Understanding two inhibitors of NF-κB: A20 and IκBβ

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Abstract

Inhibitors of NF-κB: A20 and IκBβ

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While prompt activation of NF-κB is essential for optimal immune response, it is equally important to terminate the response to avoid tissue damage and perhaps even death resulting from organ failure. This thesis describes two inhibitors of NF-κB, A20 and IκBβ. A20 is an essential inhibitor of NF-κB mediated inflammation as mice lacking A20 die from multi-organ inflammation and cachexia. Multiple biochemical approaches have suggested that A20 functions as a deubiquitinase by disassembling K63-linked regulatory ubiquitin chains from upstream adapter molecules like RIP1. To determine the contribution of the deubiquitinase role of A20 in downregulating NF-κB, we generated and characterized a knock-in mouse lacking the deubiquitinase activity of A20. However, we find that these mice display normal NF-κB activation and show no signs of inflammation. Our results suggest that the deubiquitinase activity of A20 is dispensable for downregulating NF-κB. The second part of this thesis unravels a new biological pathway mediated by IκBβ. Unlike IκBα, which functions solely as an inhibitor of NF-κB, IκBβ can both inhibit and activate NF-κB depending on the physiological context. We hypothesized that this may be because IκBβ (unlike IκBα) exists in two forms, a constitutively phosphorylated form and an unphosphorylated form. Prior work from our group has demonstrated that hypophosphorylated IκBβ complexes with p65:cRel and mediates the expression of certain inflammatory genes like TNFα . We report here that Glycogen Synthase Kinase 3β (GSK-3β) interacts with and phosphorylates IκBβ at Serine-346. This phosphorylation masks the NLS of p65 in the phospho-IκBβ:p65:cRel complex, thereby

sequestering the complex in the cytoplasm and mediating the anti-inflammatory role of IκBβ. We discovered a peptide that can inhibit this phosphorylation by abrogating the interaction between GSK-3β and IκBβ. Mice succumb to a sublethal dose of LPS when injected with this peptide because of increased production of TNFα (but not IL-6); thereby demonstrating the inflammatory role of unphosphorylated IκBβ in upregulating specific genes like TNFα. We propose a signaling model by which phosphorylation by GSK-3β can regulate the functions of IκBβ in response to LPS.

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Dedication

This thesis is dedicated to the "*Inspiration of Sachin Tendulkar*"

Some of the greatest feats of Sachin Tendulkar (including the first double century in history) happened when I was at Columbia University, away from home studying in New York city. I wrote this sonnet for him, that I want to share with the world today).

A sonnet for Sachin

At the stroke of a 1947 August midnight, half a billion awoke but slumbered.

In 1989, the walls came down, the world changed.

Out walked a young boy from the pavilion, shy and quiet

And finally a billion opened their eyes, to see daybreak…

And then for quarter of a century, he became us.

No bravado, nor pompous bluster

A sportsman who transcended the game with his karma,

Spreading delight from Mumbai to Calcutta.

Then the world unified in rapturous applause. Manchester, Sydney, Wellington,

Cape Town and Colombo became one

Human endeavor reached new heights, what was good became better

Never before did so many owe as much joy to one Little Master!

Through it all, you remained aloof. Karma was your vision!

Sachin Tendulkar, conscience of the richest Indian tradition.

(Many of my friends have asked, why dedicate a PhD thesis to Tendulkar? My friends are very dear to me and hence, a brief word will be apt here. Sachin Tendulkar is the greatest batsman the cricket world has seen in the post war era, and holds every record that we can imagine. However, this thesis is not dedicated to the batting genius of the man, an art the great Sachin Tendulkar perfected. As important as a sport is, other professions are at least as important and multiple professionals have been just as good in their respective field of enterprise. This thesis is dedicated to Tendulkar for the way he has inspired the Indian youth (and me) for the past 25 years. ***Tendulkar is an idea, an idea that will be cherished by all who believe in perfection and letting 'karma' talk).***

Chapter1:

Immune homeostasis: Activation and Downregulation of NF-κB

1.1 Introduction to immune system

Over time, we have evolved various sophisticated 'immune-defense mechanisms' to protect ourselves from other organisms, both large and small. While co-evolution of multicellular organisms and microbes have provided several essential mutual benefits for both [1], infectious diseases constitute a major threat to human health and a possible cause of death. Besides, it is also a major burden for the global economy. The sources of these infections are pathogenic organisms that include bacteria, viruses or other parasites. Hence, it is very important for the host to be able to respond to such harmful threats, and indeed our ability to clear these microbial infections is vital for survival. However, this is not easy given the enormous diversity of pathogens, and their ability to mutate, evolve and adapt rapidly to evade possible detection by the immune system. Hence, multicellular organisms have evolved several sophisticated immune recognition mechanisms to "fight" pathogens. Immune cells are present throughout the body. They are either found near the skin or gut where they can monitor the entry of foreign substances or discretely encapsulated in the spleen or thymus [2]. In vertebrates, the immune mechanism may be broadly classified as 'innate' and 'adaptive' [3].

1) *Innate immunity*

As suggested by the name, the innate immune system is evolutionarily ancient, and consists of anatomical barriers, immune cells and protective proteins that are always present right from birth. The phylogenetically conserved innate immune system is the first line of defence, initially presenting a physical obstacle to microbes, and subsequently fighting the microbes at the site of infection. The main components of the innate immune system include physical barriers (skin and epithelial layers), phagocytic cells that 'eat' microbes (neutrophils and macrophages),

dendritic cells (critically important for activation of the 'adaptive' arm of the immune system), natural killer (NK) cells, and circulating plasma proteins.

In order to recognize various molecules of microbial origin, the cells of the innate immune system (macrophage, dendritic cells) have germline encoded receptors. The innate defense system is activated when these germline encoded receptors recognize the molecular patterns that are conserved in a wide range of pathogens (PAMPs).

The first component of our immune system is the skin, which maintains a constant barrier against external infection. There are also other barrier tissues preventing harmful pathogens from getting inside the body. If the pathogens succeed in breaching the "first line of defense", and get into the body, the other cells of the innate immune system (macrophages, neutrophils) respond to the pathogens immediately, engulfing the foreign organisms by a process known as phagocytosis and killing them.

Thus, this innate, germline-encoded, one-size-fits-all immune system is always available and ready to fight any infection. In most cases, the actions of the innate immune system is sufficient to clear the infection. However, should the non-specific innate response be insufficient to kill the pathogens, it is able to control the infection for a few days while simultaneously triggering the more specific adaptive immune response.

2) *Adaptive immunity*

This is the more evolutionary recent immune arm first seen in jawed vertebrates. In general, the effector class of the adaptive immune response could be humoral (mediated by antibodies produced by B lymphocytes) or cell-mediated (mediated by T lymphocytes). A highly

diverse group of T cell and B cell receptors generated by the process of VDJ recombination and somatic hypermutation form the basis of adaptive immune recognition. Receptors with appropriate specificities are clonally selected and expanded. “Clonal selection” forms the basis of “immunological memory”, a process that lets the host ‘adapt’ and remember the pathogen, enabling a more robust immunological response in case of a subsequent attack by the same pathogen. This is a major advantage for advanced organisms over their microbial counterparts. However, there are two limitations of an adaptive response. Firstly, ‘clonal selection’ or the expansion and differentiation of specific lymphocyte clones to effector cells takes up to 4-7 days. Hence, no adaptive response can be mounted till this time, a considerable span for multiplication of fast replicating microorganisms. Secondly, since these receptors are formed randomly, they cannot by itself distinguish pathogenic antigens. Hence, the adaptive immune system does not function independently and innate immune signals activate and control the extent of the adaptive response.

Figure 1.1 shows the mechanisms of action of the innate and adaptive immune system. Innate immunity involves immediate, nonspecific response to pathogens. Thus, pattern-recognition receptors (PRRs) on macrophages and neutrophils recognize pathogen-associated molecular patterns (PAMPs) in pathogens leading to phagocytosis and opsonization. This leads to the secretion of cytokines like tumour necrosis factor α (TNFα), and interleukin-1β (IL-1β) that mediate the inflammatory response. These initial defensive acts by the innate immune system trigger the adaptive immune system. The lymphocytes (T cells and B cells) of the adaptive immune system have receptors that bind to specific antigens. The full development of the adaptive response requires selection, expansion and differentiation of the specific responder

cells. This takes time (4-7 days), but leads to memory lymphocytes which remember the specific antigen they responded to [4].

Although Elie Metchnikoff first described the innate immune system a century ago, the research in this field has been largely eclipsed by the intriguing discoveries of the many facets of the adaptive immune system. During this time, it was thought that the innate immune system only helps to keep the infection at bay while the adaptive immune system prepares to mount the specific and robust response. However discoveries in the last twenty years have clearly shown that the innate immune arm not only plays an essential role in detecting the infection and providing the immediate response, but also in simultaneously coordinating the initiation and determining the effector class of the adaptive response [5].

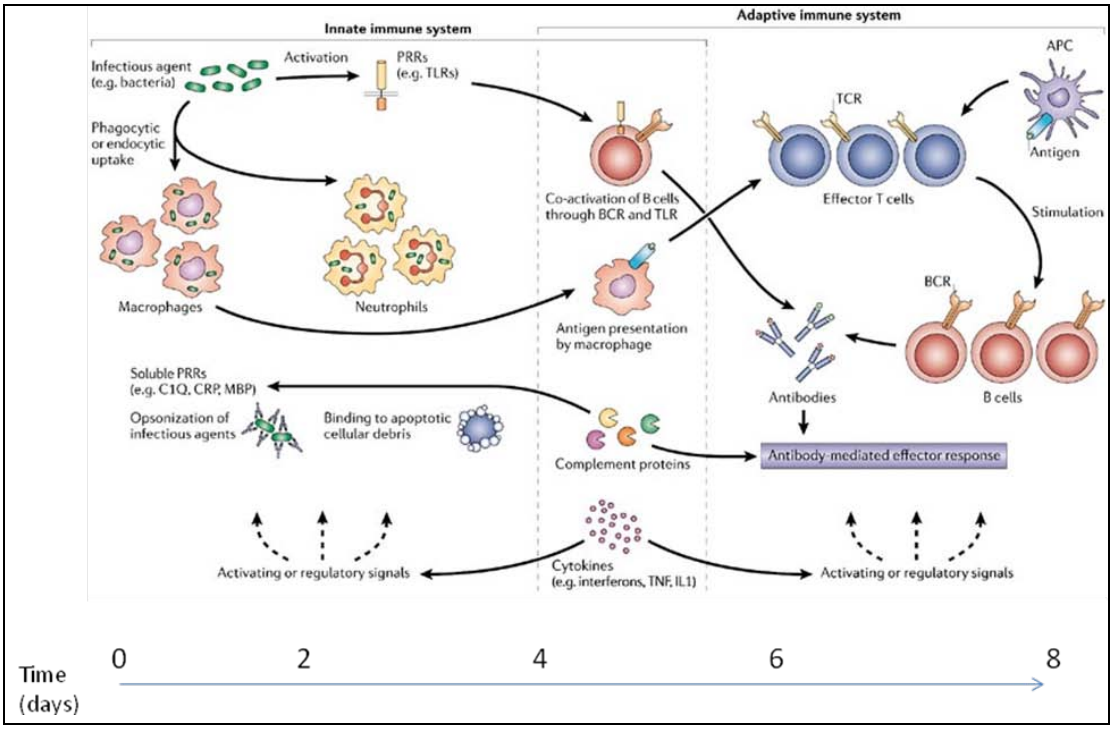


Figure 1.1: Innate and adaptive immune system (APC, antigen-presenting cell; BCR, B-cell receptor) (adopted from[4])

The fundamental strategy by which the innate immune system recognizes pathogens is by detecting those unique constitutive and conserved microbial molecules that are absent in the host. These molecules are typically part of essential microbial metabolic pathways and are required to sustain microbial life. Thus, it is impossible for microbes to live without expressing these gene products. By evolving to recognize these critical microbial products, the host gains a significant advantage. Some of these substances include lipopolysaccharide (LPS), peptidoglycan, and lipoteichoic acids (LTAs); all molecules found only in bacteria and not produced by eukaryotic cells. Hence, the hosts see these molecules as 'signatures of the pathogen' and the recognition of such foreign microbial signatures is the first signal of an infection. While different strains and species of a common class of microbes (bacteria) may have minor variations in chemical structure (O-antigen subgroup of LPS is different in different bacterial species); the common invariant 'molecular pattern' is what is recognized by the host innate immune system (main lipid-A pattern of LPS). Since the targets of innate recognition is invariant and conserved, they are known as pathogen-associated molecular patterns (PAMPs). The receptors of the innate cells that are responsible for recognition of PAMPs are known as pattern-recognition receptors (PRR), a broad class of receptors that have evolved over time and this initial recognition is primarily responsible for the specificity in immune response against the non-self [6], [7].

In order to recognize these different PAMPS, a number of PRRs have evolved in order to broadly sense the diversity of the microbial world **(Figure 1.2)**. The PRRs directly engage the evolutionarily conserved PAMPS to constitute what is the first step of the innate immune response against invading microbes. The different PRRs may be expressed on the surface of cells, in intracellular compartments, or secreted into blood or tissue fluids. PRRs may be broadly classified into, a) transmembrane PRRs and a) cytosolic PRR.

