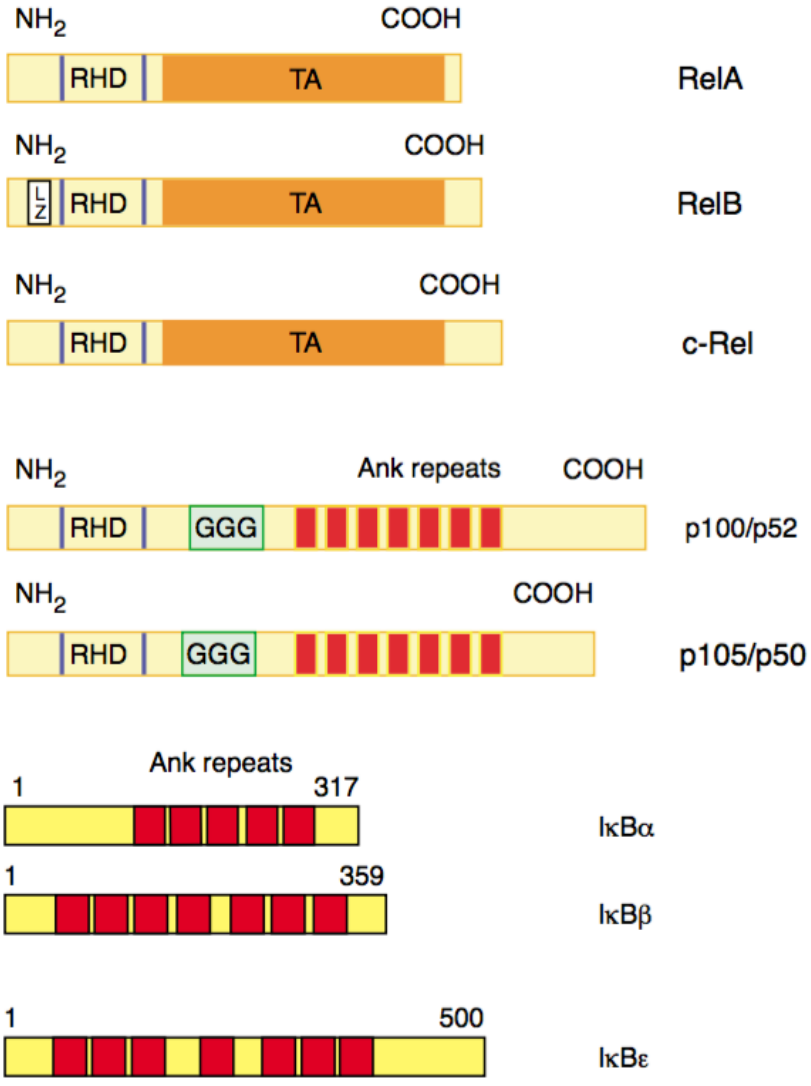

Figure 1-2: Classical NF-κB Activation.

(From Jost and Ruland, 2007)

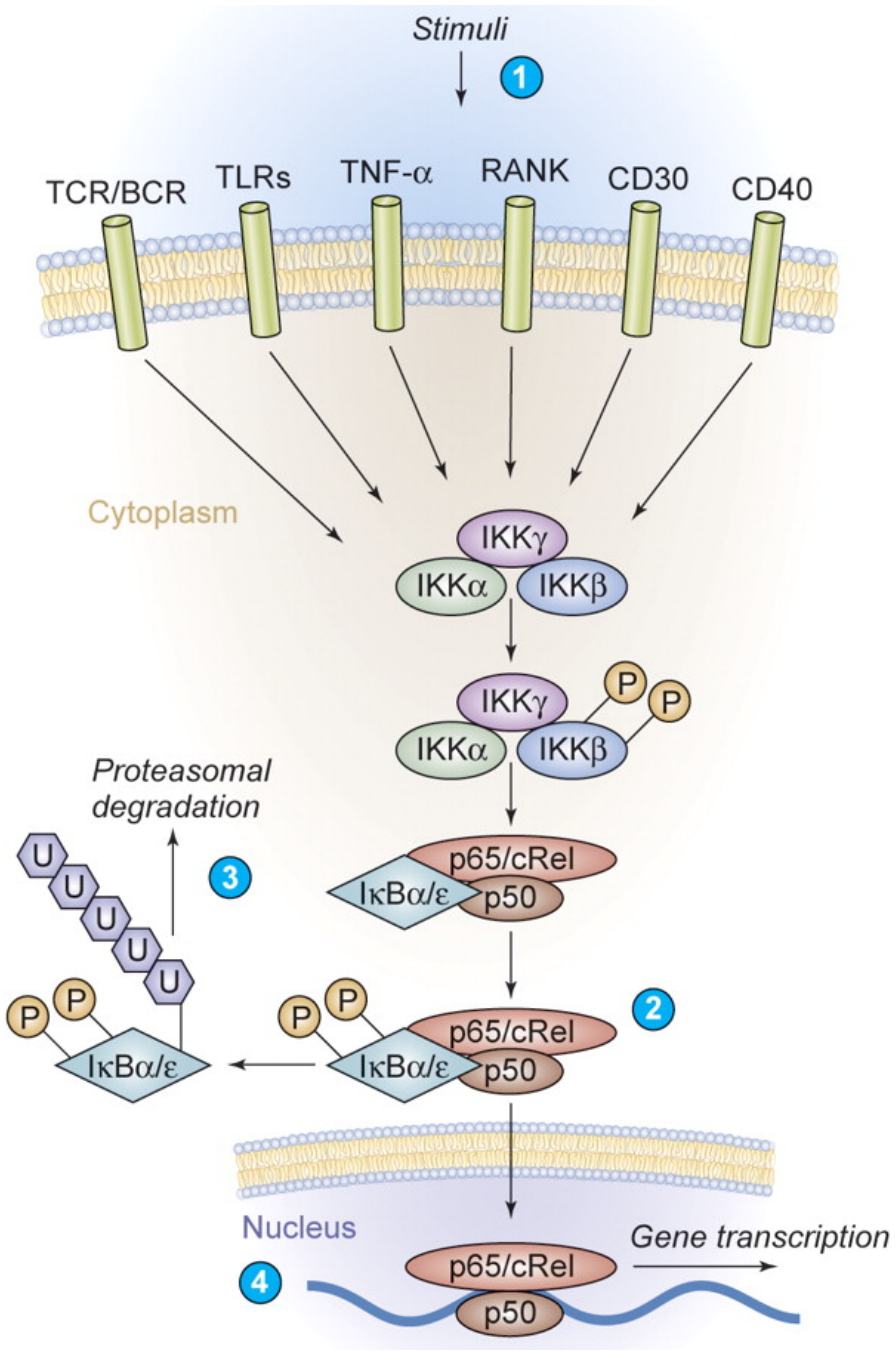

Figure 1-3: Alternative NF-κB Activation.

(From Jost and Ruland, 2007)

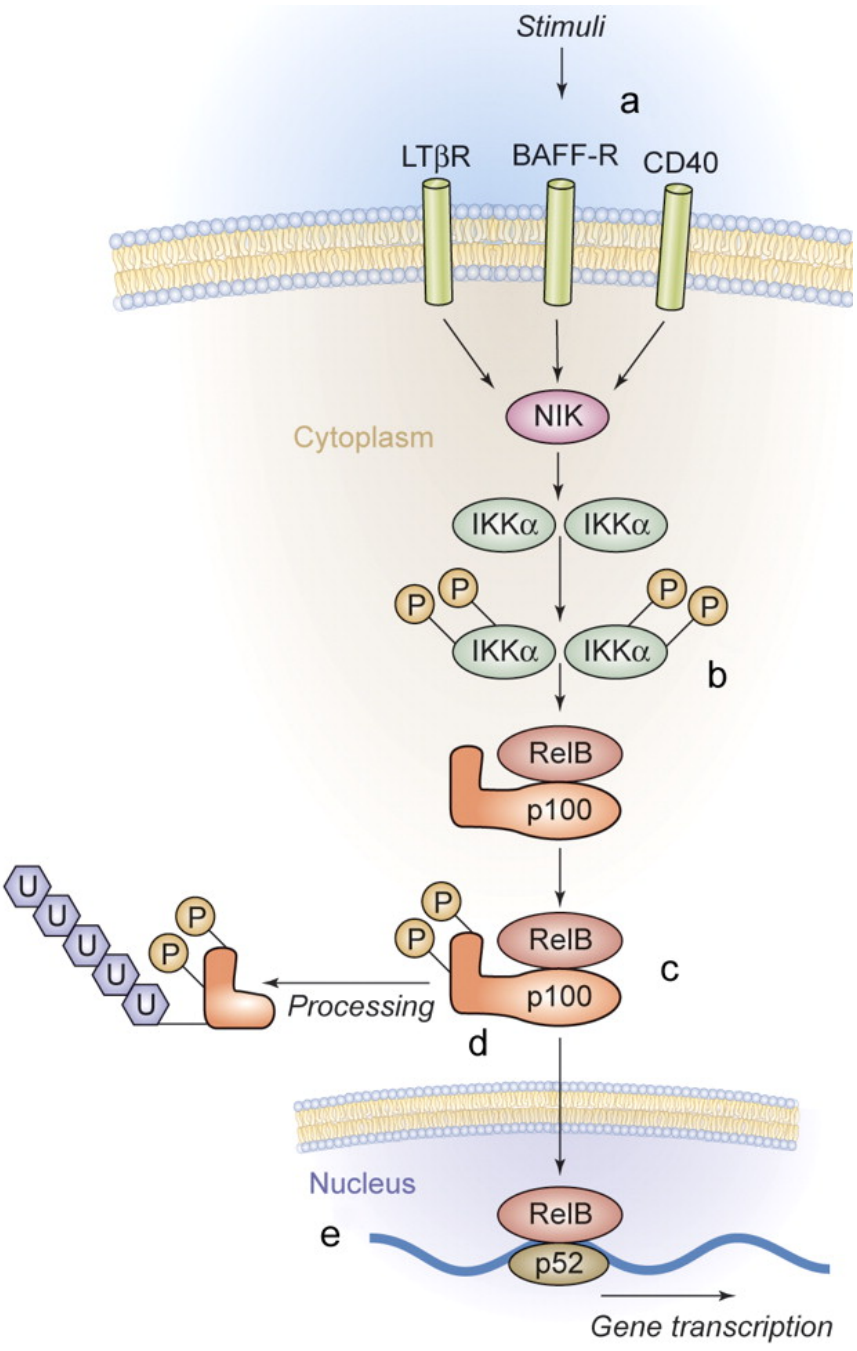

Figure 1-4: NF-κB Dimers.

(From Hoffmann and Baltimore, 2006)

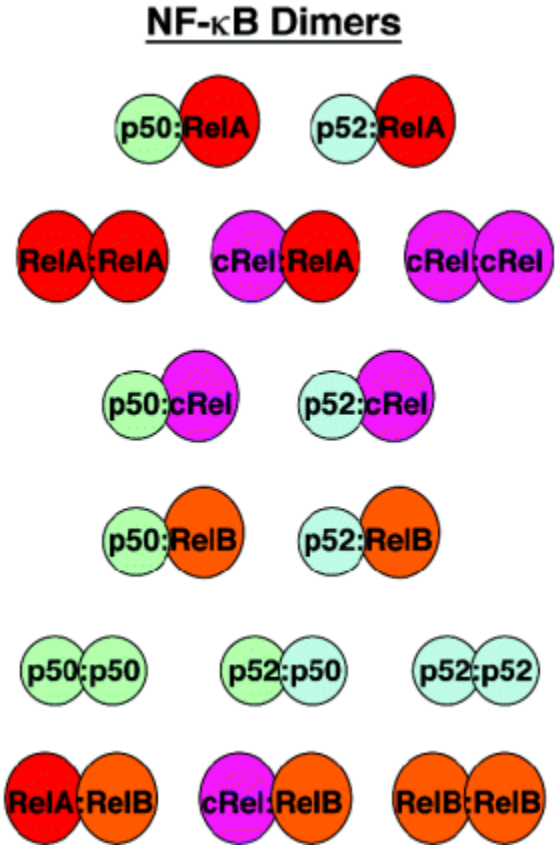

Figure 1-5: NF-κB Dimer Motif Classes.

(From Siggers et al., 2011)

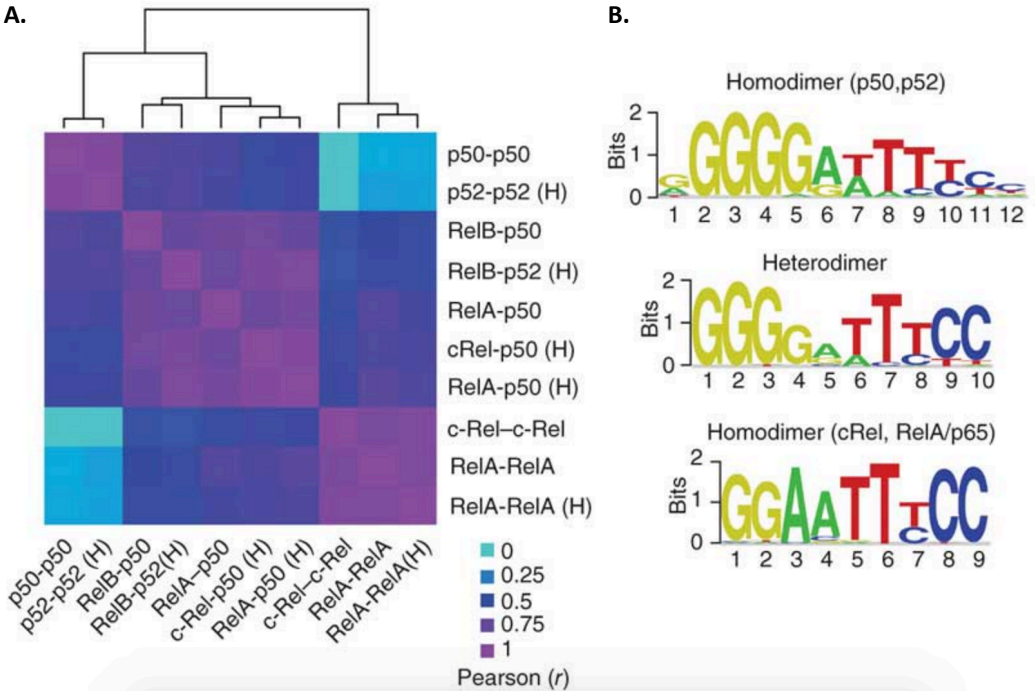

Table 1-1: NF-κB Knockout and Transgenic Mice.

(From Gerondakis et al., 2006)

Genotype	*Lethality*	*Defect/phenotype*	*Reference*
Knockouts			
nfkb1$^{-/-}$	No	B cells: marginal zone and CD5^{+} peritoneal B cells reduced; response to LPS diminished, turn over rapidly *in vivo*; defective isotype switching and impaired humoral immune response	Sha *et al.* (1995), Grumont *et al.* (1998), Snapper *et al.* (1996), Cariappa *et al.* (2000), Pohl *et al.* (2002)
		Th2 differentiation is impaired	Das *et al.* (2001), Artis *et al.* (2005)
		NK cells display enhanced proliferation and increased IFNγ production	Tato *et al.* (2006)
		Macrophages: ERK mitogen-activated protein kinase pathway activation in response to TLR signals is impaired leading to reduced expression of IL-6, IL-10 and Cox-2	Waterfield *et al.* (2003), Banerjee *et al.* (2006)
nfkb2$^{-/-}$	No	Defective secondary lymphoid organ development, impaired B-cell development; enhanced DC function	Caamano *et al.* (1998), Franzoso *et al.* (1998), Speirs *et al.* (2004)
c-rel$^{-/-}$	No	B cells: cell-cycle and survival defects; impaired isotype switching	Grumont *et al.* (1998, 1999), Cheng *et al.* (2003), Pohl *et al.* (2002)
		T cells: defects in CD4 and CD8 T-cell responses, Th1 development and cytokine production (IL-2 and GM-CSF) in CD4^{+} T-cell responses	Hilliard *et al.* (2002), Lamhamedi-Cherradi *et al.* (2003), Mason *et al.* (2004), Gerondakis *et al.* (1996), Rao *et al.* (2003)
		Reduced number of pDC, impaired IL-12 production and DC priming of CTL	O'Keeffe *et al.* (2005), Grumont *et al.* (2001), Mintern *et al.* (2002)
		Neuronal survival defects	Pizzi *et al.* (2002, 2005)
rela$^{-/-}$	Yes (~E15)	TNF-α-induced cell death: hepatocytes, macrophages and fibroblasts	Beg *et al.* (1995b), Beg and Baltimore (1996), Prendes *et al.* (2003), Senftleben *et al.* (2001), Li *et al.* (2001)
		Impaired secondary lymphoid organ development	Alcamo *et al.* (2002)
		Defects in leukocyte recruitment, T-cell-dependent responses, isotype switching to IgG3	Alcamo *et al.* (2001, 2002), Horwitz *et al.* (1999)
		Spatial learning responses	Meffert *et al.* (2003)
		Epidermal homeostasis	Zhang *et al.* (2004)
relb$^{-/-}$	No	Complex inflammatory phenotype and hematopoietic abnormalities	Burkly *et al.* (1995), Weih *et al.* (1995)
		Defects in secondary lymphoid organ structure and germinal center formation	Weih *et al.* (2001), Yilmaz *et al.* (2003)
		Lack certain DC populations, DC functional defects	Wu *et al.* (1998), Zanetti *et al.* (2003), Castiglioni *et al.* (2002)
		Generation of early NK1.1^{-} NKT cells is impaired	Elewaut *et al.* (2003), Sivakumar *et al.* (2003)
		T cells: reduced proliferation and survival of single positive thymocytes; impaired Th1 differentiation	Guerin *et al.* (2002), Corn *et al.* (2005), Weih *et al.* (1997)
nfkb1$^{\Delta CT/\Delta CT}$	No	Splenomegaly, enlarged lymph nodes and lymphoid infiltrates in various organs. Increase in B-cell numbers accompanied by hyper-responsiveness to mitogens. T cells exhibit diminished proliferative capacity	Ishikawa *et al.* (1998)
c-rel$^{\Delta CT/\Delta CT}$	No	Hypoplastic bone marrow, enlarged lymph node and lymphoid hyperplasia	Carrasco *et al.* (1998)
nfkb2$^{\Delta CT/\Delta CT}$	No	Gastric hyperplasia, hyperkeratosis in the heart, spleen and thymic atrophy, enlarged lymph nodes, lymphocytic infiltrates of various tissues and granulocytosis	Ishikawa *et al.* (1997)
Mice lacking multiple subunits			
nfkb1$^{-/-}$*nfkb2*$^{-/-}$	No	Growth retarded and craniofacial abnormalities due to osteopetrosis	Franzoso *et al.* (1997b), Iotsova *et al.* (1997)
		Severe defects in secondary lymphoid organ development	Lo *et al.* (2006)
		Lack marginal zone and mature follicular B cells	Franzoso *et al.* (1997b)
nfkb1$^{-/-}$*relb*$^{-/-}$	No	Exacerbation of organ inflammation seen in *relb*$^{-/-}$ mice	Weih *et al.* (1997)
nfkb1$^{-/-}$*c-rel*$^{-/-}$	No	Immune defects more severe than individual mutants.	Pohl *et al.* (2002)
		Markedly diminished CD5^{+} peritoneal B cells	
		B cells fail to undergo blast formation	Grumont *et al.* (2002)
		Impaired antigen-induced CD4^{+} T-cell responses	Zheng *et al.* (2003)
		Conventional and pDC markedly reduced, partly owing to survival defects; maturation of pDC in response to specific TLR signals is impaired	O'Keeffe *et al.* (2005)
nfkb1$^{-/-}$*rela*$^{-/-}$	Yes (~E13)	Defects in myelopoiesis and B-cell development in radiation chimeras	Horwitz *et al.* (1997)
rela$^{-/-}$*c-rel*$^{-/-}$	Yes (~E13)	Multiple hemopoietic defects. Radiation chimeras exhibit nucleated erythrocytes, reduced number of B cells, systemic expansion of granulocytes and a reduction in monocytes	Grossmann *et al.* (1999, 2000)
		T cells exhibit a cell-cycle block early in G1 resulting from a failure to undergo c-Myc-dependent growth	Grumont *et al.* (2004)
rela$^{-/-}$*c-rel*$^{-/-}$*tnf*$^{-/-}$	Neonatal	E18 embryos exhibit multiple epidermal defects that include a failure to form specific hair types, disorganized basal	Gugasyan *et al.* (2004)

Table 1-2: TLRs and Their Respective Agonists.

Species	PAMPs	TLR Usage	PRRs Involved in Recognition
Bacteria, mycobacteria	LPS	TLR4	
	lipoproteins, LTA, PGN, lipoarabinomannan	TLR2/1, TLR2/6	NOD1, NOD2, NALP3, NALP1
	flagellin	TLR5	IPAF, NAIP5
	DNA	TLR9	AIM2
	RNA	TLR7	NALP3
Viruses	DNA	TLR9	AIM2, DAI, IFI16
	RNA	TLR3, TLR7, TLR8	RIG-I, MDA5, NALP3
	structural protein	TLR2, TLR4	
Fungus	zymosan, β-glucan	TLR2, TLR6	Dectin-1, NALP3
	Mannan	TLR2, TLR4	
	DNA	TLR9	
	RNA	TLR7	
Parasites	tGPI-mutin (*Trypanosoma*)	TLR2	
	glycoinositolphospholipids (*Trypanosoma*)	TLR4	
	DNA	TLR9	
	hemozoin (*Plasmodium*)	TLR9	NALP3
	profilin-like molecule (*Toxoplasma gondii*)	TLR11	

Figure 1-6: TLR4 Activation.