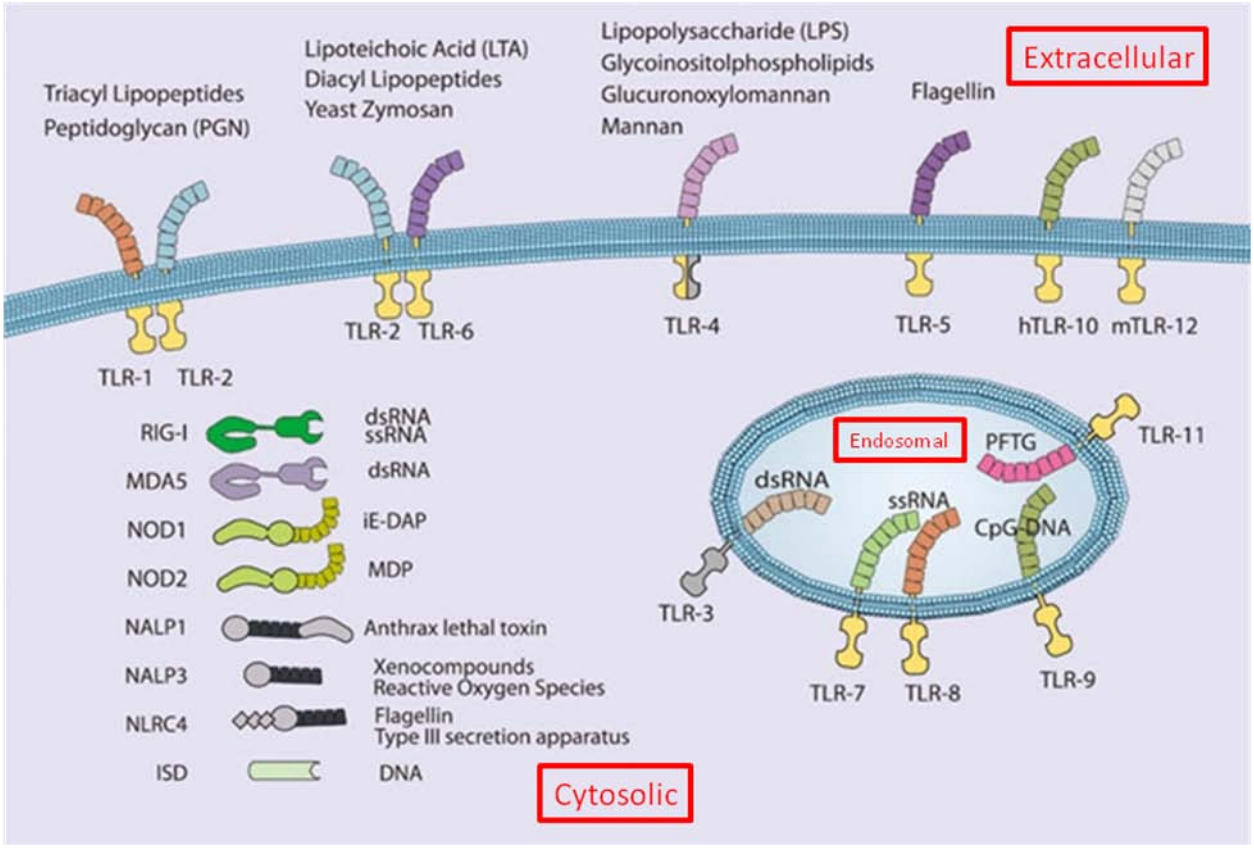


Figure 1.2: Pattern recognition receptors and their cognate ligands: PRRs may be membrane bound or endosomal in order to recognize various PAMPs in different locations. [8]

Transmembrane bound PRRs include the TLR (Toll-like receptors) family which are probably the most well known as well as the best characterized of the PAMPs. The C-type lection receptors (CLR) are another example of transmembrane receptors. The mannose receptors is a typical CLR expressed in macrophages which binds certain sugar molecules of some invading bacteria and viruses (this is especially important in the response against human immunodeficiency virus or HIV) [9].

Cytosolic PRRs recognize intracellular PAMPS. This is especially important when the invading microorganism has gotten inside the cell, either by itself or as a result of phagocytosis. They include CATERPILLAR/NOD-like receptors (NLRs) and RIG-l-like receptors (RLRs). The NLR family has around 20 members and bind to the peptidoglycans present in bacterial cell wall [10, 11], [12]. The RLRs play a crucial role in the immune response against viruses. Three different RLRs have been discovered, namely, RIG-I, MDA5 and LGP2. They sense viral replication in the host cytoplasm by interaction with dsRNA of viruses (host RNA is single stranded; a clear example of how the host immune system has evolved to distinguish between subtle differences in structures of 'self' and 'non-self') [13].

As we see, our body has an elaborate mechanism to respond to the constant challenge of various pathogenic microorganisms which trigger the immune system. The essential first line of defense involves the sentinel macrophages, sensing the various PAMPS with their PRRs. Remarkably, while binding of different PAMPs to their specific PRRs may trigger different intracellular signaling cascades, they all converge and lead to the activation of the nuclear factor κB (**NF-κB**) pathway (initially inactive in resting conditions) and the consequent expression of many proinflammatory cytokines [14] **(Figure 1.3)**. These proinflammatory cytokines are largely responsible for initiating the innate immune response by recruitment of additional professional phagocytes and subsequent pathogen clearance. They also shape the subsequent adaptive response [15]. One consequence of multiple pathways coalescing on a single transcription factor would be that dysregulation of NF-κB could be very harmful. Indeed, there are many reports associating dysregulation of NF-κB with different pathologies [16-18]. To avoid autoimmunity, it is essential that the activation of NF-κB be tightly regulated in order to limit the duration and magnitude of the response. This theses deals with two important inhibitors of NF-κB: IκBβ [19] and A20 (also known as TNFAIP3) [20].

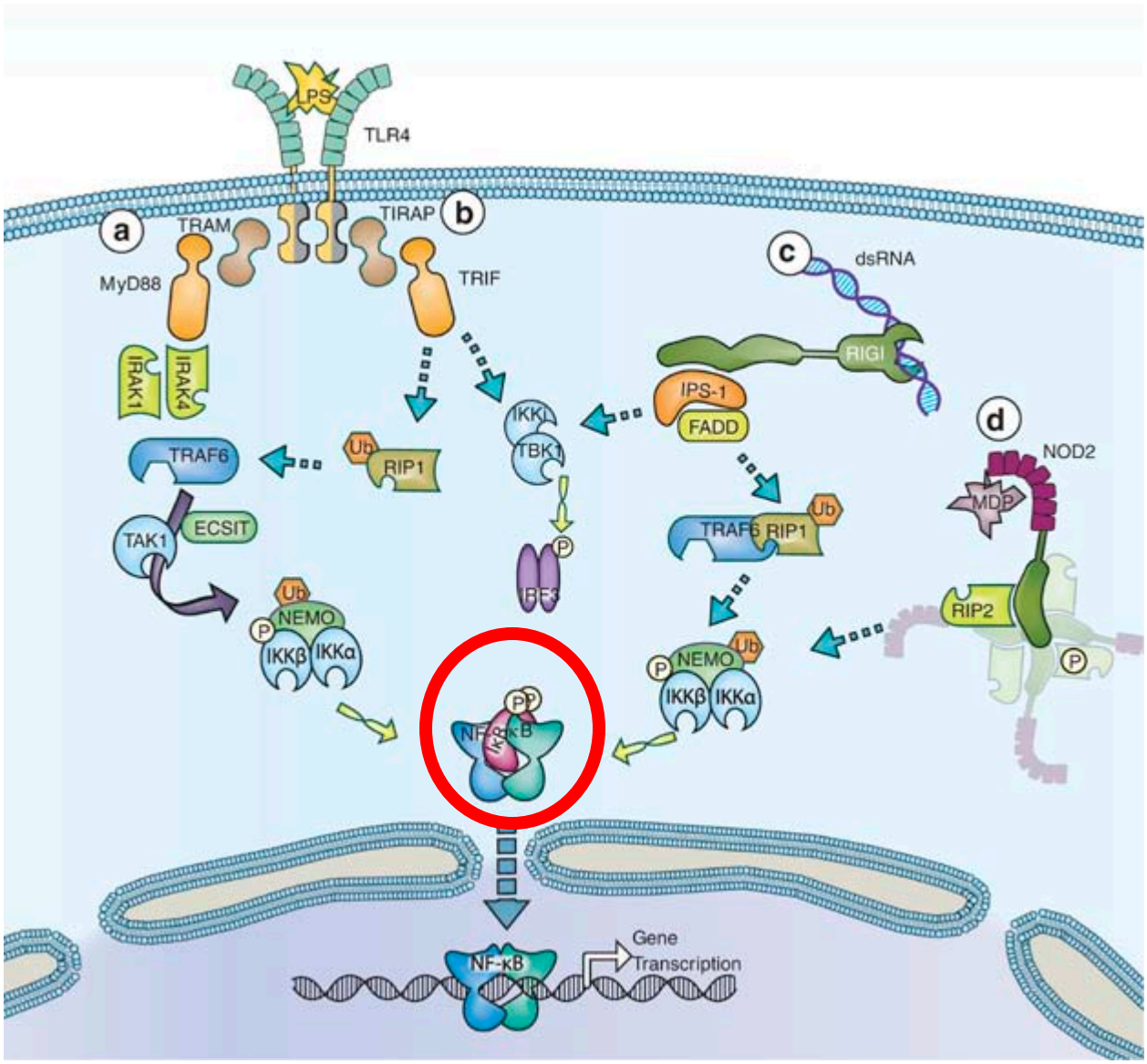


Figure 1.3: Binding of PRRs to PAMPs converges on the NF-κB pathway via the activation of IKK complex (thus, signaling through the (a) TLRs (Myd88) (b) TLRs (TRIF) (c) RIGI (d) NOD pathogen-associated molecular patterns lead to NF-κB activation.(modified from [21])

1.2: NF-κB: an overview

As seen in the last section, the mammalian immune response can be broadly subdivided into innate and adaptive responses. The first response is provided by the innate system and

begins with host-recognition of the pathogen. The subsequent responses take place at various levels of complexity at the cellular, tissue and organismal levels. The ultimate aim is to clear the pathogen. From a basic molecular point of view, it is insightful to distill the immune response into individual signal transduction events which alters gene expression at the cellular level, finally leading to a concerted immune response at the organismal level [21]. NF-κB, inactive in the basal state and activated upon sensing PAMPs, is one key transcription factor that plays a key role in mediating transcriptional changes. The gene products of the initial response include cytokines acting as 'messengers' of the 'initial recognition of pathogens', thereby propagating the immune response. Additionally, these cytokines can also activate NF-κB, thereby focusing and elaborating the immune response. Thus, we find that NF-κB mediates critical aspects of the innate and adaptive responses in a surprisingly large number of cases. The importance of this inducible transcription factor can be gauged from the volume of research done in the past 25 years since it was identified [22] as a nuclear factor interacting with immunoglobulin enhancer sequences [23], [24]. A search in PubMed using "NF-κB" alone results in more than 40,000 hits.

While the underlying interest in studying NF-κB comes from its importance in health and disease, these studies also serve as a model for studying a wide range of biological responses that depend on inducible transcription factors. Inducible gene expression plays an important role in both prokaryotes and eukaryotes. It is an important regulator of normal physiology as well as the key in allowing multicellular organisms to adapt to chemical, environmental and pathogenic stresses. Fundamental biological processes including organ morphogenesis and differentiation of both single cells and multicellular organisms depend on the paradigm of inducible gene expression.

NF-κB has been found to be amenable to a wide range of experimentation at the biochemical, cellular and organismal levels. Most of the studies of NF-κB have been done from an immunological background, as the transcription factor plays an essential and evolutionarily conserved role in responding to immune insults. However, while much of our understanding of the pathway comes from studies in immunology, it is important to appreciate that NF-κB plays an important broader role in regulating gene expression that affects cell survival and apoptosis, differentiation and proliferation [19] [25].

A brief overview of the NF-κB pathway may be helpful here. NF-κB is bound to inhibitory proteins (IκB) and is inactive in the unstimulated state. The pathway is activated by inducing stimuli acting through receptors and adapter proteins to trigger IKK (activating kinase) activation. This leads to the phosphorylation, ubiquitination, and subsequent degradation of IκB. NF-κB is now free and undergoes a series of post-translational modifications to be fully activated **(Figure 1.4)**. It translocates to the nucleus, binds to specific DNA sequences and turns on the transcription of its target genes. Thus, the basic components of the pathway are the receptors and adaptor molecules, IKK complex, IκB proteins, and NF-κB subunits. The pathway is controlled by multiple positive and negative regulatory elements [25].

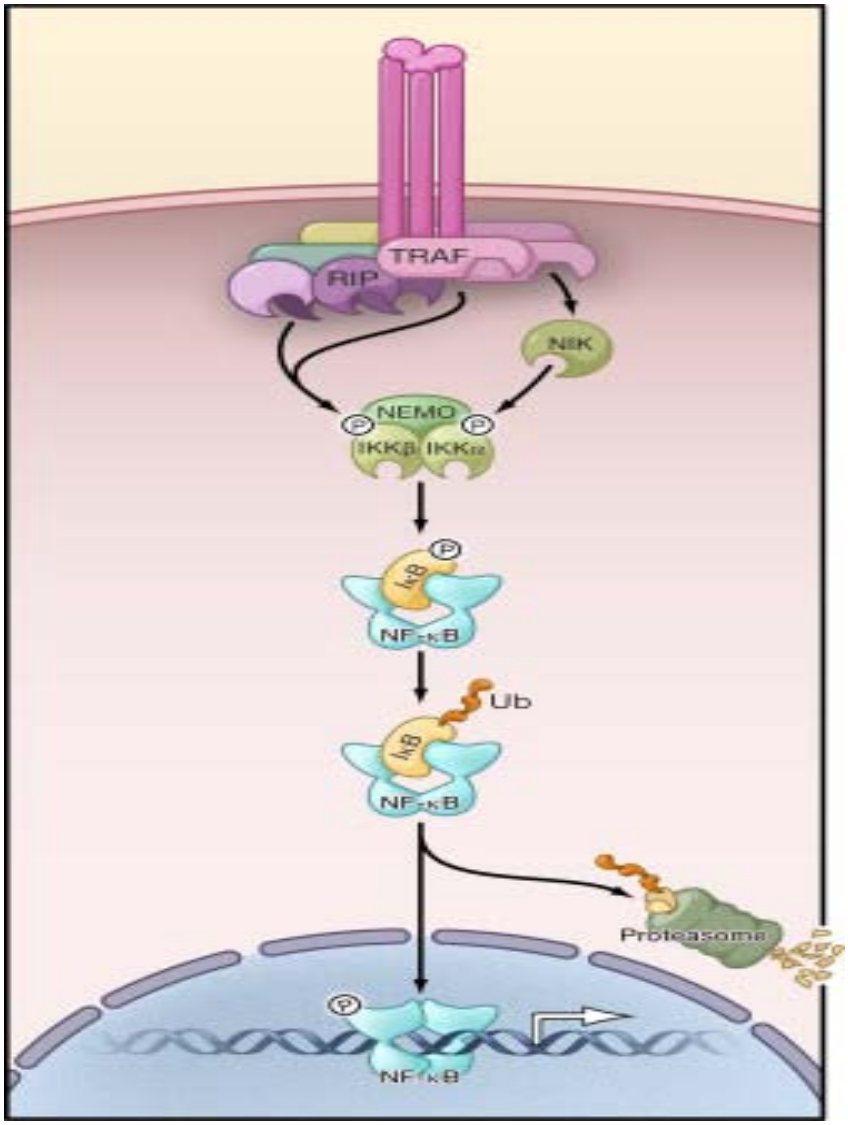


Figure 1.4: Basic NF-κB Signaling Pathway [25]

In mammals, the NF-κB family of transcription factors is comprised of five constituent monomers: p50, p52, p65 (also RelA), c-Rel, and RelB **(Figure 1.5)**. All of them have a N-terminal DNA-binding domain which is also the domain necessary for homodimer/heterodimer formation. This N-terminal domain is also known as the Rel homology domain (RHD). The

crystal structures of multiple NF-κB dimers reveal that the RHD is comprised of two immunoglobulin-like folds. Of them, one is engaged in dimerization while the other is responsible for recognition of specific sites on the DNA. Minor differences in dimer interfaces determine partner preferences and selectivity in DNA binding [26]. Multiple posttranslational modifications (phosphorylation and acetylation) of different residues in these molecules further modulate DNA binding [27]. Subsequent transcriptional activities may additionally depend on the interaction of dimers with other activator proteins [28]. Activated NF-κB dimers can bind multiple, related DNA sequences called κB promoter sites to turn on the expression of different genes. The C-terminal transcription activation domains (TADs) are necessary for activator recruitment and consequent transcription of target genes. Only RelB, c-Rel, and p65 contain the TAD. Since p50 and p52 lack the TAD, they can act as transcriptional activators only upon association with RelB, c-Rel, and p65. However, p50 and p52 can form homodimers that bind to the DNA, and repress transcription. In order to positively regulate transcription, the p52 and p50 subunits have to form heterodimers with the other factors that have TAD [25]. RelB also has a unique leucine zipper (LZ) motif in its N terminus which plays an important role in regulating transcription.

In most cells, NF-κB complexes are sequestered in the cytoplasm and are inactive. This is because they are in a complex with inhibitory IκB proteins (proteins including IκBα, IκBβ, IκBε, IκBζ, p100, p105, Bcl3, IκBns) **(Figure 1.5)**. The IκBα, IκBβ, and IκBε, are the prototypical IκBs. These proteins have multiple ankyrin repeats that are responsible for their inhibitory action. The ankyrin repeats mediate the binding of the inhibitor molecule to the NF-κB dimers, interfering with their nuclear localization signals (NLS). The C-terminus of p105 and p100 has multiple ankyrin repeats [29], allowing them to function like IκB [30]. Although the IκBs are

structurally quite similar, they may have different binding preferences [31], [32]. For example, while IκBα predominantly regulates the classical RelA-p50 heterodimers [33], IκBβ regulates the p65-cRel [34], [35] and the IκBε associates with p65-p65 homodimers as well as cRel:RelA heterodimers [31], [36, 37].

One of the basic questions at the time of discovery was to understand the process of liberation of the active nuclear factor-κB (NF-κB) from the IκB inhibitor. Initial discoveries suggested that *in vitro* phosphorylation with purified kinases released NF-κB from the inhibitory complexes. The same phenomenon was demonstrated in cell lines where it was shown that the stimulus-dependent activation of NF-κB indeed involved IκB phosphorylation [38, 39]. Thus, phosphorylation of IκBα at serines 32 and 36 [40, 41] was a prerequisite for the nuclear translocation of active NF-κB [42-46]. However, it was also evident that the phosphorylation of IκB was not enough to trigger NF-κB activation. The degradation of the phosphorylated IκB was also critical, as blocking the proteolysis of IκB could prevent NF-κB activation [47-51]. It turned out that signal-mediated phosphorylation of IκBα triggers subsequent ubiquitination and proteasomal degradation [52-55]. Multiple groups subsequently confirmed that β-TrCP was the E3 ubiquitin ligase for IκBα [56-59]. Incidentally, the first signal transduction system where ubiquitin-dependent proteolysis was shown to be essential was the NF-κB/IκB pathway [58].

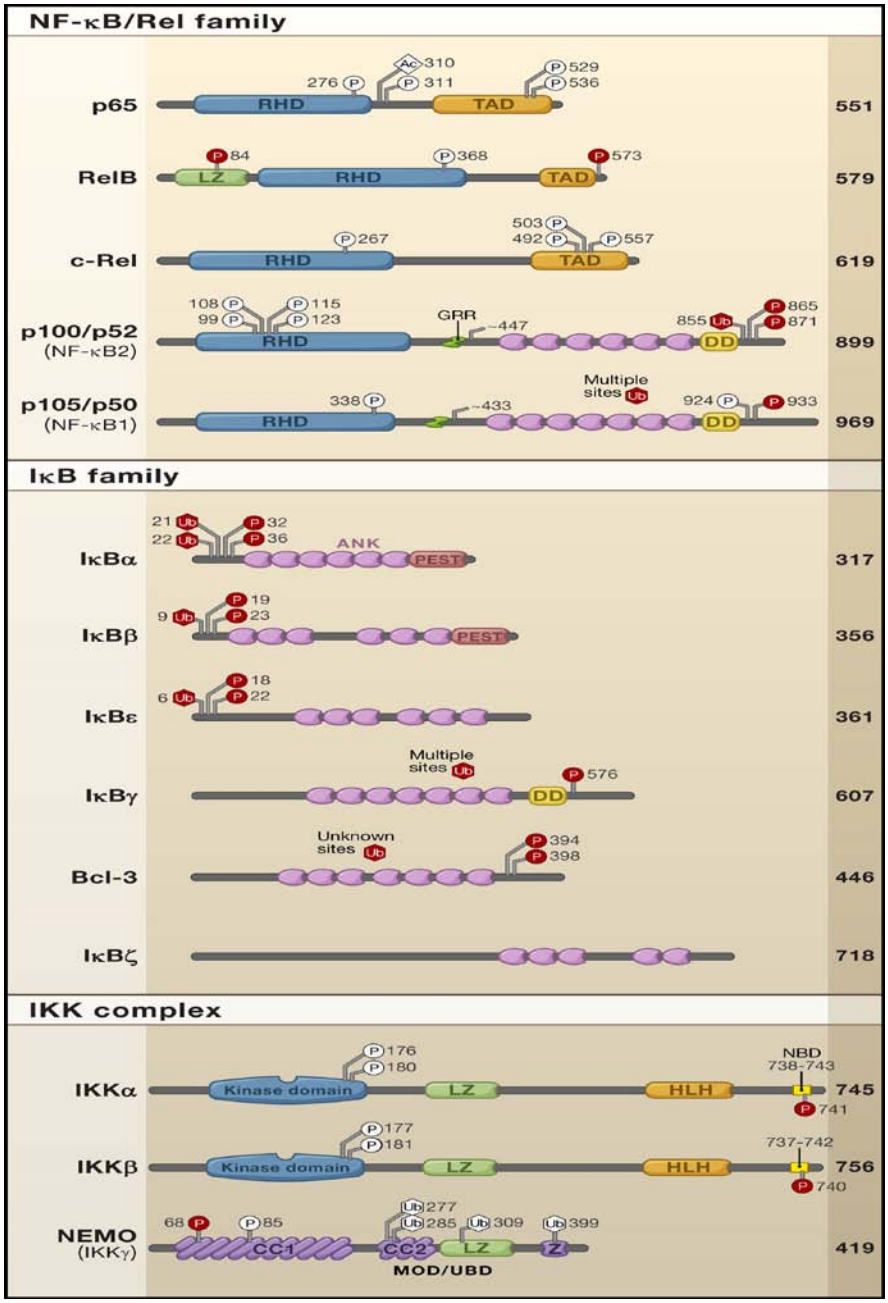


Figure 1.5: Mammalian NF-κB, IκB and IKK protein families [25] Phosphorylation, ubiquitination, or acetylation are indicated with P, U, or Ac respectively. Inhibitory events or degradative phosphorylation and ubiquitination sites on p100, p105, and IκB proteins are indicated by red Ps and Us, respectively. (RHD, Rel homology domain; TAD, transactivation domain; LZ, leucine zipper domain; GRR, glycine-rich region; HLH, helix-loop-helix domain; Z, zinc finger domain; CC1/2, coiled-coil domains; NBD, NEMO-binding domain; MOD/UBD, minimal oligomerization domain and ubiquitin-binding domain; and DD, death domain).

While all the three major IκBs undergo proteasomal degradation upon stimulation, they do so with different kinetics [60]. To exemplify, IκBα is degraded very fast in around ~15-30 minutes upon stimulation with TNF-α and lipopolysaccharide (LPS). It is then resynthesized in an NF-κB-dependent manner and demonstrates a classical negative feedback loop. The newly synthesized IκBα enters the nucleus and binds to deacetylated RelA:p50 heterodimers. The inactive inhibitor-heterodimer complex then shuttles back to the cytoplasm [61, 62]. Similar stimulation-induced degradation and resynthesis occurs for IκBβ and IκBε as well, but at a much slower rate [60]. Ultimately, these differences in kinetics in IκB degradation and resynthesis play an important role in the regulation of NF-κB activation. This topic is dealt with in the second part of the work presented in this thesis.

The kinase responsible for phosphorylation of IκB in cells was later identified and named the IκB-kinase complex (IKK), which was shown to have both catalytic (IKKα and IKKβ) and regulatory (IKKγ/NEMO) subunits [63-67]. Based on the diversity of stimuli activating NF-κB and the number of IκBs, one could have expected that multiple kinases were responsible for phosphorylation of the different IκBs. As it turned out, only one kinase complex is responsible for the phosphorylation. Besides the traditional role in IκB phosphorylation, the IKK complex has also been shown to enhance the transcriptional activity of NF-κB by phosphorylating the TADs of cytoplasmic RelA and c-Rel while still in the cytoplasm [27, 68].

1.3 NF-κB: The canonical pathway and non-canonical pathway

There are two distinct pathways for NF-κB activation, the canonical pathway and the non-canonical pathway. They are activated by different ligands and kinases. The canonical pathway mediates the typical inflammatory responses, while the non-canonical pathway plays a role in the slow differentiation and maturation of immune cells and secondary lymphoid organogenesis. The canonical pathway is dependent on NEMO, while the non-canonical pathway does not depend on it [69] **(Figure 1.6)**.

While the two pathways are quite distinct, there are evidences of crosstalk between them [69]. The pathways are briefly described here:

1) Canonical pathway: The canonical **NF-κB** signaling pathway is activated by pro-inflammatory ligands that include PAMPS, cellular danger-associated molecular patterns (DAMPs) and cytokines. The effector NF-κB heterodimers activated downstream are mainly the p65:p50 and c-Rel:p50 heterodimers. Upon ligand binding to cognate receptors, various adapter proteins trigger the activation of IKK complexes containing the regulatory NEMO subunit. Deletion of NEMO causes massive liver degeneration resulting in embryonic lethality [70], demonstrating the importance of this pathway and NEMO for life. While this pathway is characterized by the essential requirement of the regulatory NEMO subunit, the catalytic subunit of IKK that is activated and responsible for phosphorylating the inhibitory IκB is IKKβ. IKKβ is activated by phosphorylation of its serine residues at positions 177 and 181 [71]. Additional evidence for the signaling axis of NEMO-IKKβ-p65 comes from the uncanny similarity of the phenotypes of the IKKβ knockout [72] and the RelA knockout [73], both of which resemble the NEMO knockout and show apoptosis and degeneration in the liver.

A brief description of the pathway is provided here. The p65:p50 dimer is sequestered in the cytoplasm by the binding of IκBα. The IκBα:RelA:p50 trimer is mostly localized to the cytoplasm as the IκBα has a strong nuclear export signal. However, this complex also shuttles between the nucleus and cytoplasm [74, 75]. This is because the binding of IκBα to p65:p50 masks the NLS of p65 but fails to mask the NLS of p50 [33].

Upon stimulation, the activated IKK phosphorylates IκBα on Ser32 and Ser36 [76]. The phosphorylated IκBα is now polyubiquitinated by degradative Lys48-linked polyubiquitin chains at Lys19 by the Skp1, Cdc53/Cullin1, and F-box protein β transducin repeat-containing protein (βTRCP) $SCF^{Iκ B}$ E3 ubiquitin ligase complex [33]. This leads to the degradation of ubiquitinated IκBα via the 26S proteasome. The strong p65 NLS is now exposed and the p65:p50 dimers translocate to the nucleus. In addition to IκBα, IκBβ and IκBε are also substrates for IKK phosphorylation and subsequent degradation via similar pathways. [77]. That being said, the affinity of IKK to the various IκBs is markedly different. Hence, the various IκB protected dimers are degraded with different kinetics. The net consequence is that the basally inactive NF-κB transcription factors are inducibly activated by a diverse set of stimuli, all leading to the nuclear translocation and subsequent expression of NF-KB dependent genes [78, 79].