(From Watts, 2008)

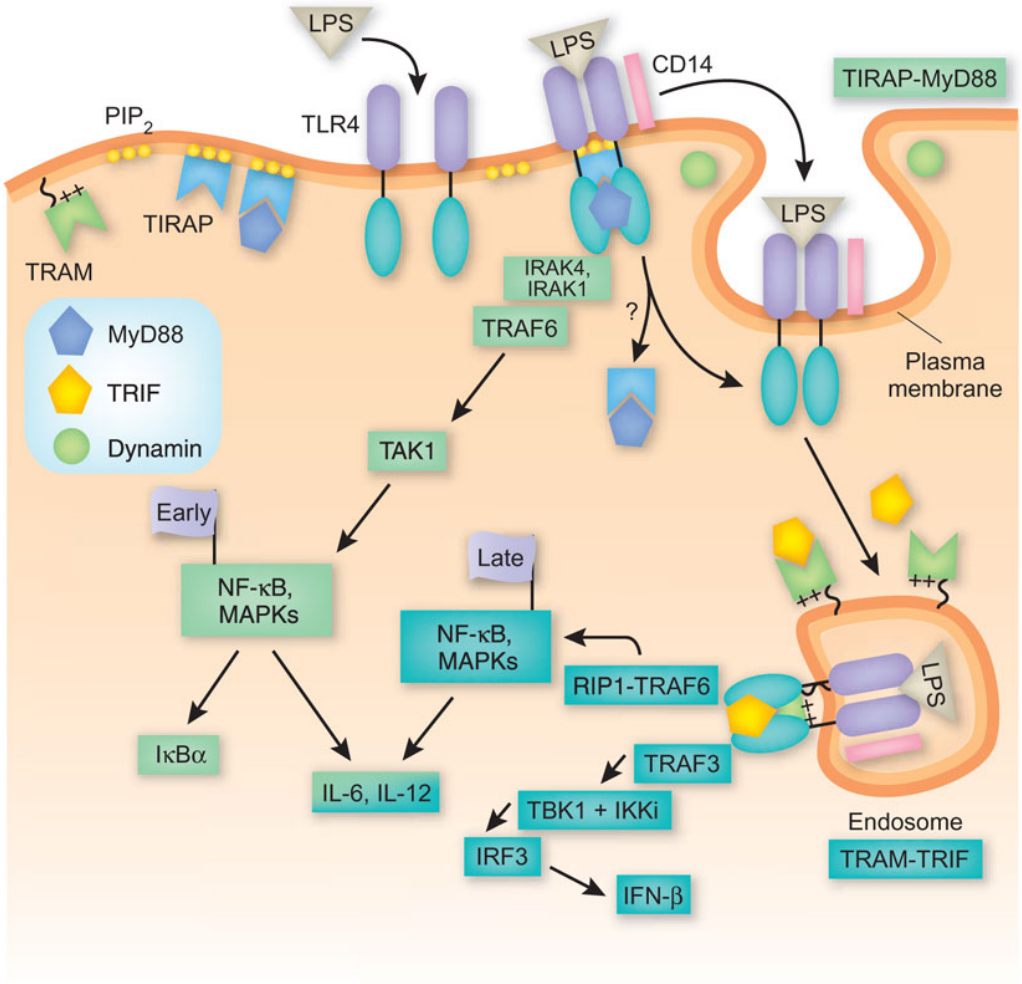

Figure 1-7: Schematic of IL-12, Il-23, Their Receptors, and Downstream Signaling Pathways. (From Teng et al., 2015)

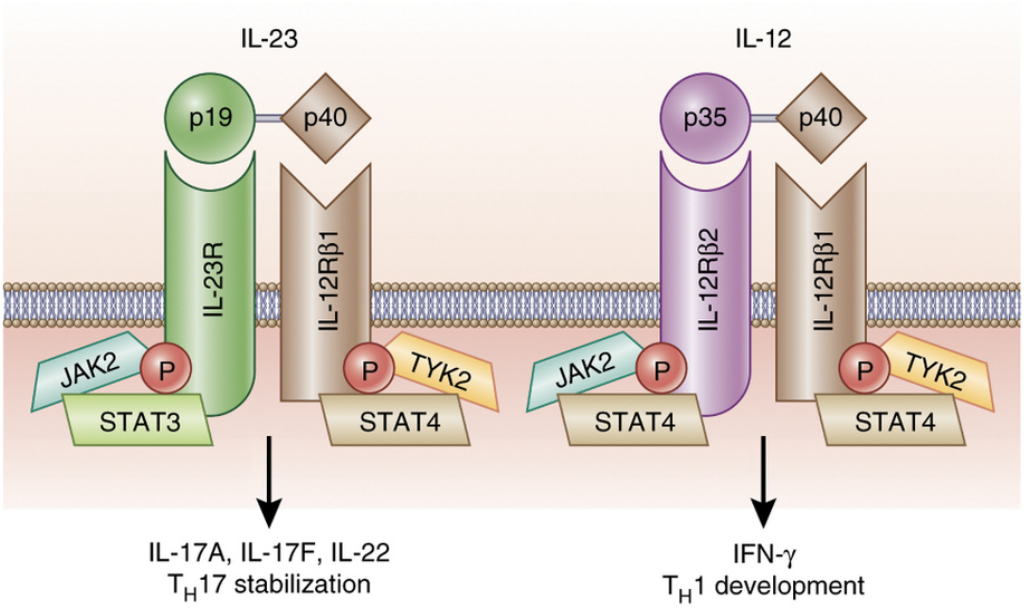

Figure 1-8: Schematic of Nuclear IκBs.

(From Chiba et al., 2013)

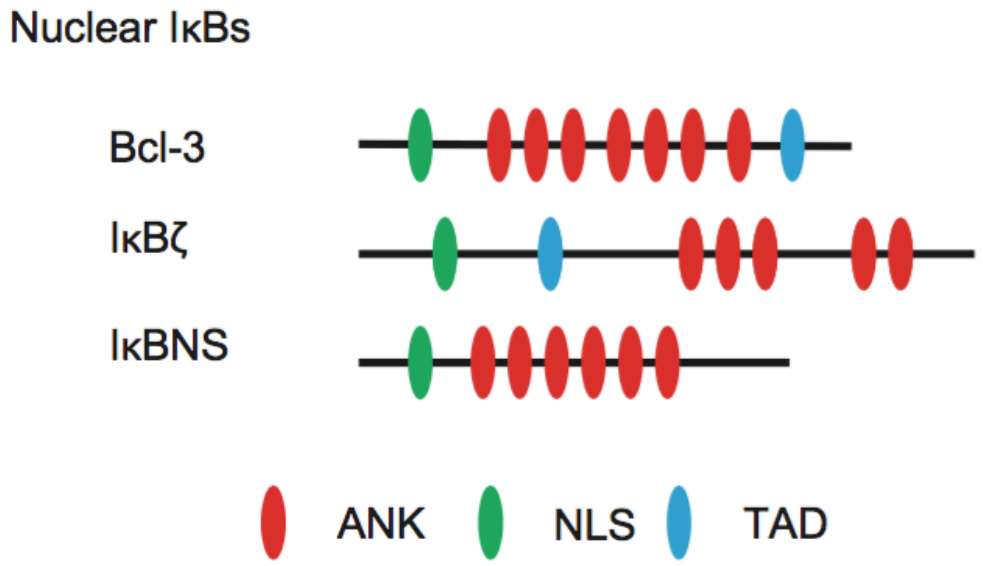

Table 1-3: Nuclear IκB Knockout Mice and Phenotypes.

(From Chiba et al., 2013)

IκB	Knockout mice	Specificity to NF-κB	Expression		Cytokine expression	Reference
			Basal	Inducible		
Bcl-3	Die earlier by multi-organ inflammation due to mTEC defect Abnormal splenic architecture and germinal center formation Sensitive to LPS-induced endotoxin shock	p50 or p52 homodimer	+	++	Inhibition: TNFα	(14, 15, 18–21, 23)
IκBζ	Atopic dermatitis-like lesion Prevents EAE development	p50 homodimer	-	+++	Activation: IL-6, IL-12p40, IL-17A	(30, 33, 34)
IκBNS	Sensitive to DSS-induced colitis and LPS-induced endotoxin shock Defect of B cell development	p50 homodimer	-	+++	Inhibition: IL-6, IL-12p40 Activation: IL-2	(37–40)

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Chapter 2

Genome-wide Exclusive Selectivity of c-Rel on *Il12b* Expression.

Abstract

IL-12B (IL12B) protein is an important pro-inflammatory cytokine that acts on T and natural killer cells and has a broad array of biological activities. It is strongly induced in LPS-stimulated macrophages and dendritic cells, which can then stimulate T helper cell 1 (Th1) differentiation or T helper cell 17 (Th17) maintenance. Our lab previously characterized the cis-elements in *Il12b* gene promoter and enhancer, and identified C/EBP and NF-κB as the key factors for *Il12b* expression (Plevy et al., 2007). Sanjabi et al. further showed that *Il12b* gene transcriptions is specifically dependent on NF-κB c-Rel and not RelA. Despite striking homologies between RelA and c-Rel NF-κB, c-Rel homodimers exhibit stronger binding affinity than RelA homodimers toward *Il12b* gene promoter. c-Rel homodimers can also synergistically bind to multiple potential NF-κB sites in *Il12b* promoter whereas RelA homodimers cannot. Finally, mRNA-seq data of LPS-induced macrophages also suggested strong c-Rel dependence in *Il12b* transcription (Sanjabi et al., 2000; Sanjabi et al., 2005, Chang, unpublished). However, whether this selective dependency is seen genome-wide at transcriptional level is unknown. Here, with novel chromatin-associated RNA-sequencing and ChIP-sequencing data, we report and confirm the exquisite selectivity of c-Rel in regulating *Il12b* transcription. Although other genes also show c-Rel dependence in their transcriptions, *Il12b* is the only gene, at genomic scale, showing strong c-Rel-dependent transcription as well as striking binding differences between NF-κB c-Rel and RelA.

Introduction

IL-12B protein exerts multiple functions due to its ability to form two different cytokines, IL-12 and IL-23. IL-12 consists of two subunit proteins, IL-12A (IL12A) and IL-12B (IL12B), linked by disulfide bonds. IL-12B is predominantly produced by inflammatory myeloid cells in response to endogenous or exogenous stimulations associated with host defense or wound healing, including LPS-induced TLR-4-mediated inflammatory response. IL-12 potently influences the development of T helper 1 (Th1) cell, albeit indirectly. It binds to IL-12Rβ1 and IL-12β2 receptor dimers of naïve CD4+ T cells and activate STAT4 transcription factors, which induce IFN-γ production (Thierfelder et al., 1996). IFN-γ subsequently activates the production of STAT1, which initiates the expression of T-bet (TBX21), the principal transcription factor that activates a set of genes to promote differentiation of Th1 phenotype (Afkarian et al., 2002). Interestingly, Th1cell potently expresses IFN-γ, thus creating a positive feedback loop for further T-bet expression. Th1 cell promotes cell-mediated immune responses and is required for host defense against intracellular viral and bacterial pathogens. By secreting IFN-γ and other cytokines, Th1 promotes macrophage antigen presentation, lysosome activity, and nitric oxide production, leading to the phagocytosis and destruction of microbial pathogens (Janeway et al., 2001).

IL-23 consists of IL-12B and IL-23A, again linked by disulfide bonds. While initially thought as an important cytokine to induce Th17 differentiation,

the finding that naïve T cells do not have receptors for IL-23 (Zhou et al., 2007) refines the role of IL-23 as to maintaining rather than inducing Th17 phenotypes (Stritesky et al., 2008). Th17 differentiation from naïve cells is identified to depend on the stimulation of TGF-β and IL-6, which induce IL-21 expression that drives Th17 phenotypes (e.g. IL-17A secretion).

Given the multifaceted role of IL-12B, it is apparent that its dysregulation is related to various diseases and defects. Th1 cell is crucial to the elimination of intracellular pathogens like *L. major* and *M. tuberculosis* (Reiner and Locksley et al., 1995; North and Jun, 2004), yet excessive Th1 cell activity, especially in IFN-γ and TNF secretion, has been implicated in causing autoimmune diseases like Crohn's disease and inflammatory bowl diseases (Powrie et al., 1994; Plevy et al., 1997). On the other hand, the regulation of IL-23 cytokines has been of great interests since the discovery of Th17 subset and its indications in various chronic inflammatory diseases such as multiple sclerosis, psoriasis/psoriatic arthritis, as well as inflammatory bowel disease (Jadidi-Niaragh and Mirshafiey, 2011; Babaloo et al., 2015; Gálvez 2014). This is evident in IL-12B-deficient mice, which are resistant to experimentally induced autoimmune conditions, including brain-derived antigen-induced paralysis, joint antigen-derived arthritis inflammation, and multiple gut disease models (Cua et al., 2003; Murphy et al., 2003; Luger et al., 2008). Currently, at least ten therapeutic agents targeting IL-12, IL-23 or IL-17A are being tested in the clinic for more than 17 immune-mediated diseases (summarized in Teng et al., 2015).

To avoid defects and mount appropriate immune responses, the expressions of IL-12A, IL-12B, and IL-23A need to be under tight control. *Il12a* is widely expressed in many cell types, albeit at low levels; most of which do not even produce IL-12 cytokines (Ma et al., 2015). In LPS-simulated human monocytes, although IL-12B is strongly induced, IL-12 secretion is positively correlated with the expression of IL-12A instead of IL-12B. Therefore, it is thought that the expression of IL-12A is the rate-limiting step of IL-12 production (Snijders et al., 1996). Likewise, for IL-23, although more studies are needed for this relatively novel cytokine, it is generally believed that the rate-limiting step of IL-23 production is the expression of the IL-23A transcript (Lyakh et al., 2008).

c-Rel and RelA proteins contain TAD domains and are considered the dominant NF-κB trans-activators in classical NF-κB activation. Although RelB also contains a well-defined TAD, it predominantly exists as RelB:p100 heterodimer in cells which requires alternative NF-κB pathway for activation. In terms of dimer species, RelA:p50 heterodimer is thought to be more abundant than RelA:RelA homodimer due to its high stability in protein-protein interaction (Huang et al., 1997). c-Rel, however, under similar conditions, does not share this preference for p50 and is thought to dimerize equally well with itself as well as with p50 (Karin, 2011). This is particularly intriguing as RelA and c-Rel share greater than 70% sequence identity within their dimerization domains and the residues responsible for dimerization are identical in both proteins. The RHD domains of c-Rel and RelA are more closely related to each other than to other NF-κB members (Li and Verma, 2002). Furthermore, c-Rel and RelA use the

same residues to make contacts with DNA backbone and specific bases within NF-κB binding sites (Chen and Ghosh, 1999; Huang et al., 2001). Despite the similarities, using purified recombinant NF-κB proteins and their potencies in activating reporter assays, c-Rel homodimers have shown to bind to slightly broader range of DNA sequences than RelA homodimers (Kunsch et al., 1992). Another study that utilized electrophoretic mobility shift assays (EMSAs) to study preferred binding sequences of each NF-κB dimer also indicated that a few c-Rel-dependent genes do favor c-Rel:c-Rel homodimer or c-Rel:p50 heterodimer bindings over RelA (Hime et al., 1996; Shapiro et al., 1997; Chen et al., 2000).

These findings provide valuable information on the differences between c-Rel and RelA. Nevertheless, most of these studies are limited to certain NF-κB site sequences or to only the few highest affinity sites. Siggers et al. addressed the issue using unbiased protein binding microarray (uPBM) and surface plasmon resonance (SPR) with 8 different dimer species, including c-Rel- or RelA-containing dimers. However, RelA and c-Rel exhibited highly correlated binding profiles in either homo- and hetero-dimer species. Interestingly, c-Rel homodimers exhibited higher binding affinity toward a large subset of non-consensus NF-κB sites than RelA. This suggests that binding affinity, and not binding specificities, may be the factor that discriminates c-Rel/RelA binding (Siggers et al., 2011).

Sanjabi et al. fist reported that *Il12b* expression is strongly dependent on c-Rel-containing complexes in LPS-induced macrophages (Sanjabi et al., 2000). To identify the region of c-Rel that is required for *Il12b* transcription, RelA/c-Rel

chimeric proteins were analyzed using the retroviral transduction assay. Forty-six residues of c-Rel N-terminus Rel homology domain (RHD) were identified essential for the induction of *Il12b* expression. These 46 residues affected the binding affinity of c-Rel homodimer to DNA, particularly towards non-consensus NF-κB sites, most likely by causing conformational changes in the overall structure of c-Rel Rel homology domain (RHD). This allowed c-Rel:c-Rel homodimer, but not c-Rel:p50 heterodimer, to bind at a much higher affinity than RelA homodimer by approximately 40 fold (Sanjabi et al., 2005).

Although c-Rel and RelA homodimers exhibit highly correlated binding specificity at high affinity NF-κB sites, via SPR studies, Chang observed that c-Rel homodimer can bind to 3' stretch of thymines (5'-GGTTTT-3') at a much higher affinity than RelA homodimer (unpublished). Interestingly, besides the two NF-κB binding sites already identified in *Il12b* promoter (Sanjabi et al., 2005), two potential sites that carry 3' stretch of thymines were identified immediately downstream. Strikingly, using EMSAs to study the binding of NF-κB dimers to these four sites, our lab observed that c-Rel homodimer strongly prefers multidimerization at these four NF-κB sites more than other dimer species (Chang, unpublished). Together, these data suggest that *Il12b* transcription is dependent on c-Rel for two main reasons: first, c-Rel homodimer can bind to DNA at a much higher affinity than RelA homodimer. Second, c-Rel homodimer can, possibly via cooperative binding, synergistically bind to multiple NF-κB sites in *Il12b* promoter. In consistence with the previous findings, in mRNA-sequencing, Chang demonstrated that *Il12b* is one of the most inhibited genes in

LPS-induced c-Rel$^{-/-}$ mouse macrophages. While the data supported strong *Il12b* dependence on c-Rel, more information is needed to further refine the claim. First, as a transcription factor, c-Rel is mostly likely affecting the expression of *Il12* at transcriptional level. mRNA-sequencing may not accurately reflect the changes happening during transcription. Second, while EMSA and uPBM/SPR results are promising, *in vivo* data of c-Rel vs. RelA binding is needed to confirm the selective binding observed in *Il12b* promoter.

Methods and Material

Chromatin-associated RNA-seq Experiments

Bone marrow cells were extracted from C57BL/6 and c-Rel$^{-/-}$ mice between four to six week-old. Bone marrow-derived macrophages (BMDMs) were differentiated from bone marrow cells by growing in L929-conditioned medium (L929-CM). For each time point per strain, one 150-mm plates, in which about 20-25 million bone marrow cells were seeded on Day 0, was used. Old L929-CM is replaced with fresh L929-CM on Day 4 of differentiation. On Day 6, the adherent macrophages were activated with lipid A (100ng/ml) (Invitrogen).

The cells were collected at 0, 0.5, 1, 2, and 6 hours post lipid A stimulation. Cells were washed with cold DPBS twice before subcellular fractionation as described before (Pandya-Jones and Black, 2009), with minor changes. The cell lysis buffer contained 0.15% NP-40, and the sucrose cushion did not contain any detergent. Western blot analysis was used to confirm fraction purity. SNRP70 (Black lab), beta-tubulin (Sigma), and histone H3 (Abcam) were

used to label nucleoplasmic, cytoplasmic, and chromatin fraction, respectively. Cytoplasmic, nucleoplasmic, and chromatin RNA RNA were purified using Qiagen RNeasy columns. Chromatin RNA was isolated using TRI-reagent (MRC), followed by further purification with RNeasy columns. RNA (500ng) from the chromatin fraction was depleted of ribosomal RNA (rRNA) using the Mouse/Human Ribominus Kit (Invitrogen).

After Ribominus treatment, RNA (60 ng) was used to generate strand-specific libraries according to the TrueSeq Stranded total RNA Library Prep Kit (Illumina), with minor changes to incorporate the "dUTP" method as described by Levin et al. (Levin et al., 2010). An Illumina HiSeq 2000 was used for sequencing with a single-end sequencing length of fifty nucleotides.

After sequencing, the reads were aligned to the mouse mm9 reference genome with Tophat (Trapnell et al., 2010). The alignments were restricted to unique mapped reads with two mismatches allowed. RPKM values were calculated as described (Mortazavi et al., 2008) using mm9 Refseq gene annotations for locus definition (Pruitt et al., 2005) in Seqmonk (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). RPKMs were calculated by counting all mapped reads and dividing by the entire length of each locus for each gene.

c-Rel and RelA ChIP-sequencing

For ChIP-sequencing experiments, BMDMs were generated as described earlier, stimulated with lipid A, and collected at the same time points. ChIP-seq

was performed as described (Barish et al., 2010; Lee et al., 2006) with minor modifications. Namely, after stimulation, cells were washed twice with cold DPBS before crosslinked with 2mM DSG for 45 minutes, followed by 1% (v/v) fomaldehyde (FA) (double crosslinking). After crosslinking, the cells were lysed and chromatin pellets were sonicated until the majority of DNA size was between 200 and 1000 base pair long, with an average size of approximately 500 base pairs (checked with agarose gel electrophoresis). ChIP-seq libraries were prepared using the Kapa LTP Library Preparation Kit (Kapa Biosystems). RelA antibody (Santa Cruz Biotechnology, sc-372) was used for RelA ChIP-seq (performed by Tong et al., 2016). c-Rel antibody (Santa Cruz Biotechnology, sc-71) was used for c-Rel ChIP-seq.

Reads were aligned to the mm9 mouse genome with Bowtie2 software. Unique mapped reads were used for peak calling and annotation using HOMER (Heinz et al., 2010). Peaks were called if they passed a false discovery rate of 0.05 and were enriched over input. Called peaks were considered for downstream analysis if peaks from at least 4 of 7 (for RelA) or 3 of 6 (for c-Rel) were overlapping within 200 bp. Peaks were than annotated to the nearest TSS.

Results

The first two sections of the result were designed and performed by Dr. Abraham Chang. However, I will discuss his yet unpublished work here briefly in order to lead up to my contributions to the overall project.

Conserved DNA elements in the *Il12b* promoter show similarity to non-consensus NF-κB sequences preferentially bound by c-Rel.

As mentioned earlier, Sanjabi investigated *Il12b* dependence on c-Rel transcription factor, and identified 46 unique residues in the N-terminus of c-Rel RHD to be crucial for its higher binding affinity in *Il12b* promoter than RelA. (Sanjabi et al., 2000; Sanjabi 2005). Later, a comprehensive cross examination of eight different NF-κB dimers in terms of their binding specificities was performed by using protein-binding microarray (PBM), which unbiasedly detected binding affinity between DNA and each NF-κB dimer to assigned a normalized z-score (Siggers et al., 2011). When comparing the binding preference of RelA and c-Rel homodimer, the initial observation indicated that the two homodimers exhibited highly correlated binding profile. This agrees with the fact that c-Rel and RelA use the same amino acid residues to make contacts with DNA backbone and specific bases within NF-κB binding sites (Chen and Ghosh, 1999; Huang et al., 2001). Regardless, closer examination revealed a subset of sequences that showed preferential binding to c-Rel over RelA. Initially, this subset was recognized as GGGGGTTTTT (Figure 2-1A), which is significantly different than the consensus NF-κB sequence, GGGAAATTCC. However, with the corroborating

data from surface plasmon resonance (SPR), in which NF-κB binding affinities towards specific DNA sequences were measured by the dissociation rates ("off rate", or k_d), it was shown that the enhanced affinity was directly related to the 3' stretch of thymines (GGGGGTTTTT) (Figure 2-1B). Whereas the 5' stretch of guanines did not show biased preferential binding to either RelA or c-Rel, 3' thymines were shown to bind to c-Rel at about 10 fold higher affinity than RelA.

The unusual preference of 3' thymines towards c-Rel homodimers intrigued the re-examination of *Il12b* promoter. Previously, two NF-κB binding sites have been identified in the *Il12b* promoter (one distal and one proximal site). *Il12b* expression is proposed to depend on c-Rel homodimer because c-Rel can recognize a broader range of NF-κB recognition sequences with high affinity. Immediately downstream to the two original NF-κB binding sites, two novel, non-consensus potential NF-κB binding sites were identified within a highly conserved 25 bp region, just upstream to the known C/EBP site. The two novel sites were not identified before, and both of them contain GTTTT motif that is 100% conserved between mouse and human (Figure 2-1C).

Cooperative binding on tandem NF-κB sites in *Il12b* promoter unique to c-Rel homodimers.

To investigate the two novel binding sites (named κB3 [upstream] and κB4 [downstream] from now on), electrophoretic mobility shift assays (EMSAs) were used to assess NF-κB:DNA binding. c-Rel homodimer binds strongly to κB3 and moderately to κB4, while RelA homodimer binds poorly to κB3 and

non-detectable to κB4 (data not shown). These observations agree with PBM and SPR data, and strongly indicate that c-Rel homodimer binds to all four sites with higher affinity than RelA homodimer.

To inquire whether the c-Rel-preferred bindings at these sites affect gene transcription, mutant p40-promoter reporter plasmids were co-transfected with different NF-κB members into 293T cells. Strikingly, co-transfection of c-Rel and reporter plasmids resulted in a greater than 100-fold transactivation in contrast to the minimal effect of RelA co-transfection (Figure 2-2). Interestingly, mutations in distal, proximal, and κB3 sites all resulted in significant decrease in promoter activity, albeit to various degrees. The fact that each binding site can be mutated and affect reporter activity strongly suggests that these mutations prevent additional c-Rel dimers from binding to adjacent binding sites in order to optimally activate gene transcription.