2) Non-canonical pathway: This alternative NF-κB pathway is activated upon stimulation by a small subset of TNF family members that are involved in differentiation, maturation and development of immune cells. These include lymphotoxin β, LTαβ, BAFF (B cell–activating factor), CD40 ligand, RANKL (receptor activator of NF-κB ligand), and TWEAK (TNF-related weak inducer of apoptosis) [80-83]. Since developmental processes require sustained signaling, it is not surprising that the kinetics of activation and deactivation of this pathway is slower as

compared to the canonical pathway. Hence the pathway has a long-lasting activity, as opposed to the rapid transient activation of p65-p50 heterodimers by inflammatory stimuli.

Biochemical characterization has shown that in unstimulated cells, RelB interacts with and is associated with p100. The RelB:p100 complex is stabilized by multi-domain interactions. Thus, p100 deficient cells show reduced levels of RelB and similarly, RelB knockout cells have less p100 protein [84, 85]. This alternative NF-κB pathway ultimately leads to the inducible proteasomal processing (as opposed to degradation) of p100 to p52 and activation of RelB:p52 heterodimers. Another major distinction with the canonical pathway lies in that this pathway does not require NEMO. Instead, the non-canonical **NF-κB** pathway is activated strictly through a NEMO-independent IKKα mediated pathway [79, 82, 86, 87]. Ligand binding activates the NIK protein, which directly phosphorylates p100 at Ser866 and Ser870, and also phosphorylates IKKα [87, 88]. The activated IKKα then phosphorylates p100 in its ankyrin domain at Ser99, Ser108, Ser115, Ser123 and Ser872 [77, 89]. The SCG/βTRCP E3 ligase complex then recognizes the phosphorylated p100 and polyubiquitinates it with K48-linked ubiquitin chains [89, 90]. This leads to the consequent proteasomal processing of p100 by the 26S proteosome [91] [87, 88] to p52. The p52 then associates with the RelB that was previously associated with p100 to form the activated RelB:p52 dimers.

Elegant genetic proof of NIK, IKKα and RelB as components of the non-canonical pathway comes from the remarkable similarity of the phenotypes of mice deficient for these genes [86], [92], [93]. They all show defective development of Peyer's patches and other lymph nodes. Additionally, the phenotype of *aly/aly* mice (carries a point mutation in kinase domain of

Nik gene leading to inactivation of NIK) is similar to $Ikk\alpha^{AA}$ mice (mice have catalytic serines mutated to alanine; as a result IKKα cannot be activated in these mice) [94], [87].

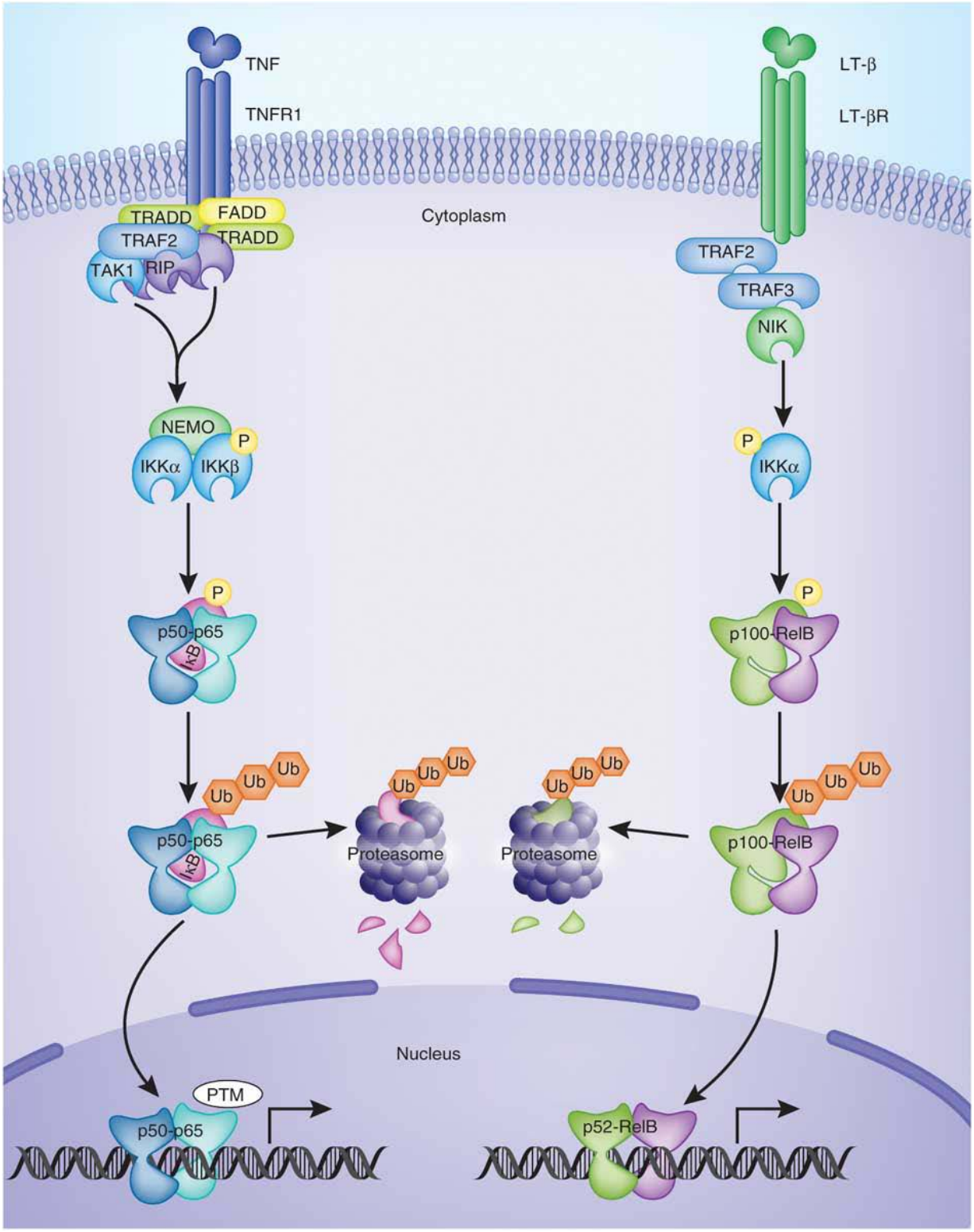


Figure 1.6: Canonical and Non-canonical NF-κB signaling [95]

1.4 A brief overview of the IKK complex

The NF-κB pathway is activated by a whole range of extracellular ligands (both membrane bound and soluble). They include members of the TLR, TNFR, IL-1R superfamilies. Additionally, NF-κB has been recently shown to respond to changes in the intracellular environment as well. These changes may be a consequence of DNA damage, elevated reactive oxygen species, and recognition of pathogens by the RIG-I and NOD family of proteins. All of these stimuli converge on the activation of the IκB kinase (IKK) complex.

The IKK complex comprises of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (NEMO or IKKγ) **(Figure 1.5)**. There is a 52% overall sequence identity between the catalytically active kinase subunits IKKα and IKKβ, while the catalytic domain are 65% identical. Amongst the two catalytic subunits of IKK, IKKβ contributes more than IKKα towards IκB kinase activity in most cell types and thus seems to be the more important catalytic subunit. Loss of IKKβ leads to lethality, thus IKKα cannot compensate for the IκB kinase activity in absence of IKKβ [96] However, loss of IKKα has little effect on net IKK activity as IKKβ can compensate for IKKα [97].

There is a plethora of clear genetic evidence pointing to the essential role of all the three subunits of IKK [98]. Mice lacking IKKβ resemble p65 knockouts. This is expected since IKKβ plays a profound role in the canonical activation of p65-containing dimers [72, 99, 100]. The embryonic lethality can be rescued by deletion of TNFR1, demonstrating a crucial role for IKKβ in TNF-mediated signaling [72, 99, 101]. IKKα knockout mice die soon after birth because of severe morphological defects in the skin and the limbs [102-104]. Initially, there was little evidence for IKKα playing a role in NF-κB activation. However, subsequent reports

demonstrated the central importance of IKKα in the noncanonical NF-κB pathway and in certain canonical pathways as well [105]. NEMO is essential in the canonical pathway; hence NF-κB cannot be activated via the canonical pathway in NEMO deficient cells. In addition, mice lacking NEMO die from severe liver degeneration. [67, 70, 106].

It is now understood that almost all NF-κB-stimulating ligands act via the IKK complex [107] as there is no NF-κB activity mice lacking both IKKα and IKKβ [107]. Surprisingly, many of the components upstream of IKK are similar, and also show significant mechanistic overlap (even in pathways that are functionally divergent). However, even 15 years after the initial characterization of the IKK complex, it is still not entirely known how IKK gets activated. One of the major reasons for this is that, in general, the receptors that lead to IKK activation lack enzymatic activity [108].

What is known is that the activation of IKK depends on the phosphorylation of serines in the activation loop of the IKKs. Thus IKKα is activated upon phosphorylation of Ser 176 and Ser 180 while IKKβ is activated upon phosphorylation of Ser 177 and Ser 181. This phosphorylation of active loop serines is essential for inducing kinase activity of the IKK complex. Treatment with phosphatases decreases kinase activity *in vitro*, and mutating the serines to alanines abolishes signal responsiveness. Conversely, mutation to glutamic acid results in IKK that is constitutively active [109], [110], [111], [112], [113].

Thus, although receptors leading to IKK activation lack kinase activity, it is evident that some kinase has to be responsible for phosphorylating and activating IKK. As described before, there is both biochemical and genetic evidence for NIK acting as the kinase to directly phosphorylate and activate IKKα in the non-canonical pathway [112], [114], [94]. For the

canonical pathway, it is hypothesized that IKK could either be activated by autophosphorylation or phosphorylation by an upstream kinase [108]. While multiple kinases can act as an IKK kinase *in vitro* ((IKK-K), they have all failed the genetic test. It has been found that many molecules that have kinase activity act as adaptor proteins in the activation of IKK. Currently, the only acceptable candidate kinase to phosphorylate IKK is TAK1. Indeed, the failure of multiple potential kinases to satisfy the gene-knockout test has supported the theory that IKK might activate itself by autophosphorylation.

Recent resolution of the crystal structure of IKKβ suggests that a dimer of IKKβ could not phosphorylate itself. This is because the active site of the one IKKβ would be distant from the 'serine activation loop' of the second IKKβ of the dimer [115]. However, the active site and activation loop are much closer in related higher-order structures that would mediate IKK activation [115]. Hence, while it seems that IKKβ could not undergo *cis*-autophosphorylation; there is indeed a possibility that two dimers could autophosphorylate and activate each other by a process known as *trans*-autophosphorylation [116]. For this to happen, the two dimers need to be in close induced proximity with the right conformation [117], [113].

These recent structural insights are in line with documented evidence suggesting that large oligomeric signaling complexes need to assemble for IKK activation. Thus, NF-κB activation might be mediated by recruitment of IKK to the receptor by adaptor proteins and signaling complexes from the TRAF/RIP (Receptor-interacting protein) family members [113], [117], [118], [119]. Upon recruitment of the IKK complex to the receptor, it either activates itself by autophosphorylation or is phosphorylated by another kinase like TAK1.

Different pathways leading to IKK activation share multiple signaling intermediates upstream of the IKK complex. These molecules are often the non-kinase adaptor molecules like the RIP and TRAF proteins. It is still an open question whether IKK activation in the canonical pathway is mediated by autophosphorylation or TAK-1. However, there is no doubt that a kinase-independent organization of adaptor molecules leading to formation of receptor signaling complexes is necessary for either of these possibilities and therefore, in the downstream activation of NF-κB [108].

1.5: Ubiquitination and NF-κB

Ubiquitin is a small protein made of 76-amino-acid that is covalently linked to its target protein [120, 121]. The protein is highly conserved across eukaryotes from yeast to humans. Ubiquitin is encoded in the genome as 'ubiquitin precursors' with ubiquitin fused with a ribosomal subunit or two ubiquitins fused together. There are four such genes encoding for 'ubiquitin precursors' [122]. Ubiquitination may affect a protein's half-life, localization or function. Ubiquitination is a post-translational modification by which the epsilon amine of a particular lysine of a protein can be covalently linked through an isopeptide bond with the carboxylic acid of the C-terminal glycine of ubiquitin. The process occurs in three consecutive steps catalyzed by three classes of enzymes. These enzymes are the Ub-activating enzymes (or E1), Ub-conjugating enzymes (or E2) and Ub ligases (or E3) [123] **(Figure 1.7).**

There are only two E1 enzymes in the genome. They catalyze the transfer of a thio-ubiquitin intermediate to a cysteine residue of a specific E2 enzyme. This transfer is an energy-

intensive process and proceeds by hydrolysis of an ATP. Thus ubiquitination requires energy and the cell uses it to mostly degrade its substrates. There are over 50 E2 enzymes which transfer the ubiquitin to an E3 ligase. There are around 700 E3 ligases and the specificity of ubiquitination partly comes from the E2-E3 interaction. Additionally, the E3 binds to the specific substrate and ligates the carboxyl terminus of the ubiquitin to the ϵ -amino group of the lysine (Lys) residue of that specific substrate [124]. Subsequently, multiple rounds of ubiquitination result in the formation of polyubiquitin chains. There are seven lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48, K63), and any one of these can participate in elongation of the polyubiquitin chain. Thus, polyubiquitin chains may have different linkages. A linear ubiquitin chain may also be formed by attachment of N-terminal amine of one ubiquitin to the C-terminal carboxyl group of another ubiquitin.

The E2-E3 combinations play a major role in determining the specific linkage of the ubiquitin chains. For example, a particular E2 (UBC5) complexes with another E3 ligase ($SCF^{\beta TrCP}$) to trigger the formation of K48 linked polyubiquitin chains. Similarly, complexing of UBC13 (E2) with TRAF proteins (E3) forms a K63-linked polyubiquitin chain [125].