In order to see if the multiple NF-κB binding sites found in *Il12b* promoter supports simultaneous or independent c-Rel homodimer binding, EMSA was again used for evaluation. Radio-labeled DNA probes that contained at least two and up to all four NF-κB sites in *Il12b* promoter were tested with increasing c-Rel homodimer concentration. Interestingly, single, double, and triple dimer complexes bound to the same DNA were observed. However, no quadruple dimer complexes were seen even when all four NF-κB sites are present (Figure 2-3A). One explanation for the absence of quadruple dimer complex is due to c-Rel's weak affinity towards κB4 sites, as shown in previous ESMA. Therefore, the c-Rel/DNA complexes formation depends on DNA sequences, which allows c-Rel

to simultaneously bind to the multiple NF-κB sites (primarily the distal, proximal, and κB3 site) within *Il12b* promoter. Importantly, when the same DNA probe that contains three NF-κB sites was tested with other NF-κB dimers (RelA:RelA, N34:N34 [chimeric RelA with c-Rel 46 residue substitution], p50:p50, cRel:p50, and RelA:p50), primarily only single dimer complex on a DNA probe was observed, with double dimer complexes on a DNA probe were only weakly observed at the highest protein concentrations (Figure 2-3B). In summary, the results indicate that only c-Rel homodimers can readily and simultaneously form triple dimer complexes with DNA in *Il12b* promoter region.

The induction of *Il12b* expression is the predominant role of c-Rel in early stage lipid A-stimulated bone marrow-derived macrophages.

The following sections are primarily designed and performed by George Yeh, with guidance from Dr. Stephen Smale.

With mRNA-sequencing data, although previously Chang showed that *Il12b* mRNA, along with several other genes, was dependent on c-Rel transcription factor for transcription, questions still remained whether c-Rel is directly affecting *Il12b* gene expression at transcriptional level. mRNA level can sometimes diverge considerably from transcription level and do not faithfully reflect real-time temporal changes. For example, it is often observed that peak mRNA level lags behind the maximal activation of transcription (Hao et al., 2009;

Rabani et al., 2011). Likewise, although microarray studies have been performed on c-Rel$^{-/-}$ macrophages and have identified several c-Rel-dependent genes, microarray tends to underestimate the magnitudes of changes in mRNA levels and limit the ability to examine dynamic changes in transcription (Marioni et al., 2008). To address the issues, wild type and c-Rel$^{-/-}$ bone marrow-derived macrophages (BMDMs) were cultured and stimulated on Day 6 of differentiation with lipid A (100 ng/ml). In order to accurately reflect the kinetics and immediate changes in gene transcription, chromatin-associated RNA (termed chrRNA from now on), instead of mRNA, was used as the starting material for strand-specific next generation sequencing library preparation. The advantage of utilizing chrRNA for transcriptome studies is that it directly captures the synthesis of nascent transcripts and in general is free from being affected by other downstream processes such as post-transcriptional regulation, accumulation of mature mRNA, and variation in mRNA half-life (Bhatt et al., 2012). In order to document the temporal changes in gene transcription, cells were collected at 0, 0.5, 1, 2, and 6 hours post lipid A stimulation. The earlier time points (0.5, 1, and 2 hour) were selected to reflect immediate transcriptional changes mediated by rapid pathways such as MAPK and NF-κB signaling, where as the 6 hour time point allows us to investigate late-expressing signals such as in *Nos2* and *Ptgs2* genes. Lastly, two biological replicates of c-Rel-deficient BMDM chrRNA-seq were performed, and each of it has a wild type biological replicate performed in parallel.

Since c-Rel is a potent trans-activating NF-κB protein, we look into its effects on genes that are up-regulated by lipid A treatment. To ensure high

confidence in RPKM values and significance in *in vivo* physiology, we set stringent criteria to generate a list of "lipid A-inducible genes". Namely, a gene has to have an average RPKM greater than 3 in at least one of the five time points to be considered expressed. In additional, in terms of average RPKM, a gene has to be induced by least 10 fold when compared to its resting state (0-hour sample) to be considered a lipid A-inducible gene. The criteria result in 306 genes being identified as "lipid A-inducible genes". While these criteria might be strict and exclude many weakly induced genes, they allow us to focus on the most potently affected genes, which much more likely will have relevant physiological impacts *in vivo*. Lastly, in stead of using the average RPKM pooled from both biological replicates for mutant-wild type comparison, we opt to compare each knockout sample with its corresponding wild type sample separately first, followed by checking whether the observations made in one sample pair is reproducible in the other one. Only the genes that meet the criteria in both sample pairs will be considered for subsequent analyses.

To identify genes that are distinctly expressed between wild type and c-Rel-deficient BMDMs, log2-transformed maximal RPKMs of each lipid A-inducible gene in wild type and mutant samples were plotted for both sample pairs with the addition of standard error (Figure 2-4). Notably, in each biological replicate, most of the lipid A-inducible genes wre expressed to about the same levels between wild type and c-Rel$^{-/-}$ cells, falling within two standard errors of differences (about 97%, see Table 2-1 for summary), indicating that most of the genes were not affected significantly in the absence of c-Rel protein. One possible

explanation for this limited effect of c-Rel deficiency is its overlapping functions with RelA. As discussed before, RelA:p50 is the most prevalent NF-κB dimer and share a highly similar DNA binding profile with c-Rel. Therefore, presumably, the absence of c-Rel-containing dimers can be compensated by the presence of RelA-containing dimers. Nevertheless, a considerable amount of genes is showing moderate differences between wild type and knockout, falling in between one and two standard errors between wild type and knockout (about 21%, see Table 2-1). It is possible that these genes are partially dependent on c-Rel-containing complexes for optimal expression, as suggested by previous observations that c-Rel homodimer could bind to NF-κB sites with higher affinity than RelA. Regardless, as most of the genes that fall within two stand errors did not show drastic differences in expressions between wild type and knockout cells, whether they affect TLR-4-mediated inflammatory responses in BMDM in a physiologically relevant way is debatable and requires further experimentation. Given the fact that c-Rel is considered a trans-activating transcription factor, one would expect most of the genes that fell between one and two stand errors to show down-regulated expressions in c-Rel$^{-/-}$ macrophages. However, in both sample pairs, the percentage of genes showing higher expression in either wild type or knockout was about equal (Table 2-1). This suggests that most of the minor differences observed within two standard errors are either sporadic or hard to be directly related to the lack of c-Rel transcription factor.

Despite highly correlated gene expression profiles between the wild type and knockout, a highly selective subset of genes (about 3%, Table 2-1) exhibits

pronounced differences between wild type and knockout as they fell outside the boundaries of two standard errors. Interestingly, although each biological replicate exhibited nine genes outside of two standard error differences, only five of them overlapped between the two biological replicates. Of note, these five genes, in both sample pairs, were the most down-regulated genes (except *Timd4*, which was the most up-regulated gene) in the 306 lipid A-inducible gene list. Furthermore, the fold differences between wild type and knockout of these five genes were highly correlated between replicates (Table 2-2A). The high selectivity and reproducibility confer high confidence to the speculations that these genes are strongly dependent on c-Rel for their transcriptional regulation, which we will discuss next in detail.

A comprehensive expression profiles of these five genes in both sample pairs are displayed in Table 2-2A and B. Of note, although *Clcf1* is the gene showing the strongest fold differences and deviations between wild type and knockout, two main reasons suggest that *Il12b* is the most significant gene of the list. First, the maximal raw reads of *Il12b* genes is on average 10 times higher than that of *Clcf1*, indicating a stronger induction of *Il12b* gene (Table 2-2B). Second, *Clcf1*, when compared to resting state, is induced to on average 60 fold whereas *Il12b* is induced to on average 2000 fold. The other two genes, *Med21* and *Il4i1*, are also strongly expressed in wild type but not c-Rel$^{-/-}$, as evident from their high raw read counts with loci that are even shorter than *Il12b*'s. Their maximal fold change, when compared to resting state, is only about 20 fold due to higher basal expression than *Il12b*. Nevertheless, both of their basal and induced

RPKMs/raw reads show high reproducibility from the two sample pairs, giving us high confidence in the observed down-regulation in c-Rel$^{-/-}$ samples. Of the five reproducible and strongly affected genes, *Timd4* is the only one that is expressed higher in c-Rel$^{-/-}$. In fact, the highest RPKM of *Timd4* in wild type is less than 0.1. The kinetic of transcription indicates the expression of *Timd4* peaks at two hours in c-Rel$^{-/-}$ cells. In contrast, the other four genes peak at either 0.5- or 1-hour post lipid A stimulation (Figure 2-5), which are considered more in line with the tempo of NF-κB binding and subsequent gene activation.

c-Re, but not RelA, selectively binds to *Il12b* promoter *in vivo*.

One issues of using NGS technology to study transcriptional differences between wild type and mutant is the lack of mechanical information to make direct correlation between the observed phenotypes and factor of interests. In terms of c-Rel, a transcription factor, Chromatin ImmunoPrecipitation-sequencing (ChIP-seq) provides the needed information by directly surveying its genome-wide protein:DNA interactions *in vivo*. The wild type BMDMs were cultured as described earlier and stimulated to the same time points as in chrRNA-seq. Also, two biological replicates were performed for each antibody. From our hands-on experience, ChIP-seq reproducibility was not as consistent as in RNA-seq. Therefore, for subsequent analyses, we only focused on reproducible peaks. Namely, after peak calling of each sample, peaks of the same antibody from lipid A-induced samples that were overlapping within 200 base pairs were considered reproducible peaks. For c-Rel ChIP-seq, we set the peak reproducibility threshold

to 3 out of 6 samples; in RelA ChIP-seq that was set to 4 out of 7 samples. Based on this reproducibility threshold, 2077 and 8455 peaks were called in c-Rel and RelA ChIP-seq, respectively, with 1863 peaks overlapped between c-Rel and RelA ChIP-seq (Figure 2-6). It is well documented that c-Rel and RelA, whether in homodimer or heterodimer species, recognize highly correlated DNA motifs. The fact that nearly 90% of c-Rel peaks were also observed in RelA peaks confirm this high correlation, although there were still 214 peaks (about 10%) unique to c-Rel. On the other hand, 6592 peaks were identified only in RelA and not c-Rel. Two possible reasons may explain this discrepancy. First, the RelA antibody exhibited higher immunoprecipitation efficiency than c-Rel in our hands, even after extensive optimizations were performed for c-Rel ChIP-seq. As ChIP-seq quality relies substantially on the quality, sensitivity, and specificity of antibodies, c-Rel antibody may be inferior than RelA in these regards. Second, it is possible that the endogenous protein level of RelA is much higher than c-Rel, given the fact that RelA:p50 is often the most abundant NF-κB dimer found in cells. A recent report in which RelA and c-Rel ChIP-seq were performed in human B cells, using the same antibodies, also reported lower immuoprecopitation efficiency and peaks identified with c-Rel antibodies than RelA (Zhao et al., 2014).

To compare the binding of c-Rel and RelA, we used a normalized value, "Reads Per Kilobase Locus per Million mapped reads" (RPKLM) for comparison. We also tested using peak scores, as annotated by HOMER software, for comparison. Peak score was calculated based on the ratio between reads within a

locus using its surrounding reads as background. Given that c-Rel ChIP-seq has substantially higher background noise than RelA, we determined that using RPKLM gives a better quantitative comparison than peak scores. Another drawback of using peak score is that a value of zero is assigned when a peak is not called. This was problematic for our subsequent analyses as we focused on comparing c-Rel/RelA binding at all peaks by calculating their binding ratio. RPKLM values allowed us to make quantitative comparison even if a peak is not called in either c-Rel or RelA dataset. To generate loci of peaks for RPKLM calculation, each peak locus was set to cover the peak widths found in both c-Rel and RelA ChIP-seq. Within each locus, mapped reads from either c-Rel or RelA were then counted and normalized to the length of locus and total mapped reads to generate RPKLM values.

We inspected the peaks found nearby the five potential c-Rel-dependent genes (*Il12b, Il4i1, Med21, Clcf1,* and *Timd4*). Interestingly, we only found c-Rel peaks in the vicinities of *Il12b, Il4i1,* and *Med21* gene bodies (Figure 2-7). The lack of peaks nearby *Clcf1* and *Timd4* can be explained by two possibilities. One, it is possible that the c-Rel ChIPs-eq quality is not good enough to capture the binding of c-Rel around *Clcf1* and *Timd4*. This was most likely the case for *Clcf1* gene, as we did see RelA peaks -888 bp to its transcription starting site. Two, it may suggest that c-Rel is indirectly affecting the expression of the genes. This was most likely the explanation for *Timd4* gene, as no peak was found in either RelA and c-Rel dataset. As mentioned before, the maximal expression of *Timd4* happens at 2 hour, which is much later than the typical binding kinetics of NF-κB

dimers and the time most of NF-κB-dependent genes are expressed. These observations suggest that, at least in wild type BMDM, c-Rel suppresses the expression of *Timd4* indirectly, most likely via the expression of another c-Rel-dependent factor. For *Med21*, although no peaks are called in RelA ChIP-seq, a weak peak of both c-Rel and RelA can be visually identified at around -1774 bp to its TSS. Both RelA and c-Rel peaks are observed at -650 base pair to *Il4i1* TSS.

The most interesting observation was with *Il12b* gene. Three peaks were found in the vicinity of its gene body (Figure 2-7). The farthest peak (termed enhancer peak from now on), about -10,000 base pair to TSS, is a well-defined enhancer to which c-Rel and RelA both bind. The second farthest peak was about -1000 bp to TSS. Interestingly, only RelA had called peak in this locus, as c-Rel peak only showed up in 2 of 6 samples and did not meet the reproducibility threshold. The third peak located in the *Il12b* promoter (termed promoter peak) was -150 base pairs away from TSS. The promoter has been extensively studied before, and is known to contain crucial binding sites for NF-κB and C/EBP, both crucial to the *Il12b* expression (Plevy et al., 1997). As mentioned before, Sanjabi et al., showed this region binds to c-Rel with higher affinity than RelA, and Chang showed that c-Rel homodimer can form triple c-Rel homodimer complexes on the NF-κB sites found within the promoter. Strikingly, we did not see RelA peaks in this locus, whereas constitutive c-Rel peaks are observed, even at resting state. This *in vivo* evidence supports previous finding that c-Rel can strongly bind to *Il12b* promoter whereas RelA cannot. Along with chrRNA-seq data, these

observations provided convincing data explaining the unique selectivity of c-Rel on *Il12b* expression.

The unusual selectivity of c-Rel binding at *Il12b* promoter prompted us to investigate if others peaks also demonstrated such discrepancy between c-Rel and RelA binding. To do so, we calculated the RPKLM ratios of c-Rel over RelA at each locus. Initially, we calculated the ratios of all peaks found in either RelA or c-Rel ChIP-seq. However, further analyses indicated that the RPKLM of c-Rel in peaks that were only called in RelA ChIP-seq was not reliable, most likely due to the aforementioned inherent high background noise. In stead, we found that the ratio calculation is most reproducible if we only focused on the peaks that were called in c-Rel ChIP-seq. The log2-transformed c-Rel/RelA RPKLM ratios of all 2077 peaks found in c-Rel, of which 1863 (approx. 90%) are also found in RelA ChIP-seq, had the mean ratio value of -2.26 (Figure 2-8A, black dash line), most likely reflecting the fact that RelA antibody immunoprecipitation efficiency was indeed higher than c-Rel. Strikingly, at genome-wide scale, *Il12b* promoter peak was the second highest peak that preferred c-Rel binding over RelA (Figure 2-8A; red square). In addition, the highest peak that preferred c-Rel binding was about 22 kilobase pairs away from the nearest gene, Arghap35, which was not inducved by lipid A stimulation. In contrast, the enhancer peak of *Il12b* showed equal binding preference between RelA and c-Rel (yellow circle). Since the unique c-Rel-biased binding was only seen in promoter peak, it further strengthens the concept that the binding of c-Rel at the promoter, and not the enhancer, was the key determining factor of *Il12b* expression. In additional to genome-wide peak

analyses, we also restricted the analysis to all promoter peaks of lipid A inducible genes, as promoter is usually the most potent transcriptional regulatory element. Indeed, when restricting the ranking to promoter peaks of lipid A-inducible genes, *Il12b* promoter peak stood out prominently as the only peak preferring c-Rel binding over RelA (Figure 2-8B). As mentioned earlier, peaks were also identified for *Med21* (-1774 bp) and *Il4i1* (-650 bp). However, their c-Rel/RelA RPKLM ratios only fell slightly above +1 standard error (Figure 2-8A, green diamond and green triangle). While this does not negate the possibility that c-Rel is an important regulator for the the expressions of these two genes, it is hard to make conclusive interpretation without the information of $RelA^{-/-}$ BMDM. In summary, by combining chrRNA-seq and ChIP-seq, we offer compelling *in vivo* data suggesting that c-Rel is unusually unique in its ability to bind strongly to *Il12b* promoter and activates its gene transcription.

Discussion

In this report, we investigated the transcriptional regulatory role of c-Rel in lipid A-induced mouse BMDM. Combining high-throughput chrRNA-seq and ChIP-seq technologies, we are able to report c-Rel's specific role in gene activation with high confidence, particularly in terms of *Il12b* gene. Although it has been reported earlier, this is the first time we are able to accurately pinpoint c-Rel's point of action at transcriptional level and provide direct *in vivo* evidence for the exclusive selective binding at *Il12b* promoter at genomic scale. Although several other genes (*Med1, Il4i1*, *Timd4,* and *Clcf1*) were also reproducibly and

potently affected in c-Rel-deficient BMDM, our information only allowed us to speculate their dependence on c-Rel transcription factor, whether directly or indirectly.

The up-regulation of *Timd4* in c-Rel knockout mice is an interesting observation, as c-Rel is considered a trans-activating transcription factor. Due to the lack of detectable c-Rel binding (and RelA binding as well) in the gene's vicinity, this suggests that c-Rel may be indirectly affecting *Timd4* expression. However, given the fact that our c-Rel ChIP-seq data was not of the best quality, it is possible that we may have failed to capture its binding event. *Timd4* is extensively expressed on antigen-presenting cells like dendritic cells and macrophages. A cell surface receptor, TIMD4 binds to phosphatidylserines on the surface of apoptotic cells and induces phagocytosis to prevent the release of noxious materials (Miyanishi et al., 2007).

MED21 is a subunit of mediator complex and interact with RNA polymerase II (Chao et al., 1996). However, given that very few genes were effectively down-regulated in c-Rel$^{-/-}$ BMDM, the lack of MED21 didn't seem to affect too much on gene transcriptions in BMDM. Since the absence of MED21 inhibits keratinocytes differentiation, it is possible that MED21 specifically affects a subset of genes in that particular cell type (Oda et al., 2010). Il4I1 recently has been shown to inhibit Th1/Th2 polarization and induce Th17 production (Cousin et al., 2015). As noted before, IL-23 is an important cytokine to maintain Th17 cell phenotype. It is possible that BMDM utilize both IL4I1 and IL-23 to properly induce and maintain Th17 cell populations *in vivo*.

Often time, the observations made with ChIP-seq data were assumed to carry physiological significance. Our data demonstrated that, on the contrary, most of the c-Rel binding did not infer functional relevance, at least in terms of gene transcription. Of the 61 peaks found within promoters (-1000 to +150 bp) of lipid A-inducible genes (RPKM > 3, fold change > 10), only three genes (less than 5%) showed a 5-fold difference in expressions between wild type and c-Rel$^{-/-}$ BMDM. However, this makes sense with NF-κB protein family, in which the absence of one family member can be compensated by the others due to their highly correlated DNA binding properties. Regardless, in the entire genome, we were able to identify *Il12b* promoter as the only locus of lipid A-inducible genes that preferred c-Rel binding over RelA by a large margin. In fact, knowing the inferior quality of c-Rel antibody, we expect that our data was an underestimation of *in vivo* c-Rel binding. Interestingly, the two promoter peaks that preferred RelA over c-Rel binding the most were of *Nfkbia* and *Nfkbib* genes, the two key inhibitors and regulators of classical NF-κB activation. As these two genes were not affected in c-Rel-deficient mice, it would be interesting to see if their expressions are affected in RelA$^{-/-}$ mice.

The unique selectivity of c-Rel on *Il12b* expression is an appealing therapeutic target. Given such specificity at genomic scale, disrupting c-Rel-mediated *Il12b* expression could potentially have significant efficacy with limited side effects. However, our studies only focused on bone marrow-derived macrophages, whereas c-Rel has also been implicated important for the normal function of B and T cells. For example, c-Rel appears to promote G1- to S-phase

cell cycle progression by inducing the expression of E2F3a (Grumont et al., 1999; Cheng et al., 2003). During CD4+ T cell activation, c-Rel is required for the efficient expression of IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF, or CSF2), which is important for the differentiation of immature T cells into regulatory T cells and effector T cells (Gerondakis et al., 1996). More over, c-Rel has been shown to directly mediate the chromatin remodeling across *Il2* promoter (Rao et al., 2003; Chen et al., 2005). In BMDM, although *Csf2* is weakly induced by lipid A (max expression RPKM is 1.6 at 0.5 hour), we also observe potent down-regulations in c-Rel$^{-/-}$ cells. *Il2*, however, is not expressed in BMDM. Overall, when targeting c-Rel-mediated *Il12b* expression, one must keep in mind of c-Rel's pleiotropic effects in different cell types. Lastly, given that IL-12B is a subunit of both IL-12 and IL-23 cytokines, for the interests of target specificity, it might be more strategically sound to find methods to target either *Il12a* or *Il23a* expression separately.

Figure Legends

Figure 2-1: c-Rel Homodimer Binds to Non-consensus NF-κB Site with Enhanced Affinity.

(A) Protein binding microarray data of c-Rel homodimer (cRel/cRel), RelA homodimer (RelA/RelA), and chimeric RelA homodimer (N34/N34), which consisted of RelA protein with 46 c-Rel residues substitution, was generated and normalized for comparison. GGGGGTTTTT showed higher binding affinity to c-Rel and N34 homodimers but not RelA homodimer. In contrast, GGGGGGAGAT, which did not have 3' stretch of thymines, bound to both c-Rel and RelA homodimer poorly. (B) Surface plasmon resonance (SPR) was used to measure the binding affinities of the same dimers towards the two aforementioned DNA sequences. $t_{1/2}$ indicated the time required to disassociate half of the proteins from the detection surface, which was coated with DNA probes which can interact with dimers. Whereas c-Rel and RelA homodimers bind to GGGGGGAGAT equally poor, c-Rel homodimer showed 10-fold higher binding affinity towards GGGGGTTTTT than RelA. (C) With PBM and SPR data, two novel NF-κB binding sites (NF-κB3 and NF-κB4) were identified just downstream of two other NF-κB sites that have been reported ealier (NF-κB1 and NF-κB2). This region is highly conserved among species, including human and mouse. Importantly, all four NF-κB sites showed higher binding affinity to c-Rel homodimer than RelA.

Figure 2-2: Recombinant c-Rel Expression Induces Strong p40 Promoter Activation, which Depends on Multiple NF-κB Sites.

Top: p40 promoter reporter plasmid, which harbored NF-κB1, NF-κB2, and NF-κB3 sites, was co-transfected into 293T cells with plasmids that either express p50, c-Rel, or RelA proteins. The expressions of p50 and RelA did not induce p40 promoter activation, whereas c-Rel potently induced its activation. Individual mutation in each NF-κB site negatively affected the c-Rel-mediated reporter activation, with the one in NF-κB2 site being the most drastically reduced. Bottom: the DNA sequences of the wild type and mutant promoter of the reporter plasmids. Yellow: NF-κB1 site; red: NF-κB2 site; green: NF-κB3 site.

Figure 2-3: c-Rel can Form Triple Dimer Complexes on a DNA Probe.

(A) Using radio-labeled DNA that contains two to four NF-κB binding sites found within *Il12b* promoter, recombinant c-Rel homodimers were added to form protein:DNA complexes. Multiple c-Rel homodimers could bind to the same DNA, depending on the number of NF-κB sites. Importantly, at most, we only observed c-Rel homodimer forming triple dimer complexes on a DNA probe even when four NF-κB sites were available (top band). (B) In contrast to c-Rel homodimer, RelA and N34 homodimers bind to DNA but did not form multi-

dimer complexes on a DNA probe. At best, only double dimer complexes on a DNA probe were observed at the highest dimer concentration.