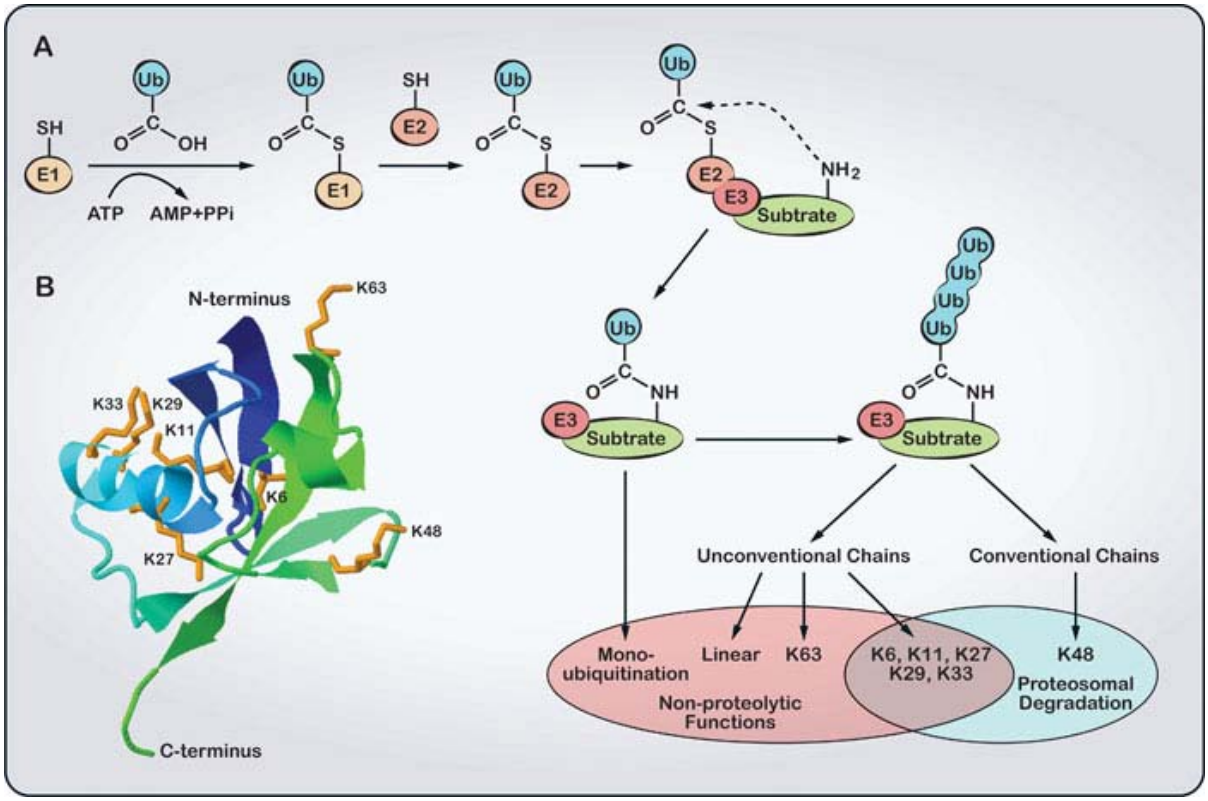


Figure 1.7: Ubiquitination and functions. **(A)** Three-step cascade that leads to ubiquitination; functions of diverse ubiquitin chains **(B)** Structure of ubiquitin (PDB code: 1UBQ), seven lysine residues are highlighted. [123]

These different kinds of polyubiquitin linkages have different structures and topologies. Ubiquitin chains are recognized by proteins having an ubiquitin binding domain (UBD). Thus, different proteins with distinct UBDs bind the specific ubiquitin linkages. There are more than 20 different types of ubiquitin binding domains (UBDs) that have been identified till now [126] [127], [128], differing substantially in their sizes (20-150 amino acids). For example, the proteasome subunits Rpn13/ARM1 and Rpn10/S5a have ubiquitin-associated (UBA) domains that preferentially bind K48 linked chains, while NEMO has a UBAN (UBD in ABIN and NEMO) motif that binds with both K63 and linear polyubiquitin chains (but not K48) [129]. Hence, different ubiquitin chains can potentially confer different fates to the substrate protein

(Figure 1.7). K48-linked ubiquitination (and K11) is known to dictate proteasomal degradation, while K63-linked chains have been proposed to function as scaffolds to assemble the different signaling complexes, thereby regulating different processes like DNA repair, chromatin remodeling, or activation of NF-κB. Thus, K63 ubiquitination is also known as regulatory ubiquitination [129], [130], [131]. UBDs can act as receptors for ubiquitin, thus in principle, it is possible for proteins that have UBDs to potentially transduce signals from substrates that are ubiquitinated to other components of the signaling pathway [128].

Like phosphorylation, ubiquitination is also a reversible post-translational modification. Thus, ubiquitination can be reversed by deubiquitination. The process of deubiquitination is carried out by proteases specifically known as deubiquitinating enzymes or deubiquitinases (DUBs). There are close to 100 DUBs in the human genome [132]. These DUBS can be subdivided into five broad families by the presence of various protease domains: they may be metalloproteases, papain-like cysteine proteases (i.e, a carboxy-terminal hydrolase), ubiquitin specific protease, joseph disease proteases or an ovarian tumor containing protease (OTU deubitinase).

The specificity of DUBs is determined by their UBDs and other protein interaction motifs. This directs them to specific ubiquitinated substrates containing a particular type of poly-Ub chain linkage [133]. If K63 linked polyubiquitin chains positively regulate NF-κB signaling, then their deubiquitinases would be expected to be critical regulators of the pathway. Indeed, A20 and CYLD have been reported to be two such critical proteins with deubiquitinase activity. However, what is not known is if the deubiquitinase activity of these proteins is actually responsible for their function, or if these proteins act by a different mechanism. Mice lacking

A20 are perinatal lethal, suffering from uncontrolled multi-organ inflammation as a result of persistent NF-κB activity [134]. Additionally, mutations or organ-specific deletions of TNFAIP3 (the gene encoding for A20) is associated with different kinds of lymphomas [135], [136], [137], [138]. Moreover, patients with the autoimmune disorder systemic lupus erythematosus also have been found to have polymorphisms in their *TNFAIP3* locus [139]. CYLD is another deubiquitinase that acts as a tumor suppressor and negatively regulates NF-κB signaling. Patients with familial cylindromatosis have been found to have multiple somatic mutations in CYLD and deletion of CYLD may cause colonic, hepatocellular, and renal carcinomas, in addition to multiple myeloma [140].

1.6: Degradative ubiquitination in canonical and non-canonical NF-κB signaling

Aaron Ciechanover, Avram Hershko and Irwin Rose elucidated the ubiquitin pathway and characterized the various enzymes (E1, E2, E3) and deubiquitination enzymes (DUBs) [141], [142]. For this, they were jointly award the Nobel Prize for Chemistry in 2004. Subsequently Alex Varshavsky and colleagues showed that ubiquitination targets proteins degradation *in vivo* [143], [144]. While there is room for abundant skepticism for the role of K63-linked polyubiquitination, there is no doubt that K48-linked ubiquitin chains play a prominent role in NF-κB signaling. Activation of NF-κB is defined by IκB degradation or by the proteasomal processing of NF-κB precursor proteins and this is mediated by degradative ubiquitination [145] **(Figure 1.8)**.

Canonical NF-κB signaling may be defined as those pathways which lead to 'degradation of IκB'. These pathways are mostly activated by proinflammatory stimuli including bacterial PAMPSs like LPS and cytokines like interleukin (IL)-1β and TNFα. Stimulation leads to the activation of the IKK complex which phosphorylates the IκB proteins. This targets the phosphorylated IκBs for K48-mediated polyubiquitination by a K48-specific ubiquitin ligase complex that consists of Skp1, Cul1, Roc1 and βTrCP [146]. The ubiquitinated IκB undergoes degradation by the 26S proteasome. This allows NF-κB to translocate to the nucleus and begin transcription of target genes.

The noncanonical pathway is defined as the pathway that leads to the 'proteasomal processing' of precursor-p100 to its mature p52 subunit [145]. This happens when some receptors (such as B-cell activating factor receptor and CD40) are stimulated in B lymphocytes. Upon stimulation, NIK activates IKKα, which in turn phosphorylates p100. p100 is now ubiquitinated by the βTrCP ubiquitin ligase complex. Under most circumstances, K48-polyubiquitination results in complete proteasomal degradation. However the ubiquitinated p100 is not complete degraded but the C-terminal ankyrin repeats are selectively degraded by the 26S proteasome. This leaves the N-terminal portion intact, and this is the mature p52 subunit. This is because a glycine-rich region in the N-terminus of p100 prevents it from degradation [147]. Additionally, the N-RHD domain is tightly folded and forms a stable dimer, making this region resistant to degradation [148].

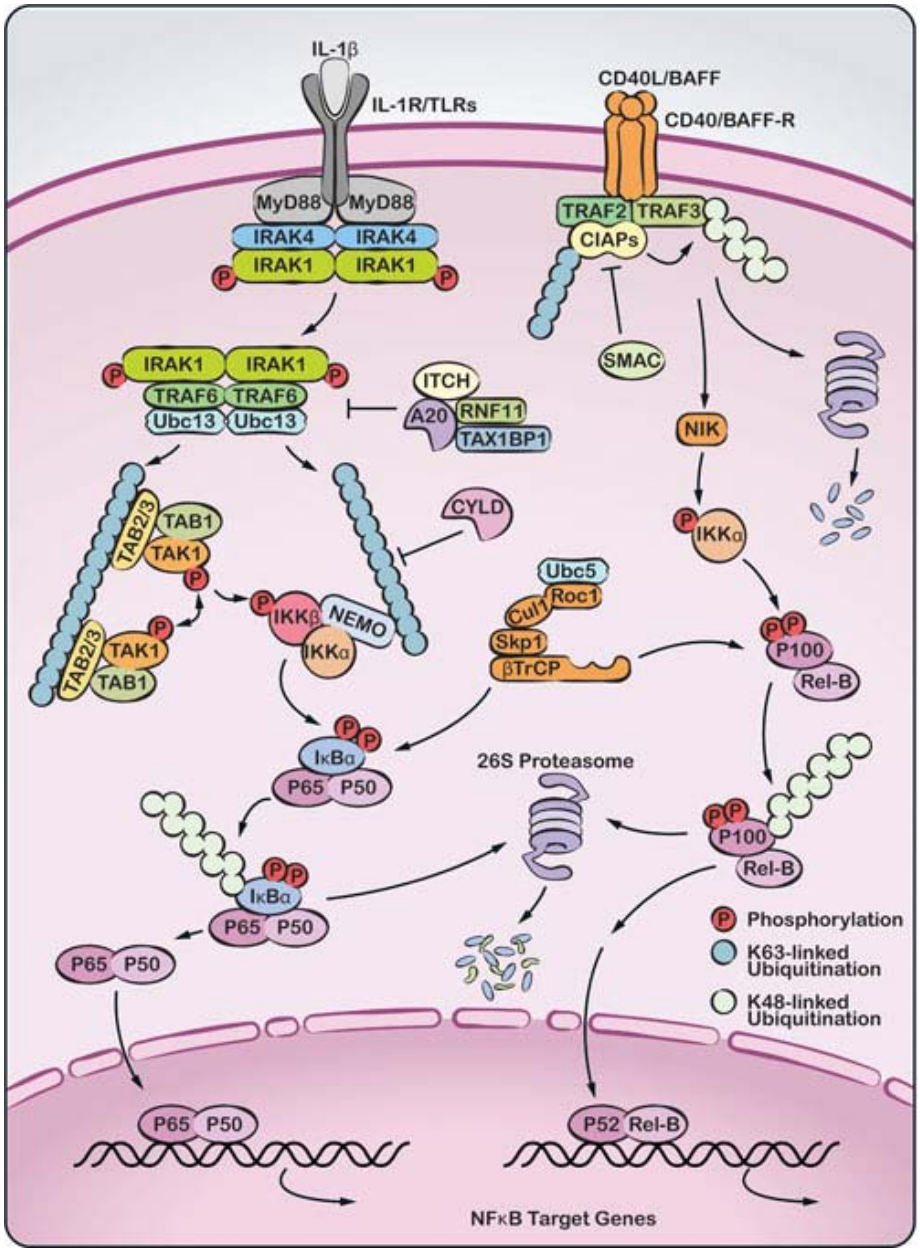


Figure 1.8: Role of ubiquitination in the canonical and noncanonical NF-κB pathways. [123]

1.7: Oligomerization in activation of IKK

A cursory look at any of the three major NF-κB activation pathways (TNF , Toll/IL-1 or T cell receptor) show that oligomerization is a shared theme in NF-κB signaling [25] **(**marked in red in **Figure 1.9)** and recruits the IKK complex to the receptor. Dimerization of the recruited IKK has been shown to be necessary and sufficient for activation of IKK [63], [117], [149].

The dimerization of IKK is mediated by NEMO, which can form higher order structures *in vitro* (dimers, trimers, and tetramers) and *in vivo* [150], [151], [152]. RIP1 was shown to have a major role in inducing oligomerization of NEMO [119], and activating IKK [118]. Mutations in the NEMO oligomerization domain can prevent IKK function. Moreover, mutated NEMO acts as a dominant negative [151], [153]. Additional proof of the role of IKK oligomerization in induction of NF-κB comes from the observation that viral proteins that activate NF-κB also proceed via oligomerization of NEMO [154], [155]. Thus, oligomerization of NEMO is essential for inducible assembly and activation of the IKK complex.

How does IKK/NEMO oligomerize following signal induction? There are two models that could explain the formation of oligomeric signaling complexes that recruit the IKK subunits and thereby facilitates IKK activation [108].

According to the first model, receptor engagement causes upstream adapter proteins like the RIPs, TRAFs, or various CARD-containing proteins to oligomerize and form higher-order structures. IKK is now recruited and binds to these adapter proteins. This causes IKK oligomerization. It has been reported that oligomerization of RIP or BCL10 and consequent binding of NEMO would recruit the IKK complex to the receptor [119].

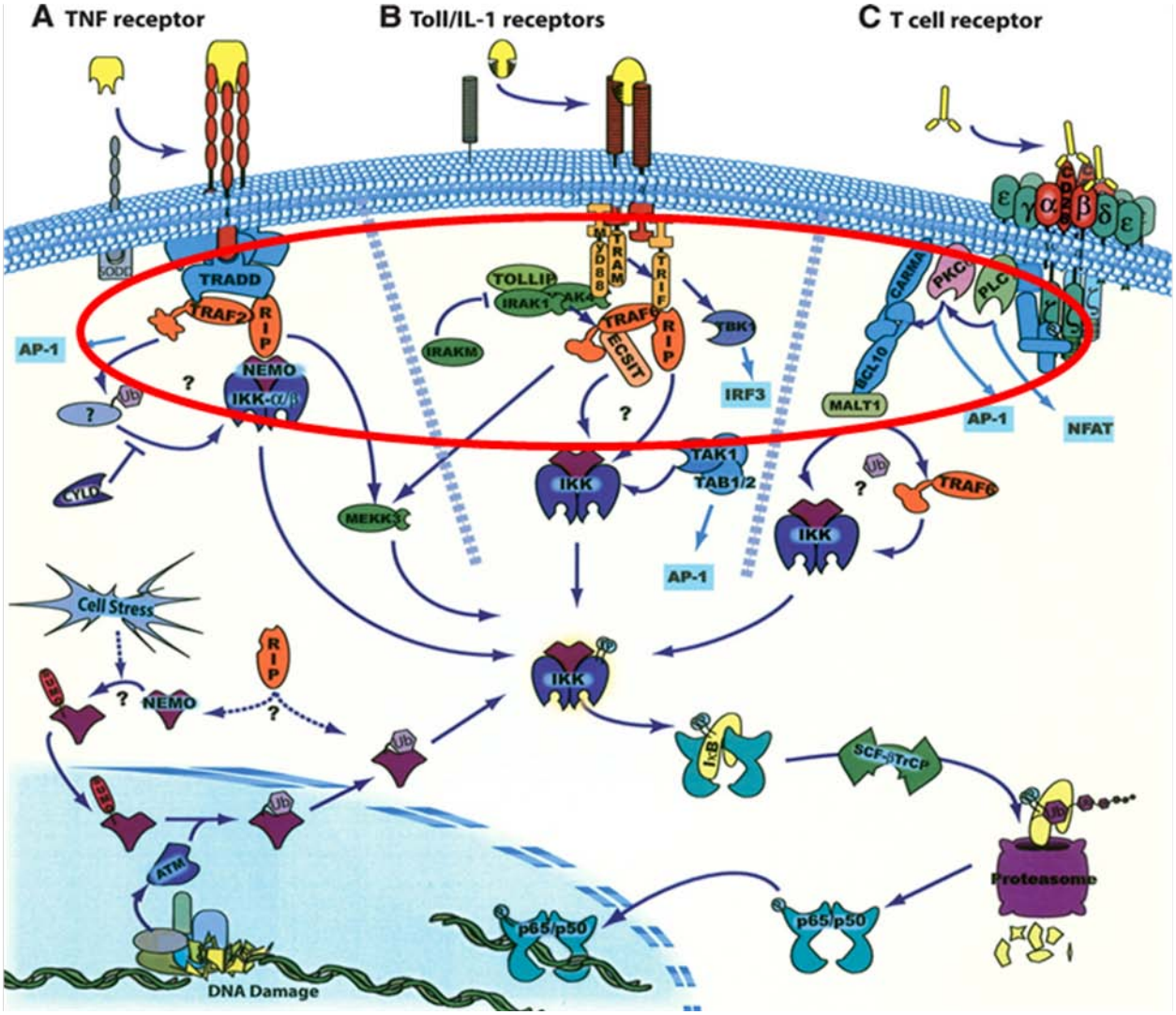


Figure 1.9: Assembly of adaptor proteins in major signaling pathways that lead to NF-κB activation (modified from [116])

According to a second model, ubiquitin chains provide the oligomeric platform and recruit the TAK1 and/or IKK complexes, thereby activating them. There are two conceivable ways in which these ubiquitin chains may act.

The ubiquitin chains may provide the oligomeric platform for another kinase to phosphorylate the proximal IKK or the chains may assist proximity-induced *trans*-autophosphorylation. Alternatively, ubiquitin chains may directly activate the IKK complex. The nature of the linkage of these ubiquitin chains has been proposed to be quite diverse. They may be K63-linked or linear. Traditionally, the ubiqutitin chains have been known to be attached to a substrate. However, in addition to these traditional ubiquitin chains, free ubiquitin chains (K63 linked) not attached to any substrate have also been proposed to activate the IKK complex. Interestingly, while K63 linked polyubiquitin chains have been proposed to play a non-degradative role in NF-κB activation, *in vitro* experiments reveal that proteins modified with K63-linked polyubiquitin chains are actually degraded by the proteasome [156]. However, quantitative mass-spectrometry suggests that following proteasomal inhibition, K48-linked but not K63-linked polyubiquitin chains accumulate in yeast cells [157].

In fact, one area in the NF-κB signaling field that has attracted much attention recently is the area of 'regulatory' ubiquitination (or signaling mediated by K63-lined and linear ubiquitin chains). Initial work showed that IKK could be activated by polyubiquitination via a proteasome-independent mechanism (thus, these polyubiquitin chains were not K48-linked) [76]. However, this publication was met with considerable skepticism as it was difficult to appreciate how these unique polyubiquitin chains would respond to a physiological stimulus. Besides, the claim was based only on *in vitro* biochemical experiments [158], and it was not clear if E2 or E3 enzymes existed that could make non-K48-linked ubiquitin chains. Then, however, TRAF6 was characterized as an E3 ligase while Ubc13/Uev1A was identified to be the E2 complex responsible for catalyzing the formation of K63-linked ubiquitin chains and inducing IKK activation *in vitro* [159]. The conclusions were partly supported when a homologous

Ubc13/Uev1A E2 complex was found in yeast and was shown to be involved in DNA repair by catalyzing the formation of K63 polyubiquitin chains [160]. This jump-started the work in the field of regulatory ubiquitination. Interestingly though, mice deficient in TRAF6 develop osteoporosis, but do not show any drastic phenotype as would be expected of mice lacking any of the crucial components of the NF-κB signaling pathway [161, 162].

Thus, mechanistically speaking IKK could be activated either by oligomerization of adapter proteins or by ubiquitin scaffolds. However, these two mechanisms may not be mutually exclusive. Indeed, proteins that play a role in assembling the signaling complexes (as adaptors) may also get ubiquitinated (like RIP1 or TRAFs). This makes it difficult to ascertain whether the ubiquitin-independent adaptor property of these proteins or the ubiquitin chains on the adaptor proteins is responsible for downstream IKK activation. Examples of adaptor proteins that get ubiquitinated include RIP1, NEMO and TRAF6, as discussed below.

RIP1 oligomerization leads to inducible interaction with NEMO and subsequent activation of the IKK complex [119]. However, RIP1 is also ubiquitinated at Lys-377 after TNFα stimulation and it has been proposed that it is through these ubiquitin chains that RIP1 binds to NEMO, thus recruiting IKK to the receptor complex (via the ubiquitin binding domain of NEMO) [163], [164]. The authors show that the K377R mutant cannot be ubiquitinated and prevents the recruitment of IKK complex to the TNF receptor. They claim that this observation demonstrates the importance of ubiquitin chains in the recruitment process. Their conclusion is however compromised by the fact that the same report reveals that K377R-RIP1 is structurally altered and cannot itself be recruited to the receptor.

NEMO oligomerization can lead to NF-κB activation [150], [151], [152]. NEMO has also been reported to be ubiquitinated in several pathways at K399 as well. However, mutation of this site did not lead to complete NF-κB inactivation [165]. Additionally, a knock-in mouse containing a mutation at this site was found to be largely normal [166]. NEMO also contains a ubiquitin-binding domain and could interact with ubiquitin chains [129]. However, it is difficult to accurately determine the domain required for interaction as the NEMO oligomerization domain overlaps with the NEMO K63-ubiquitin-binding domain (UBD).

Another protein whose autoubiquitination might lead to the activation of a possible IKK-activating kinase (TAK1) is TRAF6. TAK1 has, in turn, been proposed to be activated by TRAF6 catalyzed K63-linked ubiquitin chains [167]. TAK1 exists in a complex with TAB1 and TAB2. TAB2 and TAB3 have ubiquitin binding domains (UBD), which bind the K63 chains. An elegant mechanism was proposed in which the TAK1-TAB2-TAB3 complex bound to K63-polyubiquitin chains via the UBD of TAB2 and TAB3, resulting in activation of TAK1. TAK1 subsequently phosphorylated IKK in the two serines in the activation loop, resulting in IKK activation. Another report by the same group subsequently showed that TAK1 could also be activated by unanchored K63 linked polyubiquitin chains (these chains are not linked to any substrate but are free) [168]. From their findings, the authors proposed that K63 ubiquitin chains directly [169] regulate NF-κB activation. However, their model is not substantiated by genetic evidence. Analysis of mice deficient in TAK1, TAB2 and TAB3 shows that while TAK1 plays an essential role *in vivo*, TAB2 and TAB3 are dispensable. While TAK1 knockouts exhibit embryonic lethalality, TAB2 knockouts and TAB3 knockouts show normal NF-κB activation [169]. It is important to note that the proposed dependence on regulatory ubiquitination comes from the binding of TAB2/TAB3 to the K63 liked ubiquitin chains. This binding is important as

TAK1 does not itself have a UBD. In theory, the data with the TAB2 and TAB3 knockouts suggests that TAK1 could also be activated by recruitment to the receptor complex by various adaptors proteins; such an alternative model would preclude the requirement of the K63 ubiquitin chains. Of course, it is possible that the TAB2 is compensating for TAB3 and vice versa, but double knockouts of TAB2 and TAB3 have not yet been reported.

Many other components of the NF-κB signaling pathway have been showed to be modified by K63 linked ubiquitin chains [170]. However, what is not understood is the role these chains play in signaling. One issue with the theory of regulatory ubiquitination is with the delayed kinetics of ubiquitination, and the consistent observation that regulatory ubiquitination only targets a minuscule fraction of any protein. Thus, the hypothesis is that regulatory ubiquitination may result from aggregation of substrates with adaptors exhibiting E3 ligase activity. There are some examples of proteins that are ubiquitinated in a robust manner. But even for these substrates like IRAK1 and MALT1, it is not clear if the regulatory ubiquitination actually precedes IKK activation and IκBα degradation, as would be expected if the K63-linked ubiquitination is indeed "regulatory" [171, 172]. Since it is not clear if these substrates actually get ubiquitinated before IKK activation, there is skepticism as to whether the K63-linked ubiquitin chains are indeed necessary for IKK activation.

Thus, it has been rather challenging to understand the importance of "regulatory ubiquitination" in activating NF-κB. The central problem has been the lack of genetic data. A broad aim of my theses is trying to decipher the relevance of K63-linked regulatory ubiquitin chains in activation of NF-κB.

1.8 Negative regulation of NF-KB signaling

Most of the pathogen-sensing receptors of the innate immune system activate NF-κB and both the innate and adaptive immune system is critically dependent on NF-κB. The importance of this transcription factor can be gauged from the range of receptors that engage the pathway (receptors for proinflammatory cytokines and PAMPS, antigen receptors on T cells and B cells). Thus, the prompt activation of this transcription factor is essential for host defense. Activated NF-κB translocates to the nucleus and induces the transcription of genes responsible for survival, differentiation and proliferation of immune cells, as well as proinflammatory cytokines that provide the initial protective response against pathogens. However, since the pathway leads to the expression of multiple inflammatory genes, it is critical that the activation is transient and tightly controlled after the initial protective response, as persistent inflammation can lead to tissue damage and other autoimmune diseases. Indeed persistent NF-κB activation is one of the main causes for chronic inflammation and cancer [173], [174], [175]. Hence, multiple negative regulatory systems have evolved at different molecular levels to attenuate and terminate signaling [69], [176].

The mammalian NF-κB family consists of several members that have the transactivation domain (TAD) and are thus competent to drive transcription on their own –RelA (p65), RelB and c-Rel proteins. The family also includes the transcriptionally inactive members like NF-κB1 subunit (p50 derived from p105 precursor) and NF-κB2 subunit (p52 derived from p100 precursor) [25]. p50 and p52 need to form heterodimers with the TAD containing members to be transcriptionally active. The subunits form various homodimers and heterodimers. It is critical that NF-κB is inactive under resting conditions, otherwise the host would be overwhelmed with

inflammatory cytokines which have the potential to cause unlimited tissue damage and even death [17].

One method of negative regulation is the synthesis of more inhibitory IκB proteins. Alternatively, since the pathway converges on IKK, turning off upstream signaling molecules that activate IKK is another method of negative regulation of the pathway [116, 177]. As shown in **Figure 1.10**, activation of NF-κB induces expression of negative regulators IκBα and A20. The newly synthesized IκBα protein bind to the NF-κB dimers in the nucleus and shuttle them back into the cytoplasm. The newly synthesized A20 protein act upstream of IKK by disassembling the IKK-activating complexes.