Figure 2-4: *Il12b*, *Med21*, *Il4i1*, *Clcf1*, and *Timd4* Gene Expressions are Dysregulated in c-Rel$^{-/-}$ BMDM.

The max RPKMs (log2-transformed) of the 306 lipid A-inducible genes from wild type and c-Rel$^{-/-}$ BMDM were plotted. Black line: mean. Orange lines: ±1 standard error. Red lines: ±2 standard error. Red-texted genes were strongly down-regulated (*Il12b*, *Clcf1*, *Il4i1*, and *Med21*) or up-regulated (*Timd4*) in both biological replicates of c-Rel$^{-/-}$ BMDM.

Table 2-1: Summary of Number of Genes in Each Category Based on Standard Error.

306 lipid A-inducible genes were categorized into three groups based on their expression differences between wild type and c-Rel$^{-/-}$. SE: standard error. Genes that fall between one and two standard errors were are further divided into two sub groups, depending on whether the expression was higher or lower in c-Rel$^{-/-}$ BMDM.

Table 2-2: Detailed Expression Profiles of the Five Most Dysregulated Genes in c-Rel$^{-/-}$ BMDM.

Full time course, from resting state (0-hour) up to 6-hour post lipid A stimulation, was displayed for the top five dysregulated genes in c-Rel$^{-/-}$ BMDM. (A) Refseq ID, gene symbols, RPKMs, and max RPKM fold differences between wild type and c-Rel$^{-/-}$ are listed. (B) Raw read counts were displayed. Gene lengths, which were used in RPKM calculation, were also displayed.

Figure 2-5: Expression Kinetics of the Five Dysregulated Genes.

Both sample pairs, each included a wild type and c-Rel$^{-/-}$ dataset, were displayed in full time course in RPKM values. The time of maximal expressions of genes were different. For *Clcf1* and *Med21*: 0.5-hour, *Il12b* and *Il4i1*: 1-hour, and *Timd4* at 2-hour. Notice that *Timd4* was not expressed in wild type but induced at 2-hour in cRel$^{-/-}$ BMDM.

Figure 2-6: Schematic of c-Rel and RelA ChIP-seq Overlap.

Peaks were called if reproducible in c-Rel (3 of 6) or RelA (4 of 7) samples. Called peaks from both datasets were then combined to search for peaks that appeared in both c-Rel and RelA ChIP-seq. For peaks to be considered overlapping in both c-Rel and RelA binding, they have to overlap within 200 base pair of peak distance. Venn diagram showed the overlap between c-Rel and RelA ChIP-seq. Notice that most of the c-Rel binding loci were also bound by RelA. Very few peaks were considered c-Rel-specific.

Figure 2-7: c-Rel and RelA Binding in *Il12b*, *Med21*, and *Il4i1* Genes.

These tracks display the peaks found in the vicinity of *Il12b*, *Med21*, and *Il4i1* genes. Three peaks were found for *Il12b*: enhancer peak (-9799), promoter peak (-154), and a third peak at about -1000 bp to TSS. Notice the peak in the middle is not called in c-Rel ChIP-seq due to the lack of reproducibility (below 3 of 6 threshold). The enhancer peak was bound by both c-Rel and RelA. The promoter peak was only bound by c-Rel (although weak RelA binding is observed). *Il4i1* had one peak at -650 bp which both c-Rel and RelA bind to. *Med21* had one peak at -1774 bp, which was also bound by both c-Rel and RelA. No c-Rel peaks were found in the vicinity of *Clcf1* and *Timd4*.

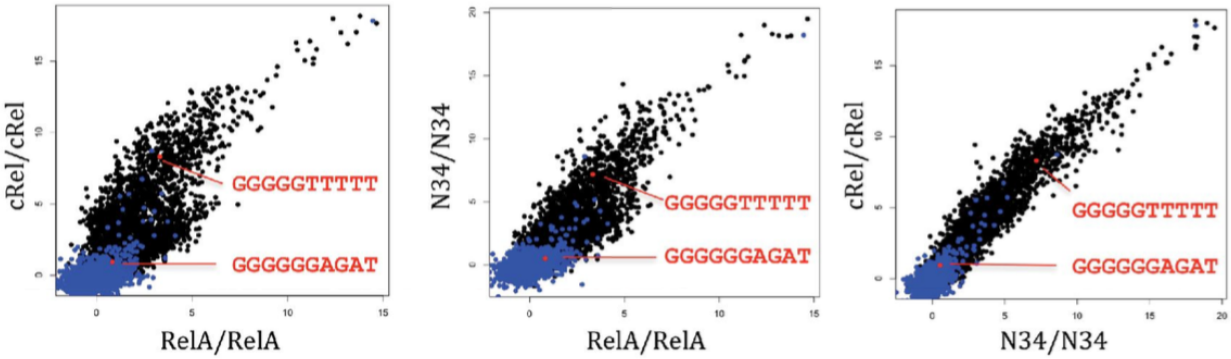
Figure 2-8: Log2-transformed c-Rel:RelA RPKLM ratios for All Peaks Called in c-Rel ChIP-seq.

All peaks called in c-Rel ChIP-seq were used to calculate the log2-transformed c-Rel/RelA RPKLM ratios. Peaks that were only called in RelA but not in c-Rel were not used because of unreliable c-Rel RPKLM values. Ratio values were ranked from the highest to the lowest, along with stand errors. (A) Red lines: ±2 standard errors. Green lines: ±1 standard error. Black dash line: mean. The fact that mean value was well below 0 (i.e. 1:1 ratio) indicated RelA's higher immunoprecipitation efficiency than c-Rel's The RPKLM ratios of *Il12b*, *Med21*, and *Il4i1* were marked. The *Il12b* promoter peak's ratio was the second highest

value of all 2077 peaks. *Med21* and *Il4i1*'s values fell between +1 and +2 standard errors. *Il12b* enhancer peak fell just above mean. (B) Promoter peaks of the inducible genes were listed here, again ranked by the log2-transformed c-Rel/RelA RPKLM ratio values. In total 61 promoter peaks. *Il12b* promoter peak was the only one that stands out in preferring c-Rel over RelA binding.

Figure 2-1: c-Rel Homodimer Binds to Non-consensus NF-κB Site with Enhanced Affinity. (From Chang, unpublished)

A



B

DNA	cRel/cRel	RelA/RelA	N34/N34
GGGGGGAGAT	4±1	4±1	8±5
GGGGGTTTTT	58±11	7±1	28±6
			$t_{1/2}$ (sec)

C

IL-12 p40 promoter sequence

NF-kB 1 NF-kB 2 NF-kB 3 NF-kB 4 C/EBP

Mouse GGAGGAACTTCTTAAAATTCCCCCAGAATGTTTTGACACTAGTTTTCAGTGTTGCAATTGAGACTAG

Human AAAGGAACTTCTTGAAATTCCCCCAGAAGGTTTTGAGAGTTGTTTTCAATGTTGCAA------CAAG

-125/-116 -115/-106 -104/-95 -92/-83 -80/-72

Figure 2-2: Recombinant c-Rel Expression Induces Strong p40 promoter Activation, which Depends on Multiple NF-κB sites.

(From Chang, unpublished)

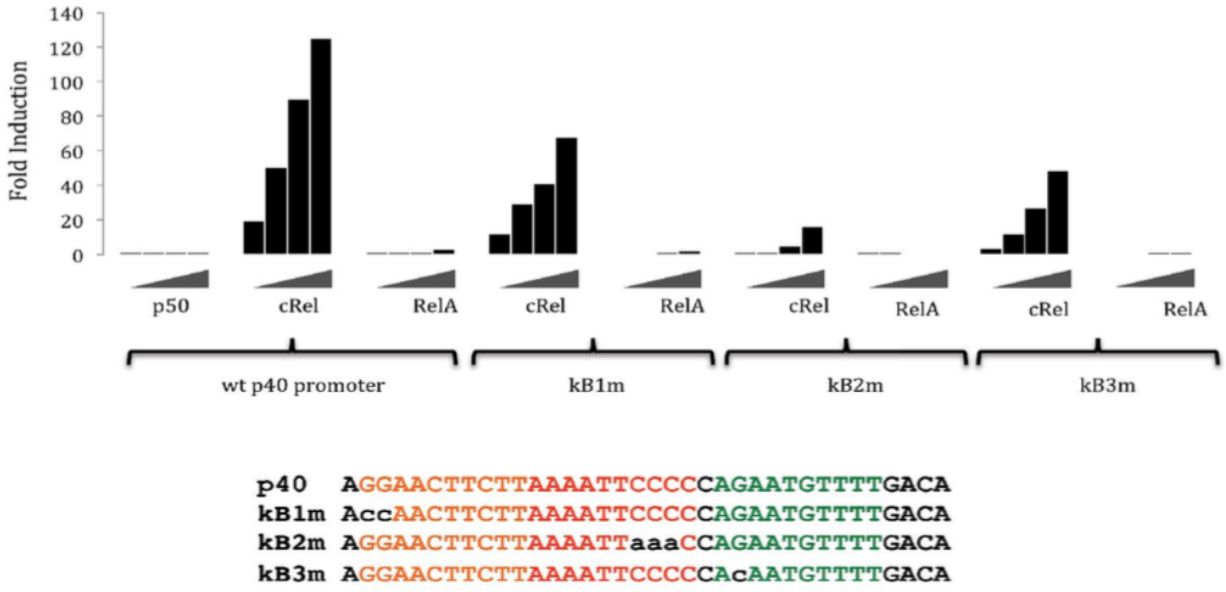
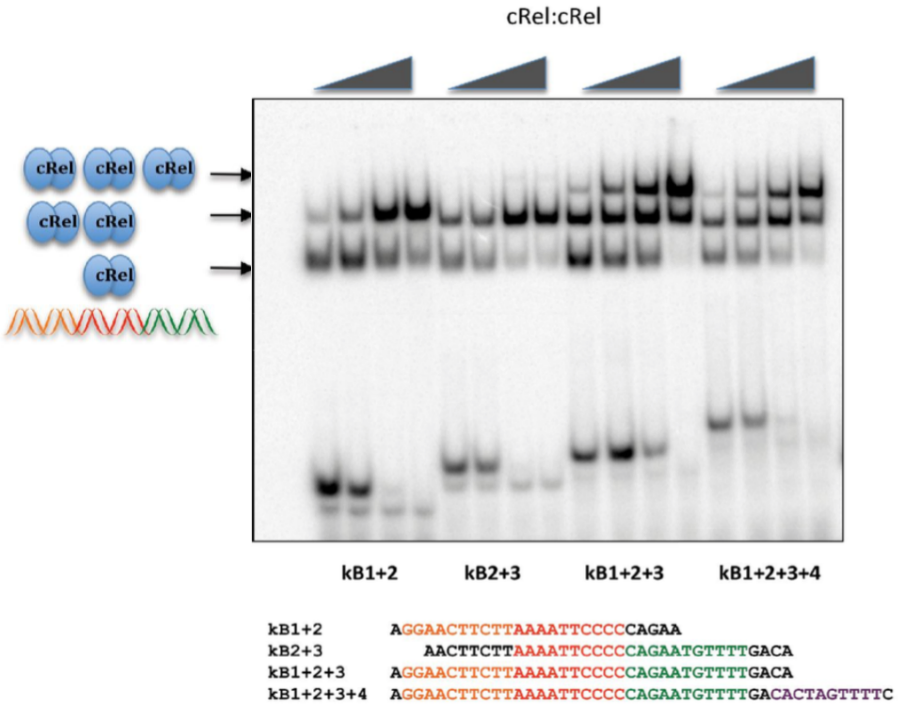


Figure 2-3: c-Rel Can Form Triple Dimer Complexes on a DNA Probe.

(From Chang, unpublished)

A



B

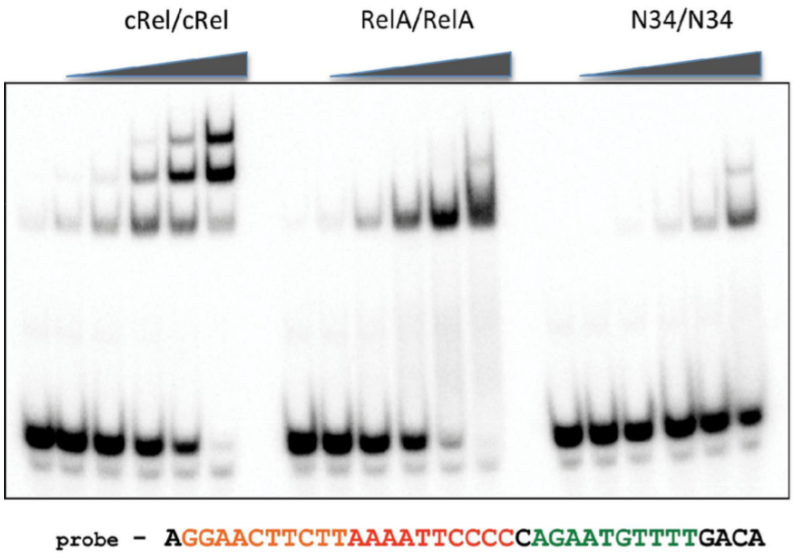
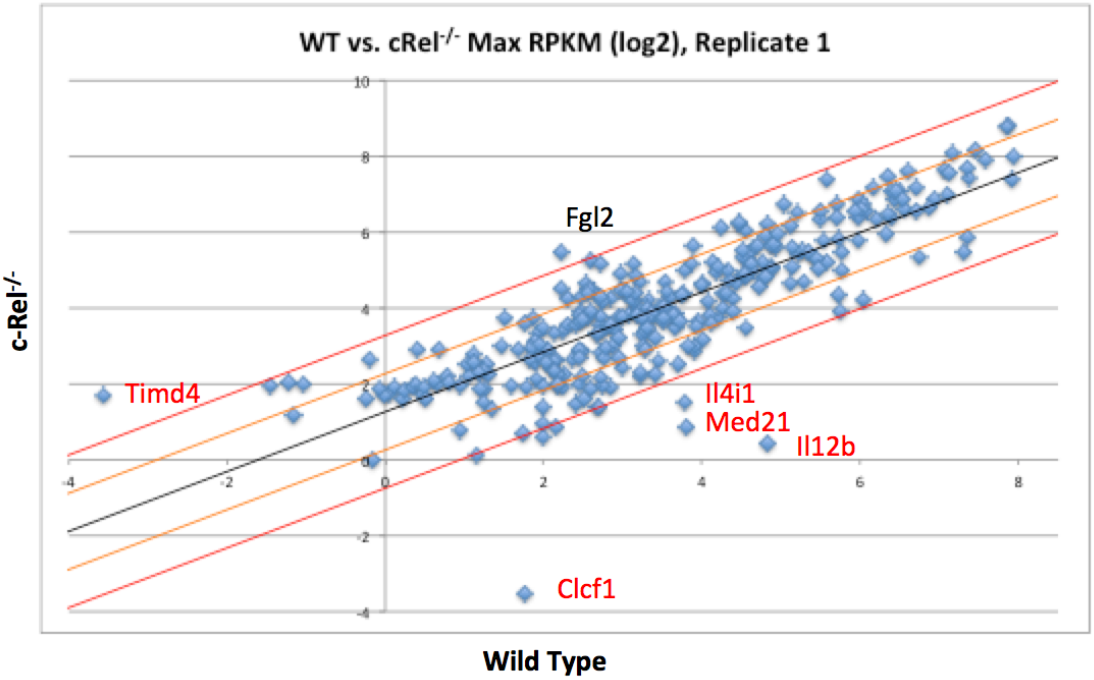


Figure 2-4: *Il12b*, *Med21*, *Il4i1*, *Clcf1*, and *Timd4* Gene Expressions are Dysregulated in c-Rel$^{-/-}$ BMDM.



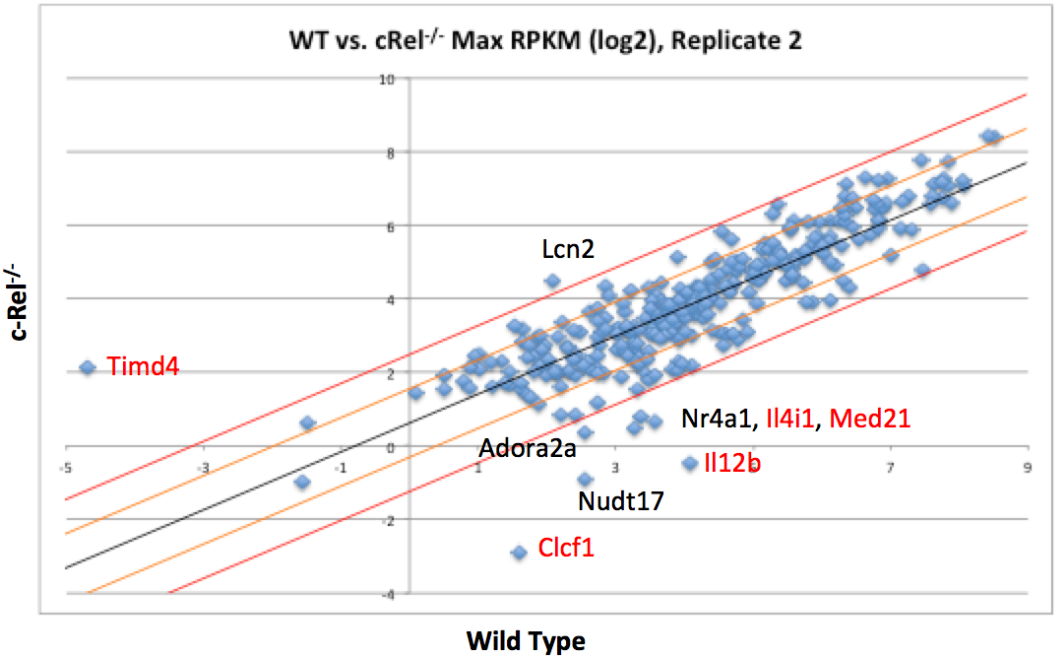


Table 2-1: Summary of Number of Genes in each Category Based on Standard Error.

	Category	Number of Genes	Percentage of 306 Genes	
Replicate 1	within 1 SE	227	74.18	
	between 1 and 2 SE	70	22.88	(37 expressed higher in c-Rel KO [33 higher in WT])
	greater than 2 SE	9	2.94	

	Category	Number of Genes	Percentage of 306 Genes	
Replicate 2	within 1 SE	234	76.47	
	between 1 and 2 SE	63	20.59	(31 expressed higher in c-Rel KO [32 higher in WT])
	greater than 2 SE	9	2.94	

Table 2-2: Detailed Expression Profiles of the Five Most Dysregulated Genes in c-Rel$^{-/-}$ BMDM.

A

(RPKM)

	Refseq_ID	Symbol	WT 0h	WT 0.5h	WT 1h	WT 2h	WT 6h	KO 0h	KO 0.5h	KO 1h	KO 2h	KO 6h	WT/KO max RPKM Fold Difference
Replicate 1	NM_019952	Clcf1	0.05	3.39	2.93	1.04	0.43	0.04	0.06	0.09	0.05	0.04	57.70
	NM_008352	Il12b	0.01	2.24	28.28	10.91	1.08	0.01	0.05	1.36	0.05	0.08	20.82
	NM_025315	Med21	0.75	13.96	1.68	1.33	0.93	0.92	1.83	0.77	1.03	1.73	7.63
	NM_010215	Il4i1	0.46	0.78	13.54	13.81	3.00	0.49	0.67	2.82	0.86	0.37	4.81
	NM_178759	Timd4	0.00	0.01	0.03	0.08	0.04	0.02	0.05	0.31	3.23	1.47	0.03

	Refseq_ID	Symbol	WT 0h	WT 0.5h	WT 1h	WT 2h	WT 6h	KO 0h	KO 0.5h	KO 1h	KO 2h	KO 6h	WT/KO max RPKM Fold Difference
Replicate 2	NM_019952	Clcf1	0.06	3.03	2.68	2.23	0.20	0.05	0.06	0.13	0.05	0.03	48.39
	NM_008352	Il12b	0.02	6.76	16.84	1.09	0.06	0.02	0.05	0.73	0.03	0.02	22.94
	NM_025315	Med21	0.59	11.88	2.45	3.34	1.60	0.95	1.17	0.33	0.56	1.58	10.18
	NM_010215	Il4i1	0.50	1.05	10.21	5.22	1.99	0.23	0.59	1.73	0.50	0.42	5.91
	NM_178759	Timd4	0.01	0.01	0.03	0.04	0.03	0.03	0.12	0.37	4.39	1.43	0.01

B

(Read Counts)

	Refseq_ID	Symbol	Gene Length	WT 0h	WT 0.5h	WT 1h	WT 2h	WT 6h	KO 0h	KO 0.5h	KO 1h	KO 2h	KO 6h
Replicate 1	NM_019952	Clcf1	8224	14	640	567	190	73	8	17	32	11	10
	NM_008352	Il12b	13955	4	719	9295	3387	311	2	26	850	18	34
	NM_025315	Med21	8022	218	2573	318	238	154	199	517	276	226	420
	NM_010215	Il4i1	4522	75	81	1442	1389	280	60	107	571	106	50
	NM_178759	Timd4	33535	4	6	21	63	31	21	63	463	2965	1492

	Refseq_ID	Symbol	Gene Length	WT 0h	WT 0.5h	WT 1h	WT 2h	WT 6h	KO 0h	KO 0.5h	KO 1h	KO 2h	KO 6h
Replicate 2	NM_019952	Clcf1	8224	18	900	716	679	48	15	19	37	12	8
	NM_008352	Il12b	13955	11	3412	7641	562	24	9	24	345	12	12
	NM_025315	Med21	8022	170	3448	640	990	365	308	346	90	145	440
	NM_010215	Il4i1	4522	81	171	1501	873	256	43	99	263	73	66
	NM_178759	Timd4	33535	13	18	34	48	33	45	144	421	4758	1664

Figure 2-5: Expression Kinetics of the Five Most Dysregulated Genes.