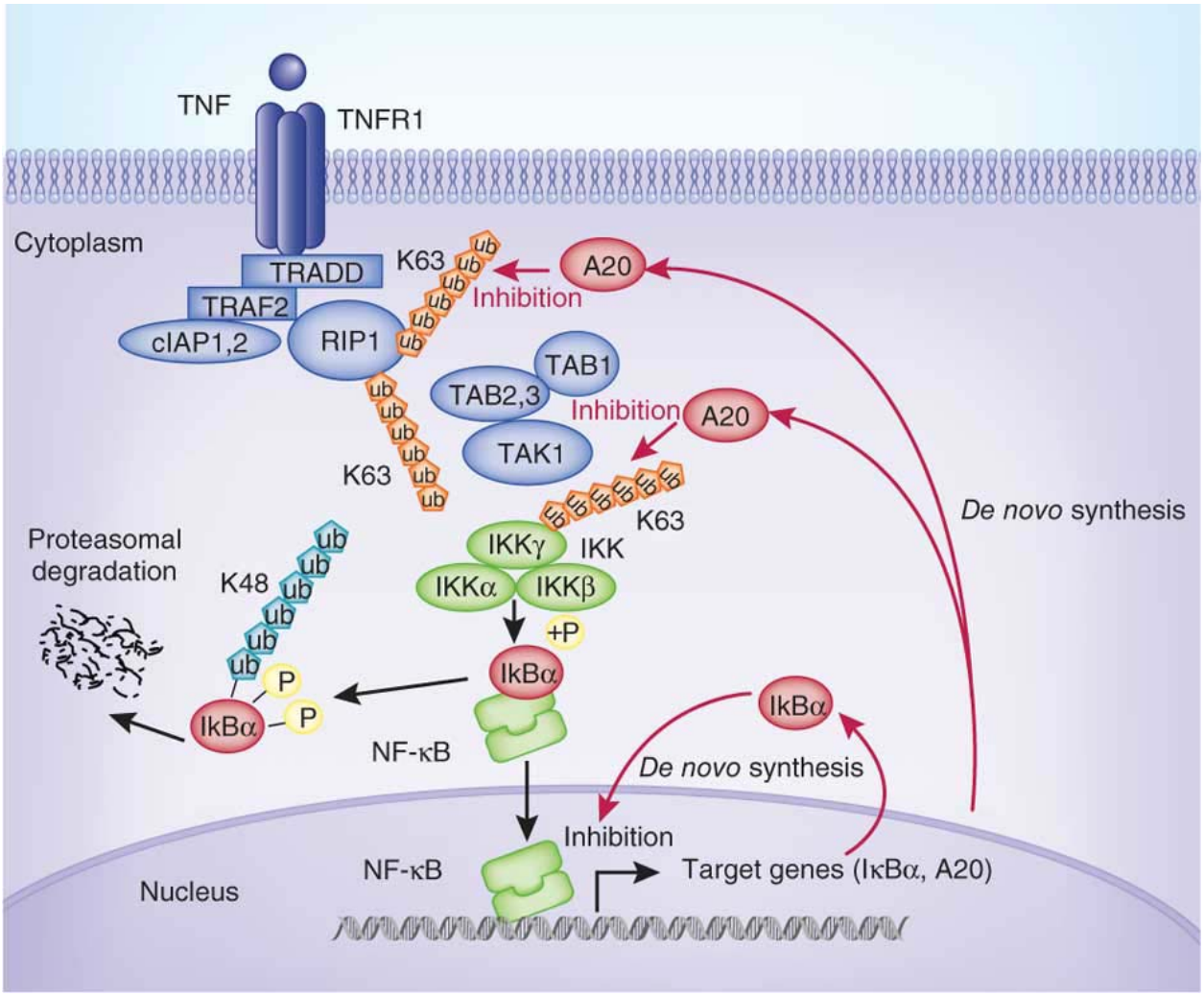


Figure 1.10: Inhibition of canonical activation of NF-κB by IκBα and A20 [176]

1.9: Inhibition of NF-κB activity by classical IκB proteins

An overwhelming amount of data points to the biological significance of IκBs as the main negative regulators in NF-κB signaling.

Upon stimulation, a cascade of events activates IKK, which then phosphorylates the inhibitory IκB proteins (IκBα, IκBβ and IκBε) at specific serine residues. The IκBs are now

proteasomally degraded. Interestingly, though the three IκBs are degraded with distinct kinetics. Moreover, their transcriptional regulation by NF-κB following degradation is substantially different [37], [60]. IκBα is rapidly degraded while IκBε undergoes the slowest degradation [37], [60]. Unlike IκBβ, genes encoding for IκBα and IκBε are under the direct control of NF-κB, enabling the two to function as negative feedback inhibitors [50], [178], [179].

Activated NF-κB induces the expression of IκBα and IκBε. The newly synthesized IκBα goes to the nucleus and associates with the NF-κB dimers which are located in the nucleus [180]. IκBα has a nuclear-export sequence, hence the IκB-NF-κB translocates from the nucleus to the cytoplasm. This constitutes a classic negative feedback loop. IκBε production is also induced by NF-κB but the expression of IκBε expression is significantly delayed as compared to IκBα expression [179]. The newly synthesized IκBε seems to dampen IκBα-driven NF-κB oscillations, as the induction of its mRNA occurs out of phase with that of IκBα

Deletion of the main inhibitor of NF-κB (IκBα) expectedly results in early lethality 7-10 days after birth. The cause of death is hyperinflammation, highlighting the importance of NF-κB in the expression of inflammatory genes [73]. Further evidence of IκBα inhibition is provided by the observation that mice which have a mutated IκBα promoter (mutated κB enhancers of the IκBα gene) and consequently produce less IκBα, have a shortened life span (13-15 months), are more sensitive to LPS-mediated septic shock and exhibit abnormal development and activation of T-cells [181]. While deletion of IκBε does not result in lethality, it does result in the increased production of cytokines [182]. Double knockouts of IκBα and IκBexhibit a more drastic phenotype than the single knockouts alone. Deficiency of both inhibitors causes neonatal lethality. B and T cells are completely absent and the number of NK cells is significantly

reduced [183]. Thus, the host has evolved these two inhibitors with overlapping functions. This is also proven by the upregulation of IκBε in IκBα -deficient cells. This upregulation disappears upon IκBα reconstitution, thereby demonstrating that a certain amount of these two inhibitors is biologically ideal [184].

Traditionally, the IκB family of proteins has been thought of as sequestering-agents of various NF-κB complexes in the cytoplasm [185]. However, it is now clear that the functions of the individual IκBs do not quite fit into this simplistic criterion. It is probably better to think of the IκB family of proteins as chaperones/cofactors of NF-κB, associating with and stabilizing the NF-κB dimers both in the cytoplasm and nucleus. The binding of IκB to DNA-bound NF-κB potentially influences the recruitment of other coactivators and the resulting transcriptional response. Thus, association of IκB with NF-κB dimers may lead to an increase or decrease of transcription, depending on the context.

An example of an IκB that serves to both activate and inhibit inflammatory gene expression is IκBβ [186]. While IκB generally inhibits NF-κB activation, IκBβ has been found to prolong the expression of certain genes like TNFα or interleukin 1β (IL-1β) [186, 187] as it serves as an essential cofactor for the expression of these genes. It might be counterintuitive to think that the same proteins can perform two opposite functions. However, this is possible as IκBβ exists in two forms that perform opposite functions: a basal phosphorylated form and an hypophosphorylated form that appears upon stimulation.

When cells are stimulated with LPS, the basally phosphorylated IκBβ is slowly degraded (as compared to IκBα). After this, IκBβ is resynthesized as a hypophosphorylated form and can be found in the nucleus [34, 35, 188, 189] with p65-cRel dimers. Thus unlike IκBα, which can

dislodge the NF-κB dimers from the DNA, IκBβ cannot promote the spontaneous dissociation of NF-κB dimers from DNA [190]. The two inhibitors are structurally different as IκBβ does not have a NES. Hence, the IκBβ-p65-cRel trimeric complex does not readily export to the cytoplasm. Additionally, the hypophosphorylated form does not mask the NLS of p65. Hence, the trimeric complex is found bound to the DNA [35, 188, 191]. This binding of the hypophosphorylated form of IκBβ to the DNA in association with NF-κB dimers is also supported by the crystal structure of IκBβ bound to homodimers of p65 [192]. In contrast, the phosphorylated IκBβ can mask the p65 NLS, thereby inhibiting binding to DNA under basal conditions [35, 188, 191].

The NF-κB:IκBβ complexes bound to the DNA are stable and resistant to newly synthesized IκBα (and IκBε). Hence, it was hypothesized that nuclear, hypophosphorylated IκBβ may actually be a transcriptional activator of specific genes [35]. This was directly in contrast to the traditional thinking of IκBs only role as cytoplasmic inhibitors of NF-κB.

After nearly a decade, the genetic evidence has substantiated the hypothesis. The IκBβ knockout (made in our lab) indeed shows that IκBβ has distinct functions in the cytoplasm and nucleus [186, 187]. In the cytoplasm, phosphorylated IκBβ acts as a traditional NF-κB inhibitor sequestering p65-cRel heterodimers. However, upon stimulation the cytoplasmic IκBβ is degraded and the heterodimers translocate to the nucleus. Hypophosphorylated IκBβ is newly synthesized and found in the nucleus bound to the p65-c-Rel heterodimers at specific κB promoter sites, accentuating expression of certain proinflammatory genes like TNF [186] and IL-1β [187]. Mice deficient in IκBβ consequently express less TNFα (or IL-1β), rendering them resistant to LPS-induced septic shock and collagen-induced arthritis.

Thus, both genetic and biochemical experiments have demonstrated that IκBβ behaves substantially differently as compared to the other IκB inhibitors.

1.10: Inhibition of NF-κB activity by deubiquitinases upstream of IKK

A plethora of biochemical work, in conjunction with mass-spectrometry, has demonstrated that multiple adaptors upstream of IKK are decorated with K63 linked polyubiquitin chains. Hence, it has been suggested that K63 linked polyubiquitin chains are essential for the activation of NF-κB. The proposition holds that ligand binding to different receptors leads to the recruitment of E3 ligases that polymerize nondegradative K63-linked polyubiquitin chains on specific adaptors [25, 125]. This leads to the activation of IKK in multiple pathways.

For example, stimulation of the TNF receptor results in recruitment of the TRADD adaptor protein, and the consequent assembly of a signalosome consisting of RIP1 and multiple E3 ubiquitin ligases like cIAP1, cIAP2 and TRAF2. This leads to the polyubiquitination of RIP1 by K63-linked polyubiquitin chains [193]. These polyubiquitin chains on RIP1 might play a role in recruiting kinases like TAK1 and IKK (via the UBD of NEMO) [163], [164].

It has been conclusively demonstrated that activation of other receptors also recruits intermediates that have E3 ligase activity. For example, TLRs recognize PAMPs and activate IKK by using various intermediates including MyD88, TRIF, TAK1 and E3 ligases like TRAF6 and IRAK1 [194]. TCR mediated signaling, on the other hand, proceeds via the recruitment of adaptor proteins like CARD11, Bcl-10, MALT1, TAK1 along with E3 ligases like TRAF2 and

TRAF6 [195]. However there is no genetic evidence to show that the ligase activity is integral to NF-κB activation.

Ubiquitination is a reversible, post-translational modification, thus deubiquitinases could disassemble the ubiquitin chains and potentially downregulate NF-κB activation. Indeed, deubiquitinases (DUBs) have been shown to be important for the downregulation of NF-κB [120]. It is important to note that by a process known as 'ubiquitin editing', DUBs can potentially collaborate with E3 ligases to initially inactivate and then degrade essential mediators of IKK activation. Thus, the DUB could initially deubiquitinate the K63 linked polyubiquitin chains, thereby disassembling the ubiquitin scaffold that organizes the signaling complex. After this, the ligase could add K48 linked polyubiquitin chains to the same protein thereby targeting it for proteasomal degradation [196], [197], [198].

As shown in **Figure 1.11**, activation of various pathways leads to recruitment of signaling complexes possibly coordinated by the K63-linked polyubiquitination of IRAK1, RIP1 and TRAF6 (grey circles). Linear ubiquitin chains may also be formed by the action of the heterodimeric LUBAC complex (linear ubiquitin chain assembly complex; a ubiquitin ligase that catalyzes formation of linear ubiquitin chains) in response to TNF-mediated signaling (tan circles). Free, unanchored Lys63-linked polyubiquitin chains may also directly activate IKK. A20 may attenuate the signaling by dislodging these activating ubiquitin chains upstream of IKK.

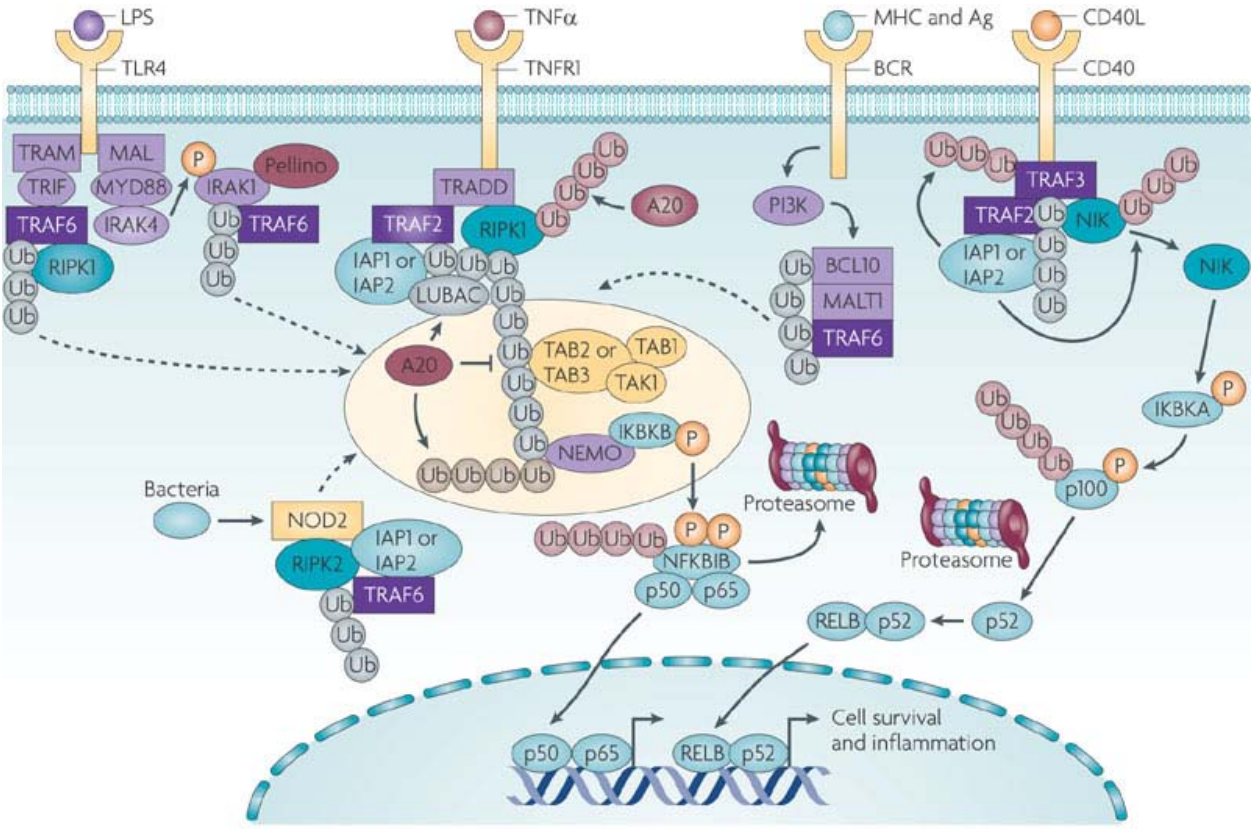


Figure 1.11: Activation of NF-κB by regulatory ubiquitination and inhibition by deubiquitinases [199]

Three common deubiquitinases are A20, Cezanne and CYLD, and mutations or deletions of these genes lead to inflammation or tumorigenesis, as would be expected if these were to function as NF-κB inhibitors. However, as with the ligases, there is no evidence that the deubiquitinase activity of these enzymes is actually responsible for downregulating NF-κB.