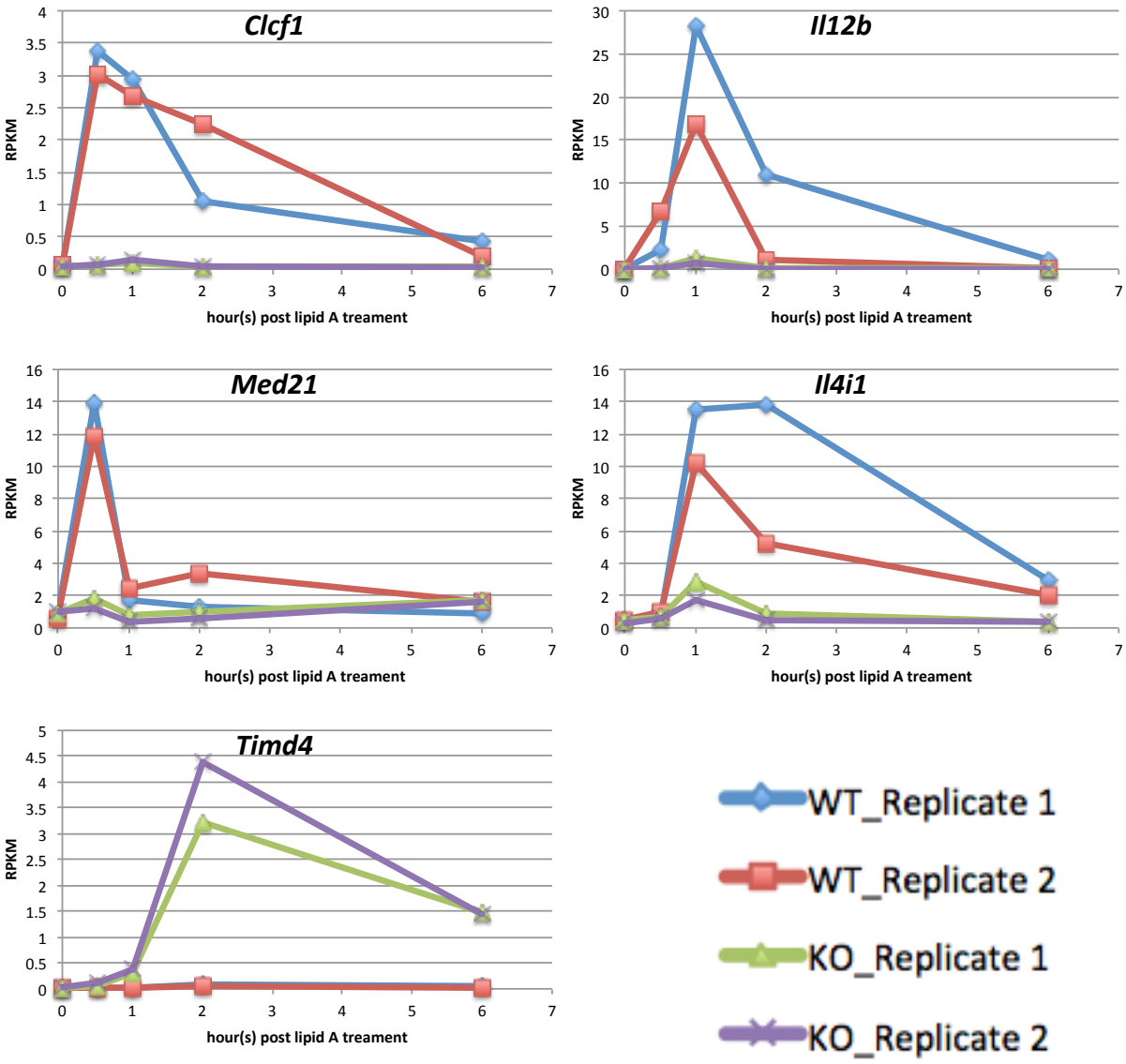
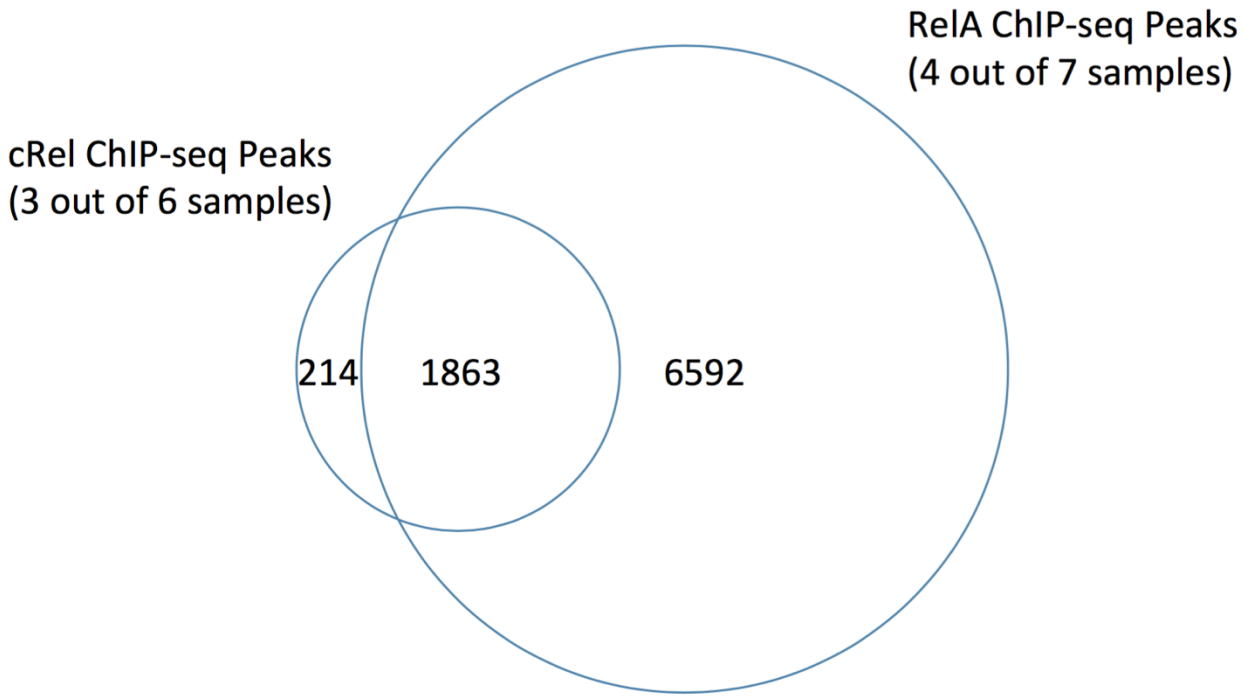


Figure 2-6: Schematic of c-Rel and RelA ChIP-seq Overlap.



Total Peaks:	8669
Peaks called in c-Rel:	2077
Peaks called in RelA:	8455
Peaks called in both:	1863

Figure 2-7: c-Rel and RelA Binding in *Il12b*, *Med21*, and *Il4i1* Genes.

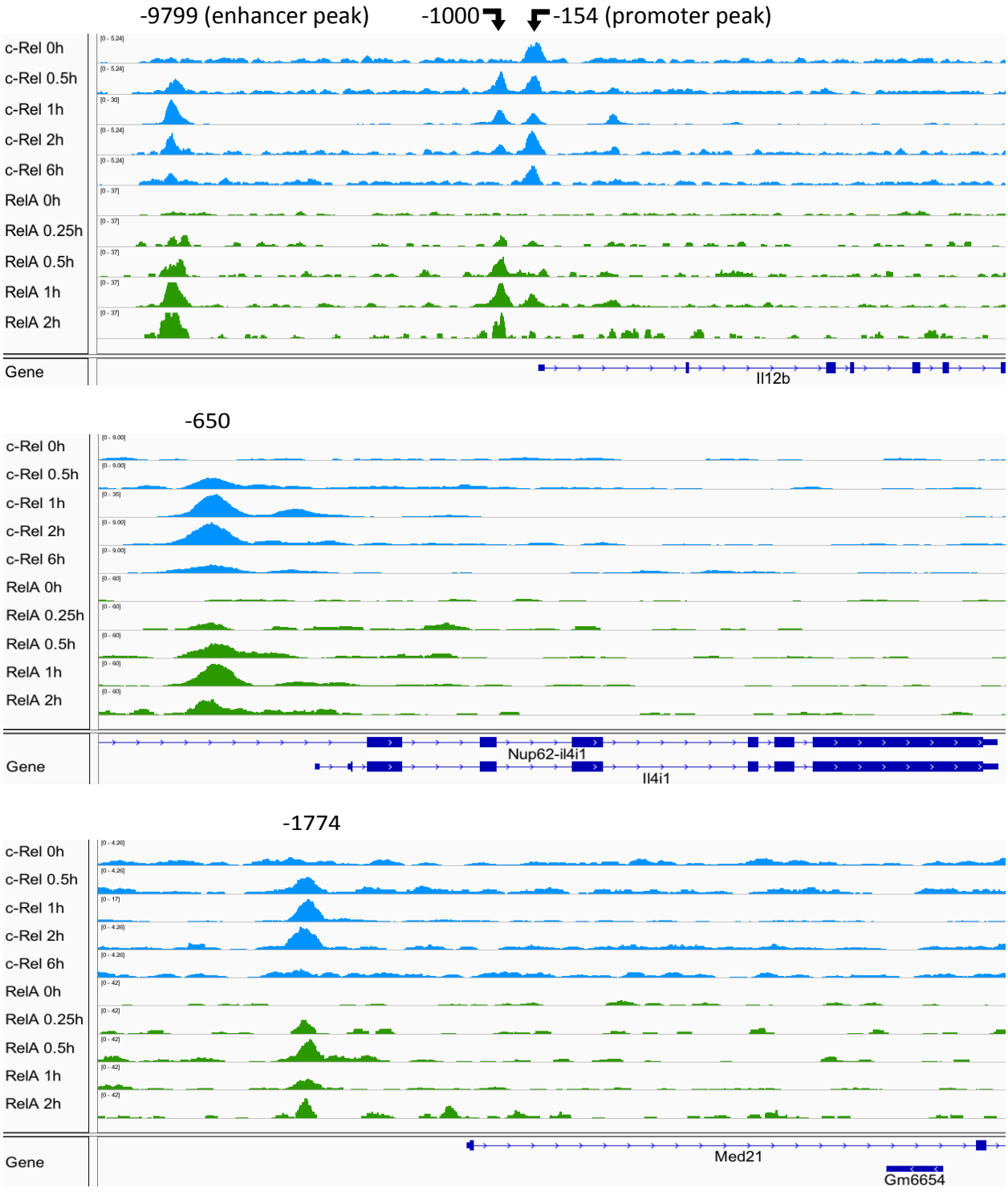
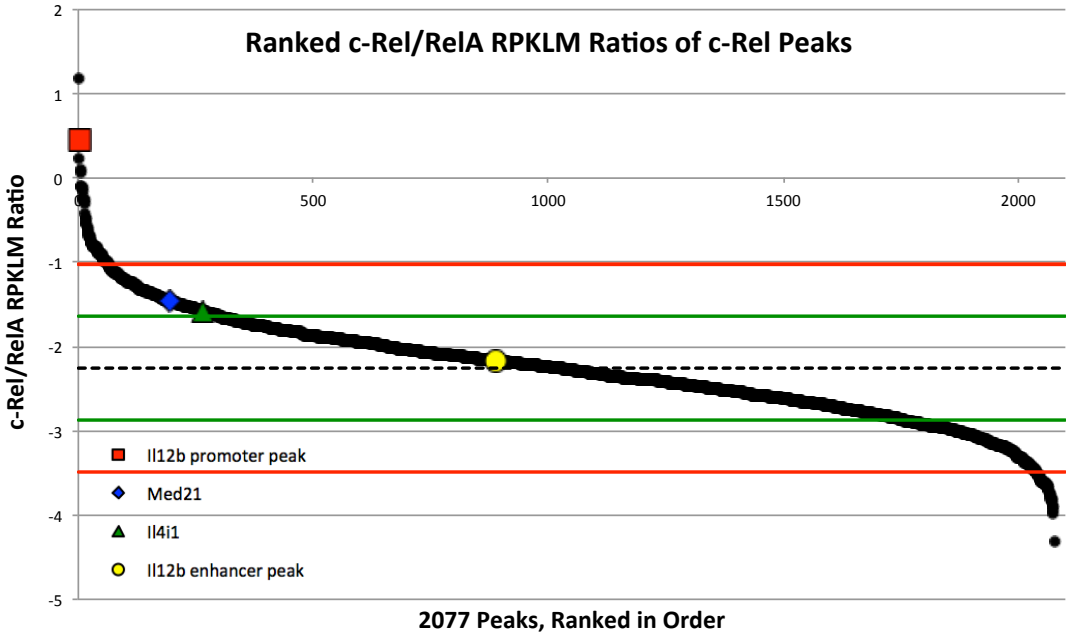
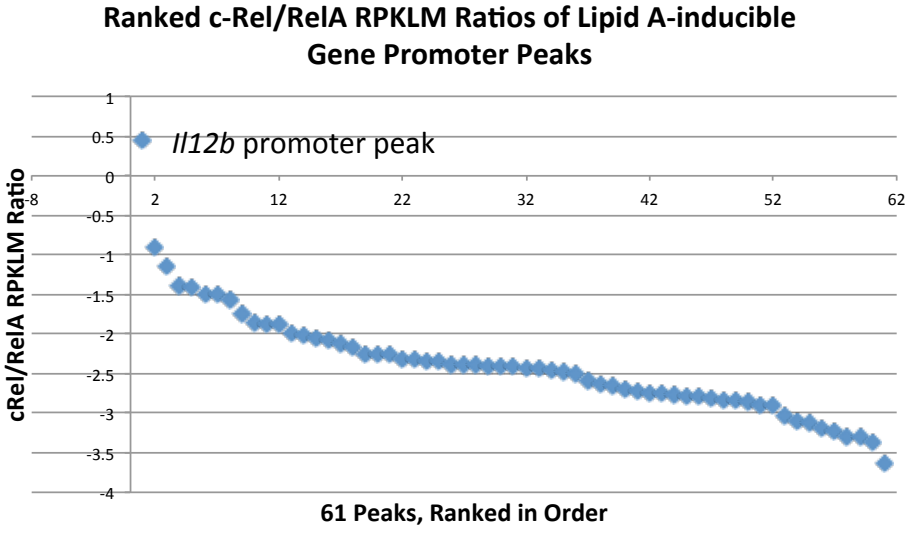


Figure 2-8: Log2-transformed c-Rel/RelA RPKLM Ratios for All Peaks Called in c-Rel ChIP-seq.

A



B



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Chapter 3

Selective Gene Regulations by Nuclear IκB and Repressive NF-κB.

Abstract

Nuclear IκB protein family (Bcl-3 [BCL3], IκBNS [NFKBID], and IκBζ [NFKBIZ]) is involved in multiple important biological processes, primarily via its interactions with NF-κB family. Unlike cytoplasmic IκB, whose main function is to sequester NF-κB in the cytoplasm, nuclear IκB resides in the nucleus and predominantly interacts with NF-κB after its nuclear translocation. Although many studies have been done with nuclear IκB family, most of them focus on phenotypic aberrations or the effects on a handful of genes. To gain better understanding of nuclear IκB's role in inflammatory gene transcriptions, we generate chrRNA-seq data of murine BMDM with each nuclear IκB knockout strain, as well as with p50/p105- and p52/p100-deficient mice. Our results suggest that IκBζ and p50/p105 regulates multiple genes during lipid A-mediated TLR-4 immune response, while Bcl-3, IκBNS, and p52/p100 deficiencies have minimal to no effects. More over, with ChIP-seq data, we report the genes that are potentially directly or indirectly regulated by IκBζ. Particularly, we find that both IκBζ and p50/p105 are indispensable for the expression of *Batf* gene, an important transcription factor responsible for Th17 cell differentiation and T/B cell class switch recombination. Overall, this study suggests that IκBζ and p50/p105 are essential to mount proper macrophage inflammatory responses.

Introduction

Since nuclear IκB family's first discovery in 1992, six year after the discovery of NF-κB, its functions were investigated in many research areas, particular for its involvement in innate and adaptive immune responses. Nuclear IκBs share the name "IκB" with the other well-characterized protein family, cytoplasmic IκBs, due to a shared common structural motif named ankyrin repeat domain (ARD). ARD determines the protein:protein interaction partners of both nuclear and cytoplasmic IκBs, directing them to interact predominantly with NF-κB proteins. This NF-κB:IκB interaction is crucial for mediating the regulatory functions of IκBs as they cannot bind to DNA directly. However, the similarities between nuclear IκB and cytoplasmic IκB end here, as these two groups of protein families are distinctly different in their functions and expression profiles.

Cytoplasmic IκBs are well-characterized as the main inhibitors of NF-κB signaling pathway. In resting cells, cytoplasmic IκBs are expressed at basal levels and actively retain NF-κB in the cytoplasm by blocking their nuclear localization sequence (NLS). In contrast, nuclear IκBs are expressed only after stimulation. Nuclear IκBs contain the NLS and reside in the nucleus after translation, where they interact with the activated NF-κBs that have been released from the cytoplasm after the degradation of cytoplasmic IκBs. Furthermore, whereas cytoplasmic IκBs predominantly interact with TAD-containing NF-κB members (RelA, c-Rel, and RelB), nuclear IκBs primarily interact with TAD-less NF-κBs (p50 and p52) (Thompson et al., 1995; Whiteside et al., 1997). Bcl-3 and IκBζ,

but not IκBNS, contain TAD domains. This is mechanistically intriguing, as Bcl-3 and IκBζ can potentially activate gene transcriptions that are otherwise repressed by p50 or p52 homodimers. Although IκBNS lacks TAD, it has been shown to inhibit gene transcriptions by stabilizing p50 homodimer at promoters. In summary, while cytoplasmic IκB are crucial for the initial NF-κB regulation and activation, nuclear IκB subsequently modulates the activities of a subset of NF-κB dimers after their translocations into the nucleus.

Macrophage is a crucial cell type of the innate immune system. It phagocytosizes and digests pathogens, foreign molecules, cell debris, and apoptotic/cancerous cells to maintain system homeostasis. Once activated, macrophage is an important secretors of cytokines including IL-6, IL-12 and TNF (TNFα) ,which are crucial to mount proper immediate innate immune response. Furthermore, as a potent antigen presenting cell to T cells, it is an important mediator between innate and adaptive immunity. The regulatory functions of nuclear IκBs in macrophages have been explored before, albeit not without complications. For example, although Bcl-3 carries TAD by nature, it has been shown as an inhibitor to the transcriptions of some pro-inflammatory genes. Bcl-3-deficient mice exhibited increased TNF expression. In contrast, lentiviral overexpression of Bcl-3 in RAW 264.7 macrophages reduced TNF expression. This is evident by the fact that Bcl-3 can interact and stabilize p50 homodimers at TNF promoter, thereby suppresses TNF expression by out-competing the binding of TAD-containing NF-κB such as RelA and c-Rel (Wessells et al., 2004; Kuwata et al., 2003). When stimulated with bacterial cell wall antigen lipopolysaccharide

(LPS), Bcl-3 expression is triggered by the expression of IL-10, an important anti-inflammatory cytokine that keeps the expressions of many pro-inflammatory cytokines in check (Opp et al., 1995). Interestingly, it has been shown that Bcl-3, once expressed, can also regulate IL-10 expression. However, it is unclear at this moment whether Bcl-3 can positively or negatively regulate IL-10 expression due to conflicting observations (Wessells et al., 2004; Carmody et al., 2007; Riemann et al., 2005). In line with the ability to block LPS-induced cytokine expression, wild type mice reconstituted with Bcl-$3^{-/-}$ bone marrow are more susceptible to septic shock when challenged with with consecutive LPS injections (Carmody et al., 2007). In contrast to its inhibitive functions, Bcl-3 has also been shown to induce gene transcription; in UV-induced DNA damage mouse model, Bcl-3 binds to the promoter of *Mdm2* and drives its expression via interactions with p50 or p52 NF-κB (Kashatus et al., 2006). Likewise, in human breast epithelia cells, Bcl-3 binds to cyclin D1 promoter and drives its expression via interaction with p52 homodimer (Westerheide et al., 2001). These observations corroborate the early assumption that Bcl-3 is a proto-oncogene; it is found to be constitutively express in B-cell chronic lymphocytic leukemia and human breast tumor tissue (Cogswell et al., 2000)

IκBNS was shown to be a negative regulator of LPS-induced pro-inflammatory cytokines such as IL-6, IL-12B, and IL-18 (Hirotani et al., 2005; Kuwata et al., 2006). Interestingly, its negative role on IL-6 and IL-12B expression juxtapose to IκBζ's positive role, suggesting that the expressions of these cytokines may be a result of competition or balance between the two nuclear

IκBs. Unlike other cell types, IκBNS is expressed at basal level in lamina propria macrophages, which suggests it may suppress excessive intestinal inflammatory cytokine expressions. Indeed, like Bcl-3, IκBNS-deficient mice exhibited higher lethality when challenged with LPS, most likely due to the excessive expressions of IL-6 and IL-12B (Kuwata et al., 2006).

Similar to Bcl-3 and IκBNS, IκBζ is induced in macrophages with LPS stimulations. Interestingly, IκBζ can also be induced by various stimuli except TNF, a potent pro-inflammatory cytokine. Interestingly, although TNF expression is not altered in IκBζ$^{-/-}$ macrophages at transcriptional level, TNF secretion is prolonged (Yamamoto et al., 2004). Previous studies indicated that IκBζ is an important co-activator for a subset of genes that requires SWI/SNF complex-mediated nucleosome remodeling for transcriptional activation. Many of these genes are key pro-inflammatory cytokines, including IL-6, IL-12B, EBI3, IL-18, and CCL17. As mentioned earlier, IL-6 is important for Th17 development while IL-12B is crucial for Th1 development and Th17 maintenance. EBI3, IL18, and CCL17 regulate Th1/Th2/Th17 cell-mediated immune responses (Batten et al., 2006; Nakanishi et al., 2001; Imai et al., 1999). Mechanistically, IκBζ has been show to bind to the promoters of IL-6, IL-12B, and CCL2, potentially via interactions with NF-κB p50 homodimers. While IκBζ is not needed to initiate SWI/SNF nucleosome remodeling, it is essential for subsequent H3K4me3 histone modifications and the formation of pre-initiation complex by recruiting RelA, pol II, and TATA-box binding protein (TBP) (Yamamoto et al., 2004; Kayama et al.,2008, Hildebrand et al., 2013). IκBζ$^{-/-}$ mice exhibited very high

mortality rate at embryonic stage (90%), with reasons unknown yet. The ones survived to birth grew normally and were of normal appearance until 4 to 8 weeks old. These mice then developed lesions in the skin of the periocular region, face, and neck that resembles the histopathologies of atopic dermatitis (AD). Shiina et al. attributed these defects to the higher serum IgE concentration as well as the overexpression of CCL11 (Eotaxin 1), CCL17 (TARC), and CCR3 chemokine in keratinocytes. This resulted in the infiltration of Langerhans cells and CD4+ T cells (Shiina et al., 2004). However, how the absence of IκBζ, presumably a trans-activating factors, results the overexpression of these chemokines still remained unknown.

NF-κB p50 and p52 are the prominent interacting partners of nuclear IκBs and therefor their functions are of great interests in nuclear IκB research. In lipid A (and LPS-)-stimulated macrophages, most of the p52 remains as p100 precursor in the cytoplasm while most of the p105 proteins are proteolyzed into p50. Indeed, depending on the cell types and microenvironment, p50 has been shown to exert its functions in close relationship with nuclear IκBs. In T cells, the expression of GATA3, a key transcription factor that drives Th2 phenotype, depends on the recruitment of Bcl-3 and p50 at its promoter (Corn et al., 2005). Likewise, in Treg development, IκBNS binds with p50 and c-Rel to the promoter of FoxP3 and drives its expression (Schuster et al., 2012). Given that p50 homodimers exist constitutively, even at resting state, it has been speculated that p50 deficiency would result in ectopic higher basal level gene expressions as well as the inability to modulate gene expression by p50 homodimer binding. Few

examples have been found to support this claim. In tolerant T cells, IL-2, an important Th1 cytokine that is required for T cell proliferation, is inhibited by p50 homodimers binding at its promoter (Grundström et al., 2003). Moreover, reduced expression of TNF in tolerant macrophages has been associate with binding of p50 homodimers to the NF-κB site of its promoter (Bohuslav et al., 1998). It is often difficult to elucidate the precise regulatory functions of p50 protein due several reasons. First, p50/p105 is involved in TPL-2/ERK/MAPK pathway, due to its ability to stabilize TPL-2. TPL-2, when activated, initiates ERK/MAPK signaling pathway, which subsequently leads to the down-regulation of IFN-β (Waterfield et al., 2003). Second, since cRel:p50, and especially RelA:p50 heterodimers are the dominant trans-activating NF-κB dimers, it is challenging to determine whether a transcriptional dysregulation is attributed to the loss of p50 homodimers or p50-continaining heterodimers.

To get a better understanding of the roles of nuclear IκB in lipid-A-induced murine bone marrow-derived macrophages, we performed chrRNA-seq with each nuclear IκB knockout strain as well as with p50/p105- and p52/p100-knockout mice. In addition, we performed IκBζ and p50 ChIP-seq to determine if genes are directly or indirectly regulated by IκBζ.

Methods and Material

For details of chrRNA-seq and ChIP-seq experiments, please refer to Chapter 2.

Mouse Strain

Mice between six- to ten-week old were used in both chrRNA-seq and ChIP-seq. BMDM were cultured as described in Chapter 2. p50/p105$^{-/-}$ mice were sourced from Jackson (stock number: 006097) with Jackson C57BL/6 mice as wild type. p52/p100$^{-/-}$ and BCL3$^{-/-}$ mice were kindly provided by Dr. Ulrich Siebenlist (Laboratory of Molecular Immunology, NIH). Since these two strains are of Taconic C57BL/6 background, Taconic C57BL/6 mice were used as wild type. NFKBID$^{-/-}$ mice were kindly provided by Dr. Ingo Schmitz (Center for Infection Research, Helmholtz) with Jackson C57BL/6 mice as wild type. NFKBIZ$^{-/-}$ mice (C57BL/6J background) were kindly provided by Giorgio Trinchieri, M.D. (Laboratory of Experimental Immunology, NIH), with wild type littermate as the control.

Antibodies

p50/p105 antibody was from Santa Cruz Biotechnology (sc-1192). NFKBIZ antibody was kindly provided by Dr. Alex Hoffmann (Signaling Systems Lab, UCLA).

Results

Nuclear IκBs and TAD-less NF-κBs do not affect the expressions of genes involved in classical NF-κB activation pathway.

To ensure the transcriptome of each knockout strain is not affected by fundamental defects in upstream classical NF-κB activation, we first investigate

the expressions of key genes that are involved in classical NF-κB activation in each knockout cells (Figure 3-1). Overall, in any knockout strain, most of the genes involved in classical NF-κB activation are not affected, with two exceptions. In p52/p100$^{-/-}$ cells, although p50/p105 expression was strongly induced at 0.5-hour post lipid A stimulation, its expression fell more quickly than others to about 50%. In Bcl-3$^{-/-}$ cells, the transcription of *Bcl3* prolongs into late time points at 2- and 6-hour post stimulation. Previous study had shown that *Bcl3* expression was dependent on IL-10 cytokines. IL-10 activates STAT3, which subsequently drives the expression of *Bcl3* (Hutchins et al., 2013). In deed, in Bcl-3$^{-/-}$ BMDM, we observed the prolonged expression of IL-10 (discussed below). Regardless, this results in the overexpression of mutant transcript, in which most of the ankyrin repeat-coding exons were replaced with a neomycin selection cassette (Franzoso et al., 1997). Northern blot analysis on RNA derived from various tissues of this Bcl-3$^{-/-}$ mice demonstrated no detectable transcript. Therefore, we postulate that the continually expressed mutant transcripts were quickly degraded and should have no relevant impact on gene transcription. Overall, this suggests that the classical NF-κB activation was intact in any knockout strain that we have used.

Analytic Schematic of chrRNA-seq datasets.

In order to thoroughly examine the effects of nuclear IκBs and TAD-less NF-κBs in lipid A-induced BMDM gene transcriptions, we outlined a comprehensive analytic scheme to evaluate gene transcription (Figure 3-2).

Specifically, genes were categorized into three major groups based on their average RPKM values in wild type samples (n =10 for each time point): 1) lipid A-inducible genes, 2) lipid A-inhibitable genes, and 3) lipid A-uninducible, constitutively expressed genes. Each major group was defined by the following criteria. For lipid A-inducible genes, their RPKM had to be induced at least 5-fold in one of the stimulated samples. In addition, the max RPKM of each gene, throughout the time course, had to be greater than 1 to be considered expressed. For lipid A-inhibitable genes, their RPKMs had to be greater than 3 at resting state, and show at least a 5-fold RPKM down-regulation in one of the stimulated samples. For lipid A-uninducible, constitutively expressed genes, their 0-hour RPKMs had to be greater than 3, with RPKM fold changes less 2-fold in stimulated samples. With these criteria, we assigned 595, 344, and 519 genes to each major group, respectively. Granted, this is a simplified cataloguing method, as gene expression kinetics can be more complicated and diverse. Regardless, our method can efficiently separate expressed gene into these three major groups, as there were only three genes (*Glipr1*, *Gdf15*, and *BC013712*) assigned to both lipid A-inducible and –inhibitable groups, and no genes in lipid A-uninducible, constitutively expressed group overlapped with the other two.

To detect perturbations in of gene transcription in each knockout, each major group was further divided into up-regulated or down-regulated subgroups. Based on the time-matched RPKM differences between wild type and knockout RPKM, a gene was only considered up-regulated if the knockout RPKM was greater than 1 and am 2-fold higher than the corresponding wild type RPKM.

Likewise, a gene was only considered up-regulated if the wild type RPKM was greater than 1 and am 2-fold higher than its corresponding knockout RPKM in both biological replicates. To display the identified genes in a kinetic-relevant way, they were sorted first by the earliest time at which the 2-fold difference between wild type and knockout was observed (termed "Time of Dependence"). Next, genes that have the same Time of Dependence were sorted base on the time of maximal RPKM expression in wild type cells. As each gene was expressed to different levels of RPKM, we transformed RPKM values into percent expression to faciliate visual inspection. Percent expression was based on the RPKMs of each gene in either wild type (for down-regulated genes) or mutant samples (for up-regulated genes), where the minimal and maximal RPKMs were set as 0 and 100 percent expression, respectively. The corresponding mutant RPKMs (in down-regulated genes) or wild type RPKMs (in up-regulated genes) were then scaled accordingly to match the percent expression.

Nuclear IκBs and TAD-less NF-κB do not affect the expressions of uninduced, constitutively expressed and lipid A-inhibitable genes.

Much of the previous reports only focused on the study of LPS (lipid A)-inducible genes. We investigate genes of other kinetic profiles for several reasons. First, as p50 and p52 homodimers are considered trans-inhibiting transcription factors, they can potentially actively mediate the down-regulation of genes in response to lipid A stimulation. Although p50 and p52 can also form heterodimers with TAD-containing NF-κB subunits (RelA, c-Rel, and RelB) and regulate

specific subsets of genes, our experimental setup could only potentially elucidate the down-regulating effects of p50 and p52 homodimers. Second, as mentioned earlier, since all three nuclear IκB have been shown to exert inhibitive functions on a handful of genes, it is intriguing to investigate whether we can identify novel genes that are potentially down-regulated in similar fashion. The study of lipid A-uninducible, constitutively expressed gene was more complicated. Since this group was already expressed in the absence of NF-κB activation and nuclear IκB expression, their transcriptional controls were most likely independent of both. However, it is known that gene transcriptions can be regulated in multiple phases due to the temporal differences in transcription factors recruitment and/or histone modification. For example, during cytomegalovirus (CMV) infections, several viral genes that are crucial for viral DNA replication are immediately expressed due to the presence of histone acetylation and pol II recruitment at promoters. Later in the course of infection, these genes are subsequently silenced due to the recruitment repressive transcription factor, some can recruit histone modifiers including histone deacytelase type 2 (HDAC2) and HDAC3 (Liu et al., 2010). Although the information about nuclear IκB's ability in recruiting histone modifier is scarce, IκBζ has been shown as a potent mediator for H3K4 trimethylation, Whether other nuclear IκBs can also mediate histone modifications requires further investigation.

Interestingly, we did not observe any gene affected from either lipid A-inhibitable or uninduced, constitutively expressed group in any knockout strain. This observation suggests several insights. For one, it suggests that the

transcriptionally inhibitive p50 and p52 homodimers are not involved in lipid A-induced gene down-regulation. Their irrelevance to the continual expressions of genes is not surprising. Since nuclear IκB depends on TAD-less NF-κB to indirectly interact with DNA, the fact that p50 and p52 were dispensable for the regulation of these two major groups suggests nuclear IκBs were also irrelevant, which was indeed the case. Notably, although Bcl-3, IκBNS, and IκBζ have been reported to take parts in gene down-regulation, those affected gene largely belong to the lipid A-inducible gene group.

IκBNS has no significant impact on all three major groups.

To our surprise, we did not see any genes affected in IκBNS$^{-/-}$ BMDM. It has been reported before that IκBNS can repress the expressions of *Il6* and *Il12b* by stabilizing p50 homodimer at their promoters, which prevented the binding of other trans-activating NF-κB dimers. However, the expressions of *Il6* and *Il12b* were only weakly elevated in IκBNS$^{-/-}$ BMDM (less than 2-fold; data not shown). Several possibilities can explain this observation. First, although speculative, it is possible that other nuclear IκBs, potentially Bcl-3, can compensate the absence of IκBNS. Second, it is possible that IκBNS is by nature specialized in fine-tuning TLR-4-induced gene expressions rather than influencing expressions by a large magnitude. Third, perhaps the mutant strains have somehow adapted to the deletion of IκBNS over time, and that other factors have been aberrantly re-regulated to accommodate for its absence.

Bcl-3 and p52 have minimal impacts on lipid A-inducible genes.

In both Bcl-3$^{-/-}$ and p52$^{-/-}$, we observed very few lipid A-inducible genes being affected. Out of 594 genes, only 3 and 2 genes showed down-regulation in Bcl-3$^{-/-}$ and p52$^{-/-}$, respectively (Figure 3-3A and C). Similarly, only 4 and 8 genes showed up-regulation in Bcl-3$^{-/-}$ and p52$^{-/-}$ (Figure 3-3B and D), respectively. By assessing the Time of Dependence with the time of wild type max RPKM expression, we could deduce whether the dysregulation was caused by the deleted protein directly or indirectly. If the Time of Dependence happened immediately prior to or concurrently with the time of peak expression, the deleted factor was most likely directly modulating the gene transcription (e.g. *G530011O06Rik* and *Pvrl2* in Bcl3$^{-/-}$; *Cxcl11* in p52$^{-/-}$). On the other hand, we could only speculate that the deleted protein was acting indirectly if the Time of Dependence occurs later than the time of peak expression.

Although it has been repeatedly shown that Bcl-3 is a potent regulator of *Il10* expression, whether Bcl-3 inhibits or activates *Il10* expression remains controversial. In Bcl-3$^{-/-}$ cells, *Il10* gene expression was up-regulated only at 6-hour following normal initial activation at 1-hour. This suggests that Bcl-3 is not needed to activate *Il10* transcription but is involved in inhibiting its expression at 6-hour. The observation that *Il10* down-regulation was only seen at 6-hour, despite the fact that *Bcl3* transcription started at 1-hour, can be potentially explained by the latency between *Bcl3* gene transcription and protein expression. In deed, in many earlier reports, although *Bcl3* mRNA was considerably expressed at 2-hour, Bcl-3 protein expression was hardly detectable until 4-hour

post stimulation. Bcl-3 protein expression, albeit slowly, often continues to increase up to 24-hour post stimulation (Ge et al., 2003; Brocke-Heidrich et al., 2006; Wessells et al., 2004; Mühlbauer et al., 2008). Our *Bcl3* expression profile also exhibited this slow but continuous production of nascent transcripts (see Figure 3-1). The late expression of Bcl-3 protein could potentially explain why so few genes were perturbed in Bcl-3$^{-/-}$ BMDM within 6-hour. In the future, it will be interesting to investigate the effects of Bcl-3 deficiency at time points later than 6-hour, which Bcl-3 would potentially take a more relevant role.

Several possibilities can explain why p52-deficiency had such a minor impact on TLR-4-mediated gene activation. For one, since the generation of p52 from its p100 precursor is predominantly dependent on the alternative NF-κB activation pathway, it is possible that even in wild type cells there is very little p52-containing NF-κB dimers to begin with under TLR-4-mediated classical NF-κB activation. An alternative explanation is that the absence of p52 is compensated by p50, as they are highly similar in terms of preferred DNA binding motif and the ability to interact specifically with nuclear IκBs. Interestingly, even with so few genes being affected, *G530011O06Rik*, a validated gene coding for RIKEN ncRNA, was severely down-regulated in both Bcl-3- and p52-deficient BMDMs at 2-hour. This strongly suggests that Bcl-3 and p52 are both indispensable for the activation of *G530011O06Rik* transcription, most likely by forming a trans-activating Bcl-3:p52 homodimer complex. Unfortunately, the functions of *G530011O06Rik* is currently unknown and requires further investigation.

IκBζ and p50 extensively affects lipid A-inducible genes.

In contrast to the minimal effects of Bcl-3 and p52, the deletion of IκBζ or p50 each exhibited an array of dysregulated genes. In summary, of 595 lipid A-inducible genes, 109 and 83 genes were down-regulated in IκBζ$^{-/-}$ and p50$^{-/-}$, respectively (Figure 3-4 and 3-5). In terms of genes that are up-regulated, 61 and 37 genes were observed in IκBζ$^{-/-}$ and p50$^{-/-}$, respectively (Figure 3-6 and 3-7). Interestingly, a substantial proportion genes were down-regulated in both IκBζ$^{-/-}$ and p50$^{-/-}$ cells (Figure 3-8). This strongly suggests that IκBζ and p50 work closely in activating a subset of lipid A-inducible genes. On the other hand, the overlap was less obvious in genes that were up-regulated in IκBζ$^{-/-}$ and p50$^{-/-}$ cells. In genes down-regulated in IκBζ$^{-/-}$ cells, we saw many genes reported before to be dependent on IκBζ for expression, including *Csf2*, *Ccl2*, *Lcn2*, *Il6*, *Il12b*, and *Ebi3*. Based on the chrRNA-seq and ChIP-seq data, we tried to identify novel genes that are directly regulated by IκBζ (discussed later). Interestingly, we saw many genes with maximal expressions at 6-hour strongly down-regulated in IκBζ$^{-/-}$ BMDM. It is known that many genes are induced late because their activation depends on nucleosome remodeling prior to the binding of transcription factors. Previous study has shown that IκBζ is dispensable for inducing nucleosome remodeling but required for subsequent H3K4 trimethylation and pre-initiation complex formation. Our result suggests that there may be a subset of late expression genes being regulated by similar mechanism.

The interpretation for genes dysregulated in $p50^{-/-}$ was a lot more difficult since p50 can bind to DNA and regulate gene transcription either as a p50 homodimer or RelA:p50 heterodimer. To overcome this complexity, the chrRNA-seq data of $RelA^{-/-}$ BMDM is needed for comparison with $p50^{-/-}$ chrRNA-seq. However, since $RelA^{-/-}$ mice are lethal at embryonic stage, a conditional, macrophage-specific $RelA^{-/-}$ may be needed to address this issue. Although the overlap between $p50^{-/-}$ and $I\kappa B\zeta^{-/-}$ was substantial, many genes were only affected in either $p50^{-/-}$ or $I\kappa B\zeta^{-/-}$. Genes that are only down-regulated in $p50^{-/-}$ but not $I\kappa B\zeta^{-/-}$ may be dependent on RelA:p50 or cRel:p50 for proper transcription. Genes that were only up-regulated in $p50^{-/-}$ but not $I\kappa B\zeta^{-/-}$ may be specifically inhibited by p50 homodimers. More than half of the genes (n = 68) that were down-regulated in $I\kappa B\zeta^{-/-}$ were not significantly affected in $p50^{-/-}$. One explanation is that IκBζ could potentially interact with other unknown transcription factors to bind DNA. For example, in Th17 cells, the expression of IL-17A depends on IκBζ binding to its promoter, which is dependent on the binding of transcription factor RORγt and RORα (Okamoto et al., 2010). Alternatively, it is possible that, in the absence of p50, p52 can compensate for the recruitment of IκBζ to promoter.

ChIP-seqs to elucidate genes that are directly regulated by IκBζ and/or p50.

While chrRNA-seq offers tremendous amount of information, we would like further validate genes that are directly regulated by IκBζ and/or p50 by showing their bindings to gene promoters with ChIP-seq data. Overall, 80% of the

IκBζ peaks (1145 out of 1414 peaks) overlapped with p50 peaks. Visual inspection of those IκBζ-exlusive peaks indicated that most of them do contain weak p50 peaks that were not called by HOMER software. We also speculated whether the binding affinity of p50 was a good indicator to predict IκBζ binding. By ranking all p50 peaks by their peak scores and cross reference with the corresponding IκBζ peak scores, we did not see higher p50 peak scores correlate with higher incidence of IκBζ binding. Two possible reasons can be derived from this observation. One, it suggests that the recruitment of IκBζ to p50 is not determined by the NF-κB binding site itself. It has been suggested that the recruitment of IκBζ depends on the C/EBP sites nearby NF-κB binding sites (Matsuo et al., 2007). Notably, in the three genes that have been shown to recruitment IκBζ at their promoters, all three of them contain a C/EBP binding site next to the NF-κB binding sites (*Ccl2*: Hildebrand et al., 2013; *Lcn2*: Kayama et al., 2008; *Il6*: Yamamoto et al., 2004). Two, it may suggest that our IκBζ ChIP-seq's data quality was less than idea and we simply failed capture most of the IκBζ binding events to reflect meaningful correlations. While unfortunate, this reasoning might be true since the IP efficiency was low for IκBζ ChIP-seq even after extensive optimization. Also, we only observed IκBζ binding at the promoters of *Lcn2* but not with *Il6* and *Ccl2* genes. Nevertheless, we found that the current ChIP-seq data could still offer valuable information on the binding of IκBζ, which will be discussed below.

Because IκBζ protein is expressed immediately after lipid A stimulation, we focused first on the the genes that are immediately expressed (max RPKM at 0.5-hour or 1-hour) with IκBζ Time of Dependence at 1-hour, as these genes will most likely be directly regulated by IκBζ without the requirement of nucleosome remodeling or histone mortifications. For this analysis, we restricted our range of inspection to the promoters and vicinity of gene body since most of the genes have no or poorly defined enhancers. Visual inspection of these genes identified 10 of them to have apparent IκBζ peaks in the promoters or vicinity of gene body, suggesting they are most likely the direct target genes of IκBζ (summarized in Table 3-1A).

Although IκBζ expression starts very early, its protein expression peaks at 2-hour. Therefore, we next inspected the down-regulated genes that had max expression within 2-hour and Time of Dependence at 2-hour, as they could also be the direct targets of IκBζ. Within this group, we identified 9 genes to show IκBζ binding in ChIP-seq data (Table 3-1B).

Since IκBζ can activate late expressing genes by inducing H3K4me3 and PIC formation after nucleosome remodeling, we next focused on the genes that had maximal RPKM at 6-hour and Time of Dependence at 6-hour. Within this group, although we identified 9 genes to show IκBζ binding in ChIP-seq data, only *Lcn2* had IκBζ peak in promoter (Table 3-1C). This suggests that the other 8 genes are not regulated in the same mechanism as in *Lcn2*.

Also, as mentioned earlier, genes that were only up-regulated in $p50^{-/-}$ but not $I\kappa B\zeta^{-/-}$ may be specifically inhibited by p50 homodimers. To identify these

potential genes, we inspected the genes that are only up-regulated in $p50^{-/-}$ cells. We identified 9 genes that fit the criterion with p50 peaks. Strikingly, *Ifnb1* is the only gene that shows p50 Time of Dependence prior to its max expression at 1-hour. The deletion of p50 inhibits the activation of ERK/MAPK signaling pathway, which leads to the up-regulation of *Ifnb1* expression. The observation that *Ifnb1* up-regulation in $p50^{-/-}$ proceeded its time of maximal expression corroborated that fact that p50 was acting up-stream to *Ifnb1* expression (Table 3-2).

To investigate whether the absence of p50 homodimer in $p50^{-/-}$ cells resulted in the up-regulations of genes at resting state. We compared between wild type and mutant cells for all genes that have RPKM > 1 in either wild type or mutant cells at 0-hour. Although only one gene (*Tifa*) showed a reproducible 2-fold up-regulation in $p50^{-/-}$ cells at 0-hour, we did not find convincing p50 binding data at 0-hour to suggest its role in down-regulating *Tifa* expression at 0-hour.

Lastly, we inspected the peaks that were dysregulated only in $I\kappa B\zeta^{-/-}$ and not $p50^{-/-}$, in hope to identify genes that may be recruiting IκBζ via factors other than p50. Interestingly, we found most of these genes were co-bound by both p50 and IκBζ, and no genes were found to be exclusively IκBζ-bound. This suggests that it is unlikely, at least in macrophages, that IκBζ is recruited to DNA by factors other than p50. One explanation as to why p50 was dispensable for the regulation of these gene is that p52 instead may be able to compensate the absence of p50 in $p50^{-/-}$ cells and recruit IκBζ.

Of the 10 most likely IκBζ direct target genes, one of them was *Batf*, a gene that has not been reported before to depend on IκBζ for expression. BAFT protein is an AP-1 like transcription factor that is crucial for the class switch recombination in T and B cells, as wells as Th17 differentiation by inducing cell type-defining RORγt transcription factor (Ise et al., 2011; Schraml et al., 2009). Interestingly, we observed 3 strong IκBζ peaks in the vicinity of *Batf* gene body, two of which resided in the introns and the third one downstream of the gene body (Figure 3-9). Strikingly, we observed all three peaks contain highly conserved NF-κB binding sites. This is unusual because introns are normally not under heavy selective pressure, resulting in higher mutation rate than exons or promoters. We postulate that these two intronic binding sites could potentially be ultra-conserved elements (UCE) that were crucial to the expression of *Batf*. Although BAFT has been shown as a crucial transcription factor that induces the activation of RORγt (not expressed in BMDM), it is unknown whether it is essential for the expression of other genes in BMDM, or how it contributed to the gene dysregulations seen in IκBζ$^{-/-}$ and p50$^{-/-}$ cells.

In earlier study, IκBζ$^{-/-}$ mice are found to be depleted of Th17 cell population, which can be partially explained by the lack of IL-23 cytokines. (Okamoto et al., 2010). Our finding suggests that IκBζ has a broader impact in Th17 cell differentiation and maintenance, being able to critically affects two key proteins that regulate the differentiation, expression profile (via BATF) and maintenance (via IL-23) of Th17 cell population.

Discussion

In this Chapter, we comprehensively analyzed the regulatory functions of Bcl-3, IκBζ, IκBNS, p50, and p52 in TLR-4-mediated inflammatory response. By comparing the Time of Dependence with the maximal RPKM time in wild type, we identified genes that are potentially either directly or indirectly regulated by factors of interests. Interestingly, the perturbations caused by the deletion of each factor were highly variable. The absence of IκBNS had no significant effects on 1458 expressed genes of all three major groups that we have examined. Few genes were affected in Bcl-3- and p52-deficient BMDM. The deletion of IκBζ or p50 had the most substantial effects in gene transcription. In addition, we observed a substantial overlap of the affected genes between IκBζ$^{-/-}$ and p50$^{-/-}$ BMDM, suggesting their collaborations in regulating a specific subset of genes in macrophages. In IκBζ$^{-/-}$ cells, most of the down-regulated genes were maximally expressed at 6-hour, way after the initial expression of IκBζ. This indicates that these late genes are most likely indirectly regulated by IκBζ. In the future, it will be interesting to perform ChIP-seq with H3K4me3 in IκBζ-deficient cells to investigate whether the deletion of IκBζ extensively affects H3K4me3 histone modification at genome-wide scale. Many affected genes were only seen in either IκBζ or p50. It will be interesting to investigate why there exists such selectivity while a substantial number of genes depends on both factors. Using IκBζ ChIP-seq data, we were able to identify genes that were most likely directly regulated by IκBζ due to their early expression as well as early down-regulation in IκBζ$^{-/-}$

cells. Our result indicated that IκBζ plays a critical role in TL-4-mediated inflammatory response by being able to affected both early and latent gene expression. Finally, we identified *Batf* gene strongly dependent on both p50 and IκBζ for its expression. Depending on the functions of BATF, this yet unreported relationship adds information to the roles of IκBζ in mediating immune responses.

Figure Legends

Figure 3-1: Genes Involved in Classical NF-κB Signaling Pathway are not Strongly Perturbed by Knockouts.

The expression profiles of all key proteins mediating the activation of classical NF-κB is displayed here. It includes: 1) IKK complex proteins: the major kinases that mediate the phosphorylation and subsequent degradation of cytoplasmic IκBs, 2) cytoplasmic IκBs: the key inhibitors that sequester NF-κB proteins and prevent their translocation into the nucleus, 3) the five NF-κB transcription factors that can bind to DNA and modulate gene transcriptions, and 4) the nuclear IκBs that can modulate NF-κB-mediated gene transcription by interacting with NF-κB proteins. The average RPKM values were calculated from two biological replicates.

Figure 3-2: Analytic Scheme of chrRNA-seq Datasets.

The schematic summarizes the methodology used to interrogate differential gene expressions in each wild type:mutant pair. Genes were assigned to one of the three major groups based on their average RPKM calculated from 10 wild type sample. By definition: 1) "lipid A-inducible genes" were up-regulated at least 5-fold following stimulation, with maximal RPKM throughout the time course (0-, 0.5-, 1-, 2-, and 6-hour) greater than 1.2) "lipid A-uninducible, constitutively expressed genes" were expressed at 0-hour with RPKM > 3; their subsequent RPKM fold change, in comparison to resting state, do not surpass 2-fold. 3) "lipid

A-inhibitable genes" were expressed at 0-hour with RPKM > 3. Their expressions were then down-regulated by at least 5-fold following stimulation. Within each major group, genes were further assessed and assigned to either "up-regulated gene" or "down-regulated gene" group based on their expressions in mutant cells. To be considered up-regulated or down-regulated in mutant cells, a gene had to show at least a 2-fold RPKM difference between time-matched wild type and mutant samples in both biological replicates.

Figure 3-3: Genes Affected in either Bcl-3$^{-/-}$ or p52$^{-/-}$ Macrophages.

Genes that displayed changes in expression profiles in Bcl-3$^{-/-}$ or p52$^{-/-}$ samples are displayed in full time course. In down-regulated genes, RPKM values are transformed into Percent Expression based on the wild type minimal and maximal RPKM as 0% and 100% expression, respectively. In up-regulated genes, RPKM values are transformed into Percent Expression based on the mutant minimal and maximal RPKM as 0% and 100% expression, respectively. Time of Dependence is defined as the earliest time point at which the 2-fold difference is observed between wild type and mutant samples. Genes are first sorted based on their Time of Dependences, followed by the second sorting based on their time of maximal expression in wild type sample. A) Genes that were down-regulated in Bcl-3$^{-/-}$. B) Genes that were up-regulated in Bcl-3$^{-/-}$. C) Genes that were down-regulated in p52$^{-/-}$. D) Genes that were up-regulated in p52$^{-/-}$.

Figure 3-4: Down-regulated Lipid A-inducible Genes in IκBζ$^{-/-}$ BMDM.

The expression profiles of the genes that were down-regulated in IκBζ$^{-/-}$ BMDM are displayed. Blue columns indicate the percent expression values in the WT:IκBζ$^{-/-}$ pair. Red columns display their corresponding gene expression profiles in the WT:p50$^{-/-}$ pair for comparison. Genes that are down-regulated in both IκBζ$^{-/-}$ and p50$^{-/-}$ are highlighted in yellow. The percent expression is scaled by setting wild type's minimal and maximal RPKM as 0% and 100%, respectively.

Figure 3-5: Down-regulated Lipid A-inducible Genes in p50$^{-/-}$ BMDM.

The expression profiles of the genes that were down-regulated in p50$^{-/-}$ BMDM are displayed. Red columns display the gene expression profiles in the WT:p50$^{-/-}$ pair. Blue columns indicate the corresponding percent expression values in the WT:IκBζ$^{-/-}$ pair for comparison. Genes that are down-regulated in both IκBζ$^{-/-}$ and p50$^{-/-}$ are highlighted in yellow. The percent expression is scaled by setting wild type's minimal and maximal RPKM as 0% and 100%, respectively.

Figure 3-6: Up-regulated Lipid A-inducible Genes in IκBζ$^{-/-}$ BMDM.

The expression profiles of the genes that were up-regulated in IκBζ$^{-/-}$ BMDM are displayed. Blue columns indicate the percent expression values in the WT:IκBζ$^{-/-}$ pair. Red columns display their corresponding gene expression profiles in the WT:p50$^{-/-}$ pair for comparison. Genes that are up-regulated in both IκBζ$^{-/-}$ and p50$^{-/-}$ are highlighted in yellow. The percent expression is scaled by setting mutant samples' minimal and maximal RPKM as 0% and 100%, respectively.

Figure 3-7: Up-regulated Lipid A-inducible Genes in p50$^{-/-}$ BMDM.

The expression profiles of the genes that were up-regulated in p50$^{-/-}$ BMDM are displayed. Red columns display the gene expression profiles in the WT:p50$^{-/-}$ pair. Blue columns indicate the corresponding percent expression values in the WT:IκBζ$^{-/-}$ pair for comparison. Genes that are up-regulated in both IκBζ$^{-/-}$ and p50$^{-/-}$ are highlighted in yellow. The percent expression is scaled by setting mutant samples' minimal and maximal RPKM as 0% and 100%, respectively.

Figure 3-8: Venn Diagram Summary of Genes Dysregulated in p50$^{-/-}$ and IκBζ$^{-/-}$.

A summary of the genes affected in each mutant sample is displayed here. There are 595 genes designated as lipid A-inducible genes. A) Of 595 genes, nearly 25%

of them were down-regulated in either $p50^{-/-}$ or $I\kappa B\zeta^{-/-}$. Substantial overlap between $p50^{-/-}$ and $I\kappa B\zeta^{-/-}$ were seen in down-regulated genes. B) Genes that were up-regulated in either $p50^{-/-}$ or $I\kappa B\zeta^{-/-}$ are displayed here. The overlap between $p50^{-/-}$ and $I\kappa B\zeta^{-/-}$ in down-regulated genes was only about 20%.

Table 3-1: Summary of Genes Potentially Regulated by IκBζ.

Genes down-regulated in $I\kappa B\zeta^{-/-}$ were further interrogated to distinguish direct target genes from indirect target genes. To support the claim of direct targeting, genes showing down-regulation were examined for IκBζ binding. The information of the peaks of each gene is displayed here. Notably, all IκBζ peaks were bound by p50 as well. Only peaks in promoter or gene body were considered, unless there is a well-defined enhancer. In some scenarios, a potential intergenic peak was considered if no other genes were nearby. A) For a gene to be considered a direct target of IκBζ, the gene's Time of Dependence needed to be at 1-hour, at which IκBζ was expressed. These genes are most likely to be the direct targets of IκBζ. B) Since IκBζ expression peaks at 2-hour, we also look into genes that showed Time of Dependence at 2-hour if the maxima RPKM expression occurs prior and not later. These are also likely the direct target genes of IκBζ, although not as promising as the genes in A. C) Because IκBζ can activate late expressing genes by mediating H3K3me3 histone modifications and PIC recruitment, we looked into genes with both Time of Dependence and peak expression at 6-hour. *Lcn2* is the model gene here, showing IκBζ binding only at

6-hour, presumably after nucleosome remodeling. Although IκBζ peaks were found in other genes, their bindings all appeared earlier than 6-hour time point. Therefore, we cannot confidently claim these other genes were also regulated in the same mechanism as in *Lcn2*.

Table 3-2: Summary of Genes Potentially Inhibited by p50 Homodimer.

Genes that were up-regulated in $p50^{-/-}$ and not $I\kappa B\zeta^{-/-}$ are potentially inhibited by p50 homodimer in wild type cells. 37 genes that were only up-regulated in $p50^{-/-}$ were inspected for p50 binding. Only 8 genes were found to have p50 peaks, which are reported in this table. Of note, only *Ifnb1* exhibited Time of Dependence prior to the maximal expression time. This correlates well with the fact that p50 can mediate the activation of ERK/MAPK pathway, which subsequently down-regulates *Ifnb1* expression.

Figure 3-11: IκBζ and p50 binds to *Batf* introns and nearby intergenic region.

Batf was potently down-regulated in both $p50^{-/-}$ and $I\kappa B\zeta^{-/-}$ (bottom right panel). Both p50 and IκBζ bind to two intronic loci and one downstream intergenic locus after lipid A stimulation. Bottom panel of DNA sequences: showcasing the strong conservation in these three loci. The NF-KB binding site in each locus is highlighted in blue.

Figure 3-1: Genes Involved in Classical NF-κB Signaling Pathway are not Strongly Perturbed by Knockouts.

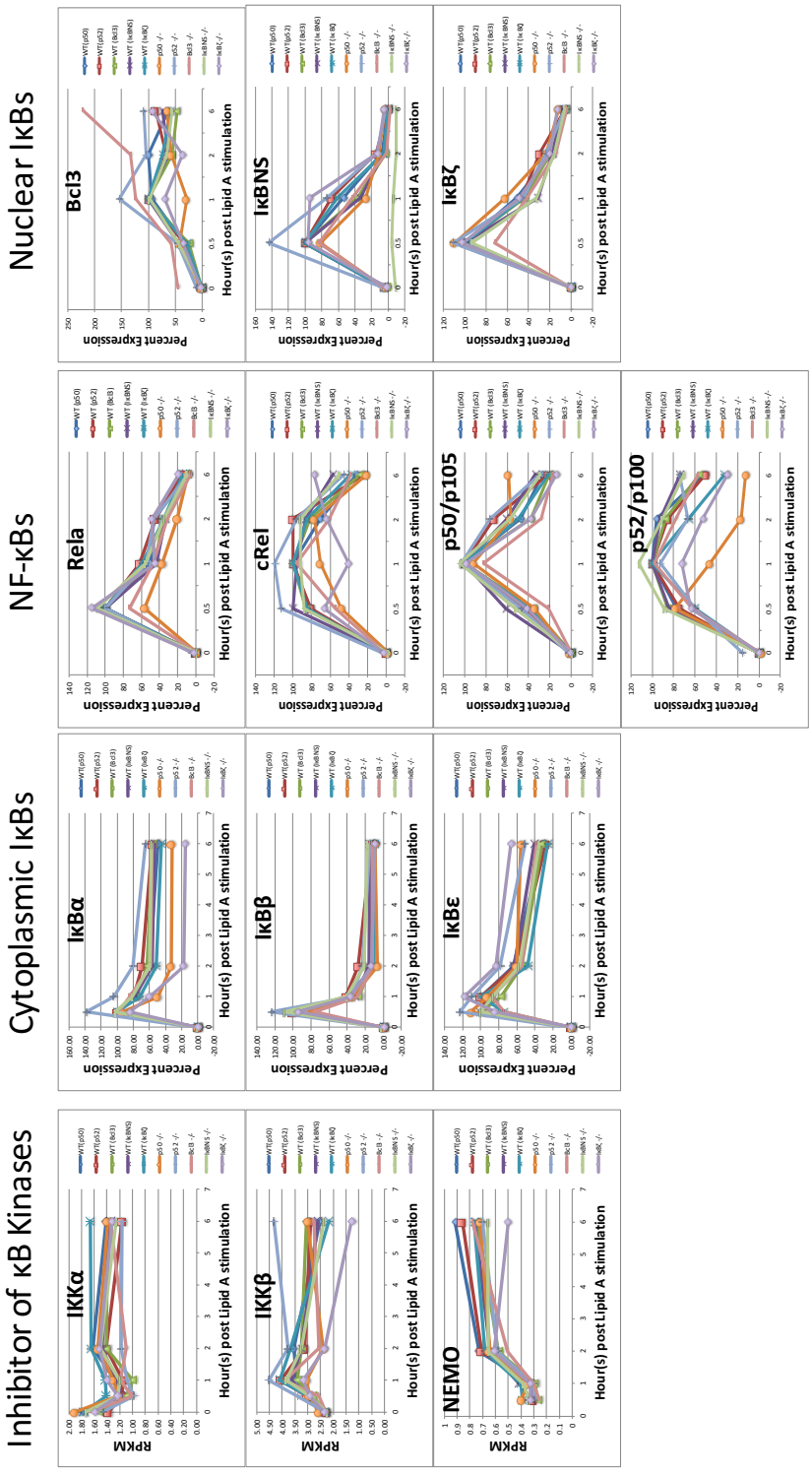


Figure 3-2: Analytic Scheme of chrRNA-seq Datasets.

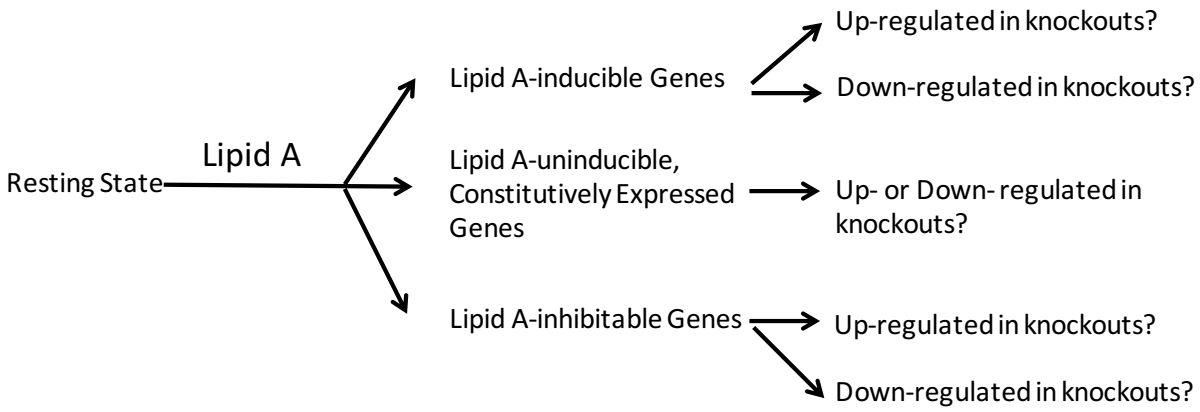


Figure 3-3: Genes Affected in either Bcl-3$^{-/-}$ or p52$^{-/-}$ Macrophages.

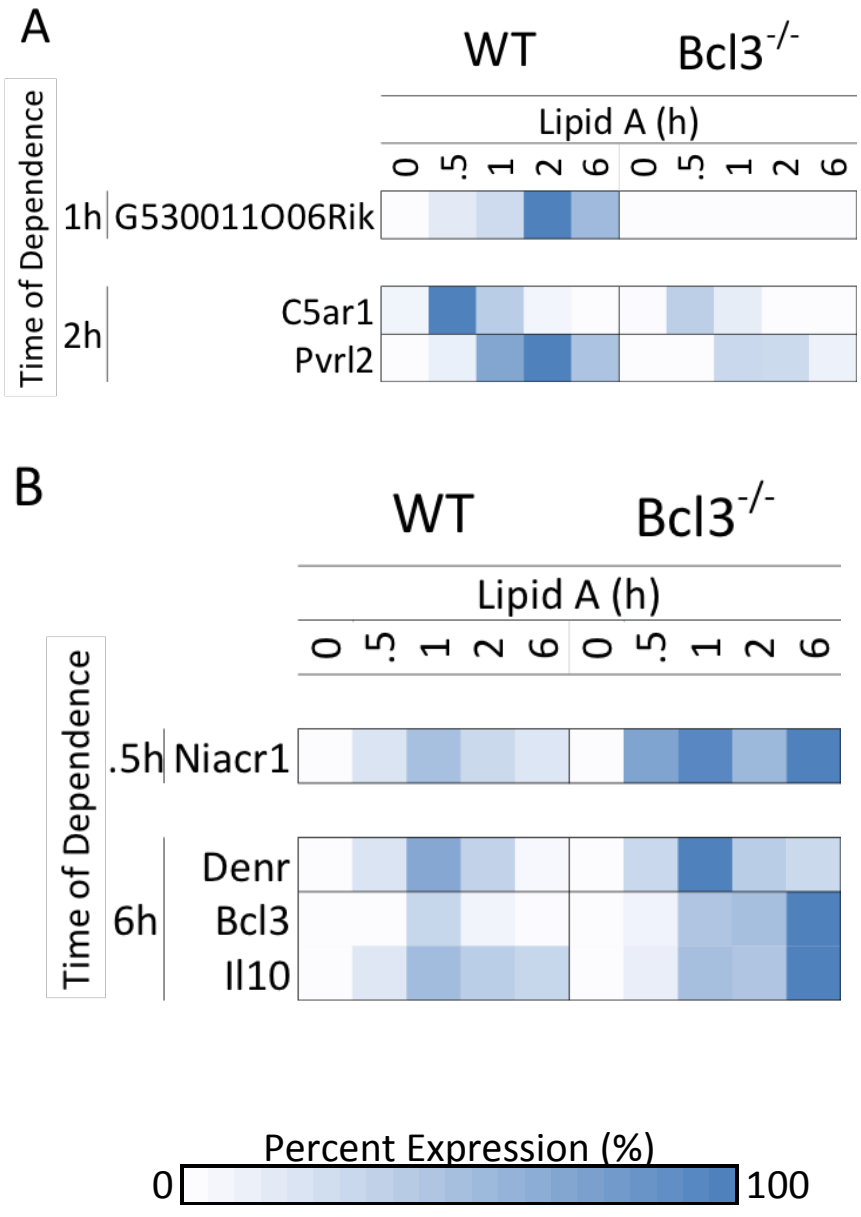


Figure 3-3: Cont'd.

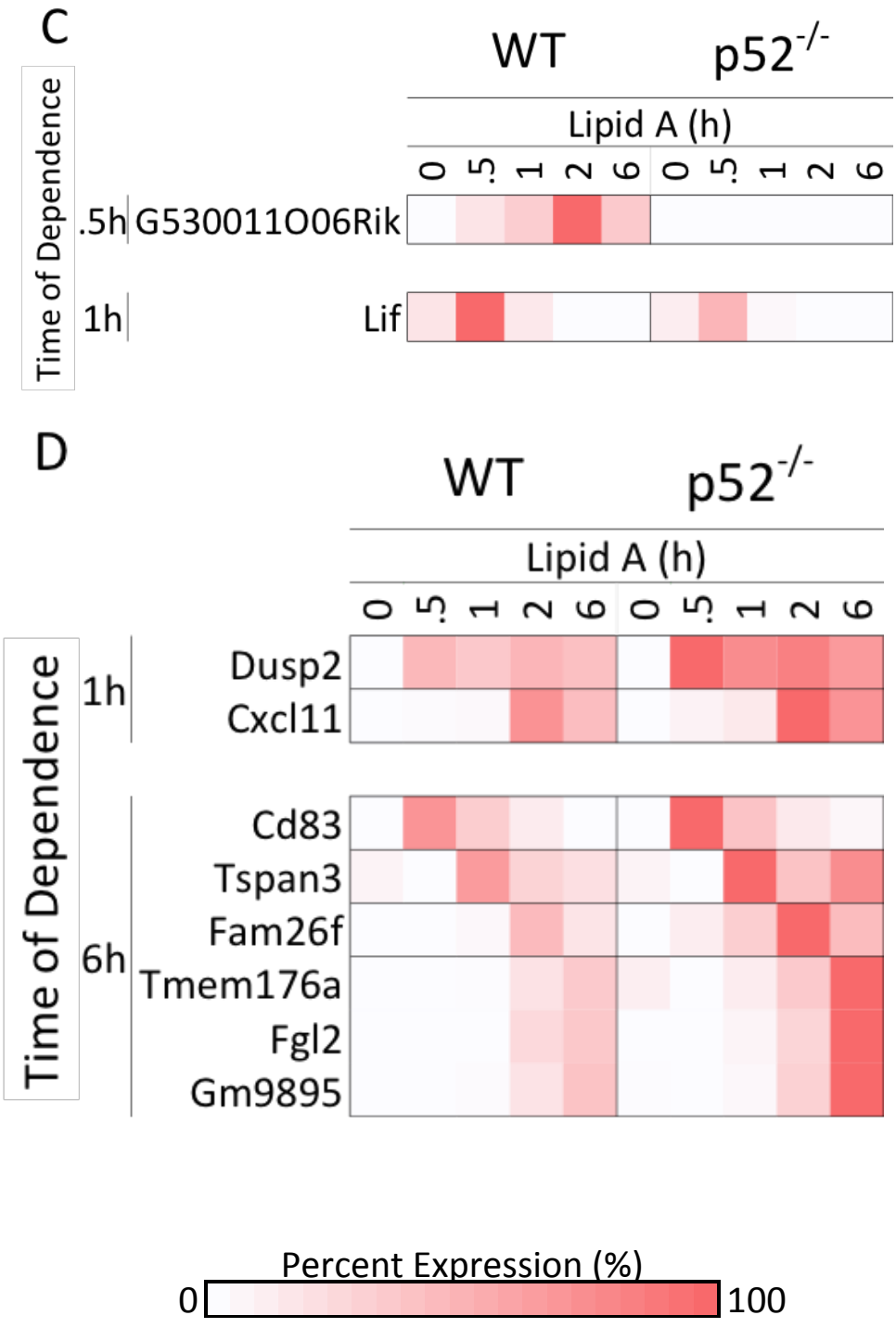


Figure 3-4: Down-regulated Lipid A-inducible Genes in IκBζ$^{-/-}$ BMDM.

Figure 3-5: Down-regulated Lipid A-inducible Genes in p50$^{-/-}$ BMDM.

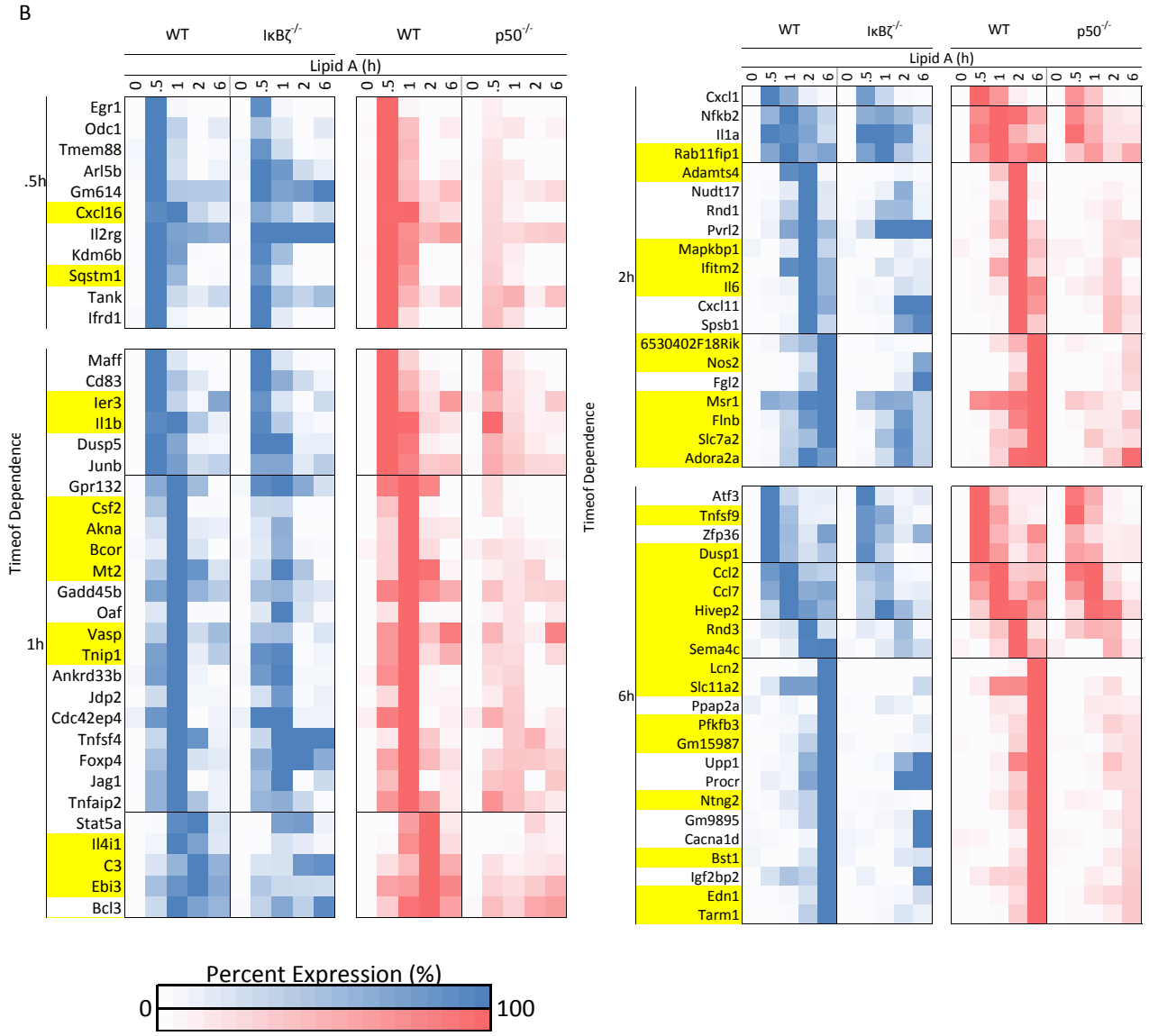


Figure 3-6: Up-regulated Lipid A-inducible Genes in IκBζ$^{-/-}$ BMDM.

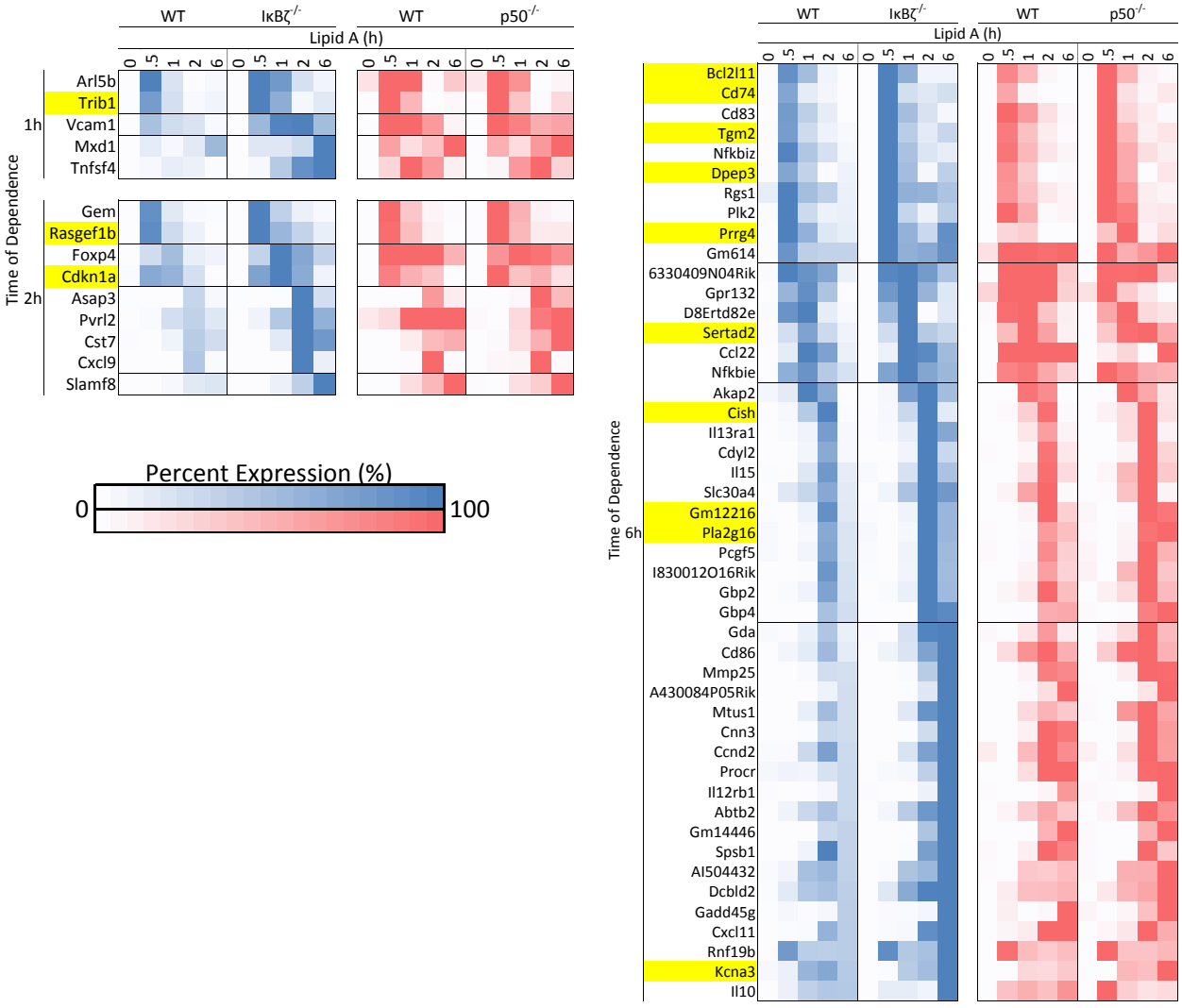


Figure 3-7: Up-regulated Lipid A-inducible Genes in p50$^{-/-}$ BMDM.

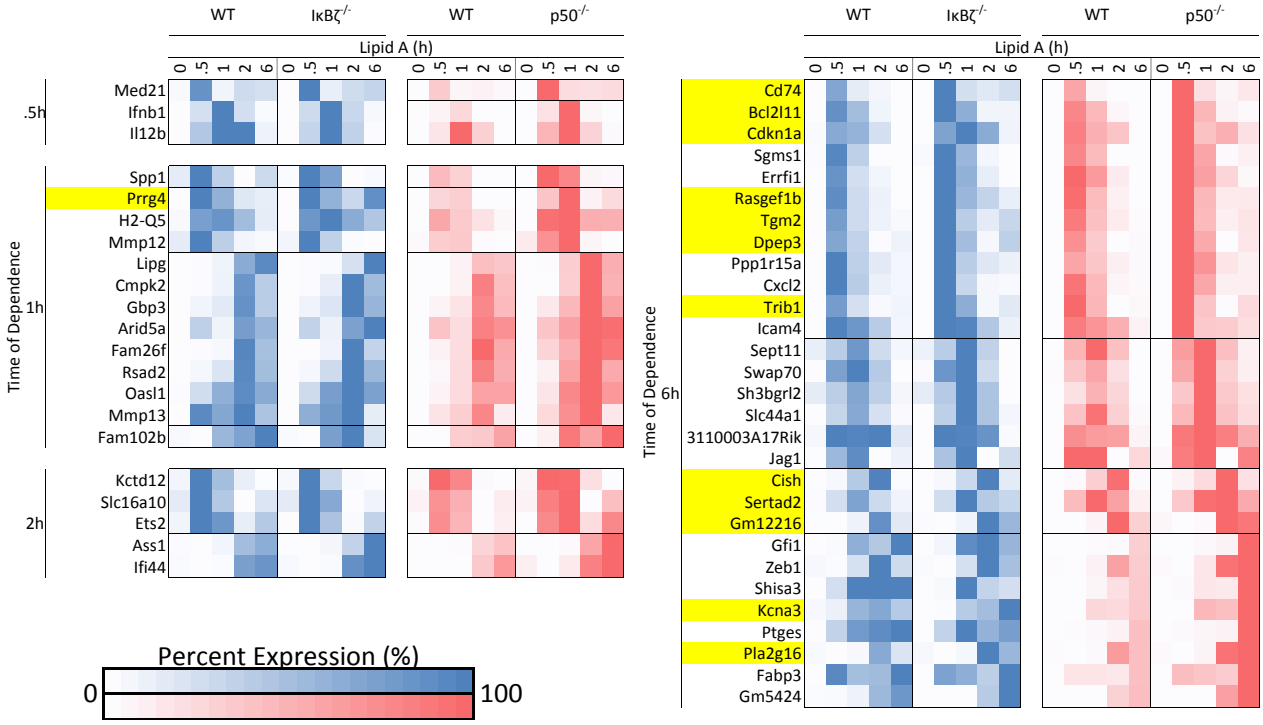


Figure 3-8: Venn Diagram Summary of Genes Dysregulated in $p50^{-/-}$ and $I\kappa B\zeta^{-/-}$.

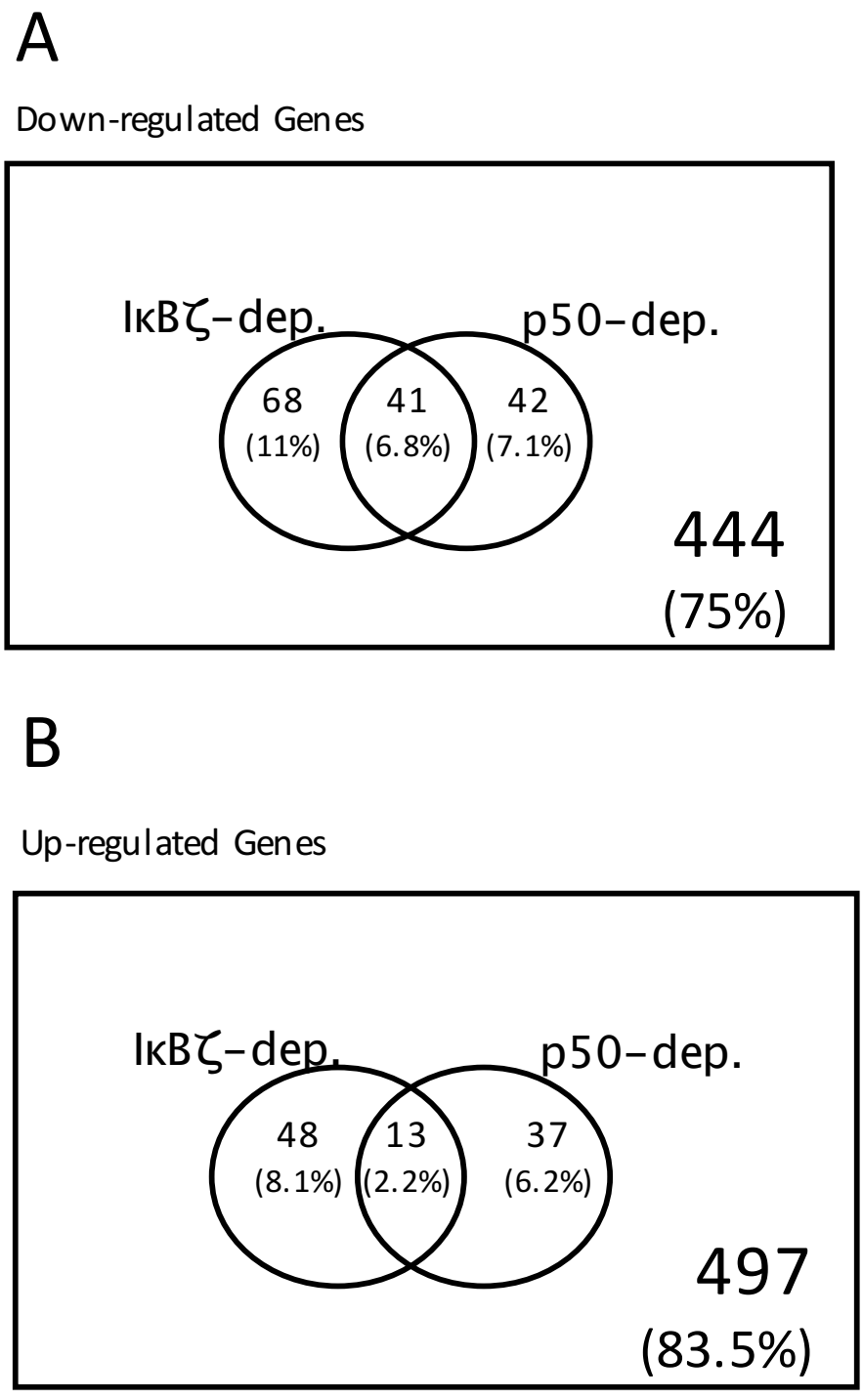


Table 3-1: Summary of Genes Potentially Regulated by IκBζ.

A

Symbol	Max. Exp. Time (h)	Time of Dep. (h)	Max IκBζ-/- %Exp.	Number of IκBζ Peaks	Peak Location(s) to TSS	Category	p50 Peak?
Stap1	0.5	1	81.47	1	7080	intron	Yes
Sqstm1	0.5	1	83.37	1	-2274	intergenic	Yes
Ccl3	0.5	1	53.72	1	-60	promoter	Yes
Il12b	1	1	18.89	2	-9939; -116	enhancer; promoter	Yes
Cpd	1	1	23.91	1	1137	intron	Yes
Il4i1	1	1	22.50	1	-717	promoter	Yes
Il1b	1	1	94.16	1	-121	promoter	Yes
Adamts4	2	1	6.78	2	-1434; -22	intergenic; promoter	Yes
Ebi3	2	1	43.53	1	-1	promoter	Yes
Batf	6	1	21.93	3	16916; 19065, 24010	intron; intron; intergenic	Yes

B

Symbol	Max. Exp. Time (h)	Time of Dep. (h)	Max IκBζ-/- %Exp.	Number of IκBζ Peaks	Peak Location(s) to TSS	Category	p50 Peak?
Ier3	0.5	2	85.87	1	-8225	intergenic	Yes
Ets2	0.5	2	83.52	1	26550	intergenic	Yes
Nfkbia	0.5	2	84.24	1	-115	promoter	Yes
Tnip3	0.5	2	99.25	1	-137	promoter	Yes
Tnip1	1	2	95.16	3	8689; 26648; 28595	introns	Yes
Vasp	1	2	50.11	1	5707	intron	Yes
Denr	1	2	43.22	1	-641	promoter	Yes
Ifitm2	2	2	23.05	1	-617	promoter	Yes
Dusp2	2	2	78.50	1	-713	promoter	Yes

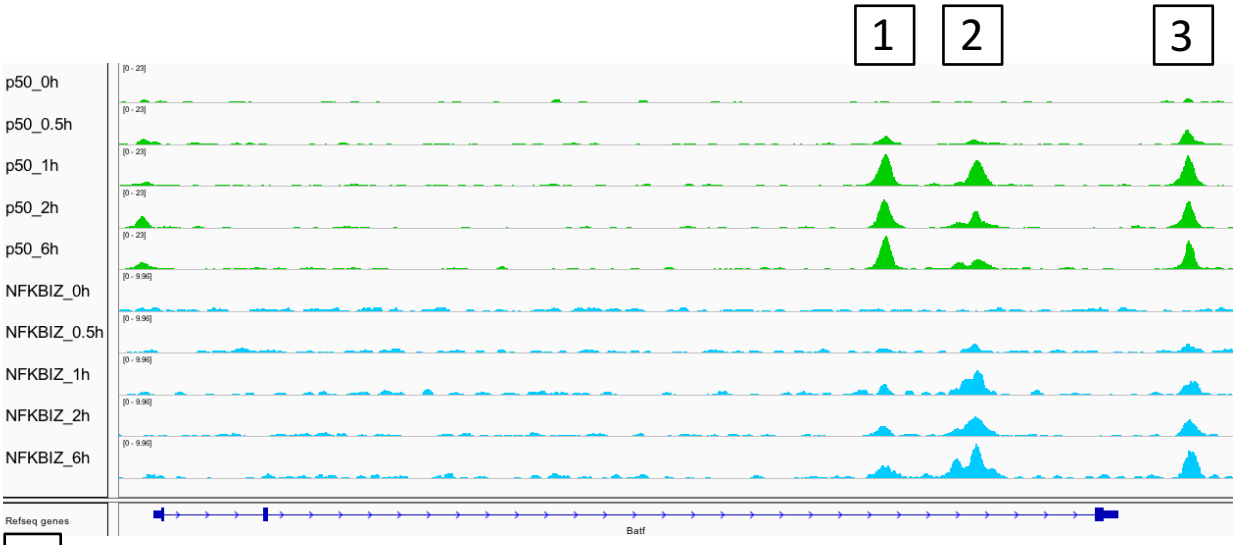
C

Symbol	Max. Exp. Time (h)	Time of Dep. (h)	Max IκBζ-/- %Exp.	Number of IκBζ Peaks	Peak Location(s) to TSS	Category	p50 Peak?
Lcn2	6	6	0.57	1 (only at 6-hour)	-204	promoter	Yes
Sipa1l1	6	6	43.49	1	55933	intron	Yes
Dtx2	6	6	13.53	1	40203	intergenic	Yes
Pfkfb3	6	6	14.41	1	-28147	intergenic	Yes
Sod2	6	6	70.39	1	2437	intron	Yes
Slamf1	6	6	21.35	1	-5127	intergenic	Yes
Slc11a2	6	6	28.75	1	1604	intron	Yes
Mefv	6	6	33.82	1	-5373	intergenic	Yes
Mmp14	6	6	34.13	1	11108	intergenic	Yes

Table 3-2: Summary of Genes Potentially Inhibited by p50 Homodimer.

Symbol	Max. Exp. Time (h)	Time of Dep. (h)	Max WT %Exp.	Number of p50 Peaks	Peak Location(s) to TSS	Category	IκBζ Peak?
Ifnb1	1	0.5	24.58	1	-116	promoter	no
Cmpk2	2	1	84.64	1	-3396	intergenic	no
Gbp3	2	1	79.3	1	5	promoter/TSS	no
Fam26f	2	1	135.43	1	-104	promoter	no
Rsad2	2	1	84.73	1	-51	promoter	no
Slc16a10	0.5	2	72.4	1	42768	intron	no
Ass1	6	2	39.03	1	-23988	intergenic	no
Sept11	1	6	120.29	2	42393; 43493	introns	no

Figure 3-9: IκBζ and p50 Bind to *Batf* Introns and Nearby Intergenic Region.



1

```
Mouse          gctgagggactagcctggggattcccctgtcactcaggagctc--tgagtgacctcctctgcagcccctccaattggggatttcccagaatgttcccacacctcaacgc
Rhesus         ggtgagggaactgcctggggattcccc-attgttgaagcactctttgagtgacccccacctaagcccctccagcaggggatttcccagaatgttcccacacccccagga
Marmoset       ggtgagggaactgcctggggattcccc-gtcgttgaagagttc---gagtgacccccctcctaagcccctccagtaggggatttcccagaatgctcccacgccccccagga
Dog            ggtaagagaactgtgtggggattcccc-atcattcaagagctctttgagtgacccccct-ttcagcccctccagtagggaatttcccagaatgttcccacacccccatga
Cow            gggaagggacctgcctggggattcccc-atcattgatgcactctac-agtgacccccctgcttggcccctccactagggaatttcccagaatgttcccacacccccaaga
Armadillo      ggtaagggaactgcctggggattcccc-accgttcaagaactccttgagtaacctcctccttggcccctccaggaggggatttcccccgaatgttcccacacccccatgg
Elephant       agtaagggaattgcctgggggttcccc-atcattcaagagatgtttgagcgacccccctcctgggcccttccagtaggggatttcccagaatgttcccatacccccattc
X. tropicalis  ==========================================================================================================
Chicken        ==========================================================================================================
```

2

```
Mouse          cc---gt-----------cctccttgctgccttggggtttgcccaggctcttcggagcacgga
Rabbit         cg---gc-----------cctcccttctggctcggggtttgcccaggctcttcggagcacaga
Marmoset       -c---gg-----------cctcccttctgcctcggggtttgcccaggttcttcagagcacaga
Rhesus         -c---gg-----------cctcccttctacctcggggtttgcccaggctcttcagagcacaga
Human          -t---gg-----------cctcccttctgcctcggggtttgcccaggctcttcagagcacaga
Tree shrew     -c---gggcctaccccggcccctcccctcgtgccttggggtttgcccaggctcgacggagcacaga
Dog            cc---ac-----------cctcccttctgcctcggggtttgcccaggctctttagagcacaga
Cow            cc---gc-----------cctcccttctgcctcggggtttgcccaggctcttcggagcacaga
Armadillo      cc--agc-----------cctcccttcagccgcggggtttgcccgggctttcgcagtacaga
Elephant       ca--ggc-----------cccgccctccagccttggggtttgcccaggcgcttcagagcacaga
X. tropicalis  ==========================================================
Chicken        ==========================================================
```

3

```
Mouse          agcatggtgatttcccccctagaaa-tccc-gcacaagacacatga-ctg
Rabbit         tgcgctgtgattccccccagaaa-ccccgcgcagaaggcacatgacccc
Human          agcactgtgatttt-ccccagaaa-ccccacacagaaggcacatgacttc
Rhesus         agcactgtgatttttccccccagaaa-ccccacacagaaggcacatgacctg
Marmoset       ggcactgtgattttccccccagaaa-ccccatagaaggcacatgacctg
Tree shrew     agcactgtgattcc-ccccagaaa-tcccacacagaaggcacatgacctg
Cow            agctctgtgatttccccccagaaaccccccgcaggaggcacatgaccag
Dog            agcactgtgactttccccccagaaa-gcccacacagaaggcacatgacctg
Armadillo      agcgctgtgatttccccccgagaaa-ccccacaggcagcacatgaccaa
Elephant       agcactgtgattttccccctgaaa-gcccacacagaaggcacatgactgc
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X. tropicalis  ==================================================
Chicken        ==================================================
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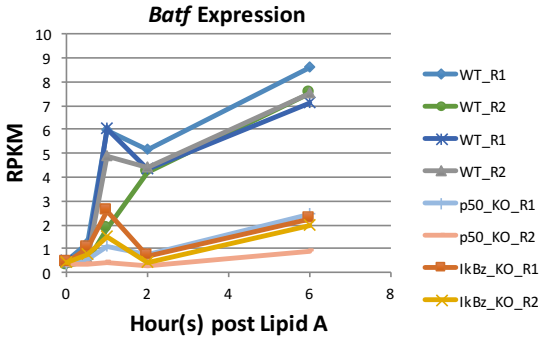

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Chapter 4

Concluding Remarks

Although NF-κB protein family has long been recognized as an important regulator in many biological processes, particularly in immune system, our understanding of how each of the family member mediates specific functions largely remains unknown. This is complicated by the fact that substantial functional overlap exists among NF-κB proteins and the observation that NF-κB-mediated regulations are diversely affected by cell types, chromatin structure, histone modifications, and microenvironments. Occasionally, rare chances arise in which an unique phenotype is only seen in a specific NF-κB knockout strain, giving us an opportunity to unravel the key elements that define the differences between the five NF-κB proteins. In our case, the significant down-regulation of *Il12b* in c-Rel$^{-/-}$ murine BMDM allowed us to uncover the key differences between c-Rel and RelA. Surprisingly, at least in BMDM, only *Il12b* exhibited such dependence and selectivity on c-Rel in terms of gene activation and promoter binding. This suggests that gene regulation can in fact be highly specific, which is promising in terms of designing therapeutic strategies with high efficacy and low off-target effects. However, as c-Rel's important functions in other cell types are known, similar studies in other cell types are needed before it can be seriously considered as a potential target. Also, although all key selective elements are conserved in human genome, it is a priority to investigate whether c-Rel dependency on *Il12b* is also observed in human cells. Lastly, it is noteworthy to mention that, after 30 years since NF-κB's discovery, so far we still lack a strategy to specifically inhibit a particular NF-κB dimer in terms of its binding and recruitment of co-factors without affecting other NF-κB dimers. Finding a

way to specifically target and modulate a specific NF-κB dimer will not only provide tremendous tool in terms of targeted therapy, but also a promising tool in future studies to further delineate the key differences between NF-κB proteins.

While nuclear IκB's regulatory functions have been of great interests for many years, our knowledge of this protein family still remains largely unknown. Although tremendous progress has been made in the past, many of the phenotypic studies were done in earlier times when technologies were not as advanced and capable as today's. Here we report the first effort to investigate the functions of all three nuclear IκBs in murine macrophages in parallel with the next-generation sequencing technology. Although our results indicated very minor effects in terms of transcriptional regulations mediated by Bcl-3, IκBNS, and p52, applying the similar research methodology to other cells types, particularly in B or T cells, could potentially yield significant findings, as suggested by the previous phenotype studies done with each knockout strain. Nevertheless, we identified IκBζ and p50 both as the key regulators of TLR-4 mediated inflammatory response. Not only did we confirm their effects on previously reported genes, we also identified many potential novel target genes that worth further studies and could have significant impact in the future. As the legacy of NF-κB continues with the advancement of technologies, it is promising that soon we will have a much better understanding and the knowledge to harness the therapeutic potentials of this protein family