1) A20 (TNFAIP3): The best known deubiquitinase is A20. A20-knockouts die from unrestrained inflammation [134], [200]. Multiple autoimmune diseases like systemic lupus erythematosis are associated with polymorphisms in the A20 locus **(Table 1.1** and **Figure 1.12)**.

A20 also acts as a tumor suppressor in B-cell lymphoma. Recent evidence also implicates dysfunction of A20 as a risk factor for multiple autoimmune diseases [135], [136], [139], [138],[201]. Thus, A20 is essential for terminating inflammatory responses. Hence, it is very important to understand the mechanism by which A20 functions. A20 is virtually absent under resting conditions and is then rapidly induced by NF-κB [202]. In fact, A20 is also called TNFAIP3 or 'TNFα induced protein 3' as it was initially identified following induction with TNFα [203]. Expression of A20 mRNA is under the direct control of NF-κB (much like IκBα). Thus upon stimulation, A20 rapidly accumulates and negatively regulates NF-κB by potentially deubiquitinating K63 chains upstream of IKK. Much like IκBα, this constitutes a classic negative feedback loop.

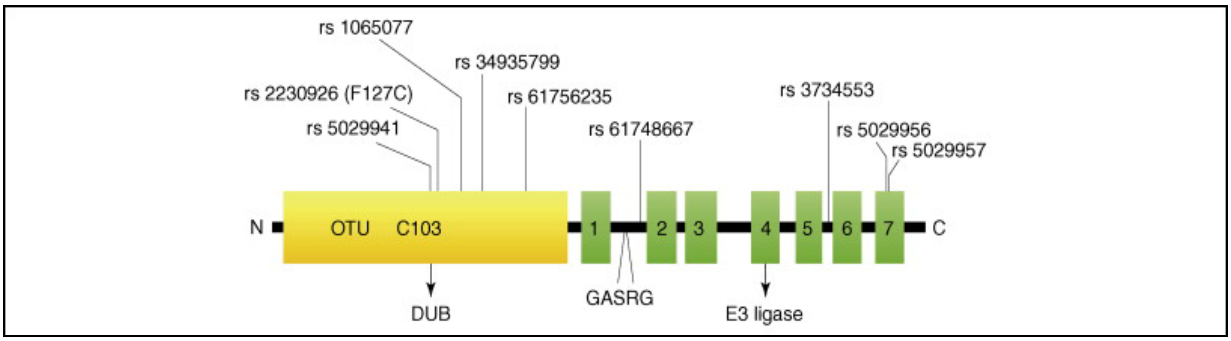


Figure 1.12: Location of SNPs in A20 protein [201]

Disease	**Mutation in *A20* or SNPs identified**
Atherosclerosis in mice	E627A (mouse)
Diabetes	rs 5029930
	rs 610604
Crohn's disease	rs 7753394
Coeliac disease	rs 2327832
Rheumatoid arthritis	rs 10499194/rs 13207033
	rs 6920220
	rs 5029937
Systemic lupus erythematosus	rs 5029939
	rs 10499197
	rs 7749323
	rs 13192841
	F127C (rs 2230926)
	rs 6922466
Type 1 diabetes	rs 10499194
	rs 6920220
Psoriasis	rs 610604

Table 1.1: Polymorphisms of A20 associated with autoimmune diseases. [201]

A20 has a DUB domain followed by a number of C2-C2 zinc-finger domains and the fourth zinc-finger domain (ZF4) is known to have E3 ubiquitin ligase activity [196], [204]. This should, in theory, allow dual ubiquitin-editing function, i.e, A20 should be able to act both as a ligase and a deubiquitinase. It has been biochemically demonstrated that upon stimulation with TNFα, A20 accumulates and disassembles the K63-linked polyubiquitin chains from RIP1, attenuating NF-κB signaling (the deubiquitinase function is mediated by Cys103 in the OTU domain). In the next step, the E3 ligase domain adds degradative K48-linked polyubiquitination chains to RIP1, leading to proteasomal degradation of RIP1 and terminating NF-κB signaling

[196]. Incidentally, the DUB activity of A20 is not specific to RIP1 as A20 generally deubiquitinates other substrates upstream of IKK. These include TRAF6 [200], NEMO [205], RIP2 [206] and MALT1 [207] **(Figure 1.13)**.

The deubiquitinase activity of A20 has been proposed to be essential in downregulating NF-κB on the basis of *in vitro* work. However, certain critical biochemical data are also difficult to reconcile with this. For example, overexpression of a deubiquitinase mutant of A20 has been shown to inhibit NF-κB [204], [208]. It is also paradoxical in the light of the proposed mechanism that the deubiquitinase activity has been found to be non-specific for K63 chains. Indeed A20 has been shown to only weakly deubiquitinate K63 polyubiquitin chains, while it robustly disassembles K48 polyubiquitin chains [209], [210].

Recently, the crystal structure of the OTU domain of A20 has been solved [209], [210] and it is not clear how A20 would distinguish between K48 and K63-linked chains. A20 reacts with polyubiquitinated TRAF6 to yield polyubiquitin chains but not monoubiquitin linkages [210]. This shows that A20 might derive specificity from directly interacting with TRAF6. However, A20 does not cleave monoubiquitinated TRAF6. Thus understanding the mechanism of action and specificity of A20 will require additional studies.

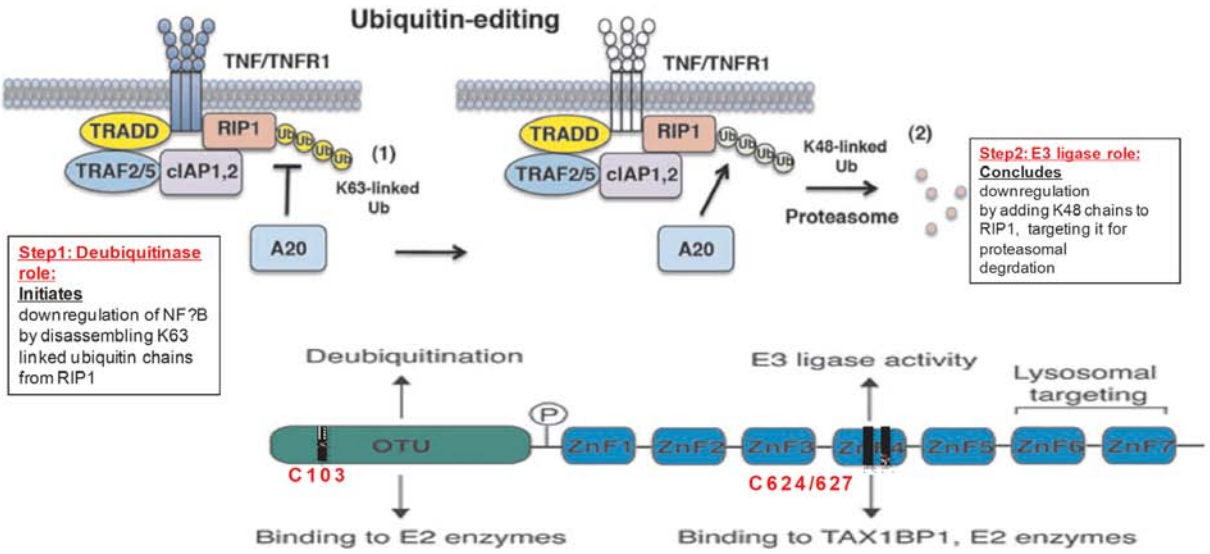


Figure 1.13: Mechanism of action of A20 [211] (modified)

2) Cezanne: Along with A20, there is another cysteine-protease DUB called Cezanne (cellular zinc finger anti-NF-κB) which also attenuates NF-κB signaling by deubiquitinating K63-linked chains from RIP1 [212]. Like A20, the expression of Cezanne increases after TNF-α stimulation. The deubiquitinase action of Cezanne may be biochemically important as the catalytically inactive mutant cannot inhibit NF-κB activation. However Cezanne is not specific to K63 chains and preferentially deubiquitinates K11-linked polyubiquitin chain [213]. Thus, the biological significance of the deubiquitinating activity of Cezanne is unclear.

3) CYLD: The third well known deubiquitinase is CYLD (cylindromatosis gene). The deubiquitinase action of CYLD is mediated by the conserved Cys601 and mutating this residue leads to persistent NF-κB signaling [214]. It has been biochemically established that CYLD negatively regulates NF-κB [215], [214], [140]. However, CYLD is a promiscuous

deubiquitinase as it removes ubiquitin chains from multiple substrates including RIP1, TRAF2, TRAF6, TAK1 and IKKγ [216], [217], [140], [214], [218]. In this regard, CYLD lacks specificity and cleaves both K63-linked polyubiquitin chains as well as linear ubiquitin chains [219], [220]. It can also cleave K48-linked chains from adaptor Lck in T cells [221].

The expression of CYLD is not directly induced by NF-κB (unlike A20 and Cezanne) [222]. Thus, CYLD is present at basal levels and might play a role in suppressing NF-κB activation in resting cells. In contrast, A20 is expressed at low levels under basal conditions and rapidly induced in response to proinflammatory stimuli to downregulate excessive NF-κB activation. This may be the reason why the two DUBs do not compensate for each other as their mechanisms of action are temporally distinct. The activity of CYLD has to be depressed for the cells to become activated. Hence, its activity is controlled by subcellular localization in addition to phosphorylation on multiple serine residues by IKK (in a NEMO dependent manner) [223], [224].

Since CYLD deubiquitinates multiple upstream activators of IKK, the expectation was that the CYLD knockout would show a drastic phenotype. Accordingly, multiple groups embarked on the project and mice deficient in CYLD were generated independently by various groups. Contrary to expectations, *Cyld*$^{-/-}$ mice show no obvious defect in growth or survival [221]. However, they do develop some defects in the immune system with age. Additionally, the knockouts generated by different groups sometimes have conflicting phenotypes. In general, these genetic knockouts show that CYLD plays a role in immune cell development, osteoclastogenesis and spermatogenesis. Thus, *Cyld*$^{-/-}$ mice made by one group have reduced numbers of CD4(+) and CD8(+) single-positive T cells in the thymus and periphery [221].

Cyld$^{-/-}$ mice spontaneously develop inflammation in the colon[218], osteoporosis [225], B cell hyperplasia and lymphoid organ enlargement [226]. Studies also show that CYLD plays an important role for survival of immature NKT cells [227] and spermatogenesis [216]. However conflicting genetic data from another group showed that development of T cells and myeloid cells was normal in CYLD-knockouts [217]. A third group showed that Cyld deficient mice are more prone to skin tumors [223]. Mice expressing a naturally occurring variant of CYLD that did not express exon 7 and 8 (thereby lacking binding sites to TRAF2 and NEMO) were found to have enlarged spleens as a result of dramatic accumulation of mature B cells [228].

1.11: Perspectives on inhibitors of NF-κB and broad aims of this thesis

25 years of research has made it clear that appropriate down-regulation of NF-κB signaling is as important as NF-κB activation. IκB proteins are the most well documented inhibitors of NF-κB in the physiological setting, as borne out by both biochemical and genetic studies. However along with the IκBs, there is a plethora of biochemical evidence to suggest that deubiquitinases may also function as negative feedback regulators of NF-κB signaling. This thesis examines the mechanism of two inhibitors of NF-κB: (i) A20, which downregulates activation of NF-κB upstream of IKK in stimulated cells and (ii) IκBβ which downregulates NF-κB under basal conditions.

1st broad/specific aim: Examine the role of regulatory K63-linked polyubiquitin chains in NF-κB activation (specifically, I examined the role of deubiquitinase activity of A20 by generating knock-in mice selectively lacking the K63-deubiquitinase function)

While multiple adaptor proteins have been shown to be covalently linked with K63-linked polyubiquitin chains, it is not clear if such regulatory polyubiquitination is essential for activation of IKK in the physiological context. This is because of an acute lack of genetic complementation experiments. Ultimately, the importance of regulatory ubiquitination mediated by K63-linked polyubiquitin chains has to be demonstrated in knock-in mouse models (that have point mutations selectively disrupting regulatory ubiquitination). The need for this genetic evidence can be understood by studies done on NEMO. NEMO knockouts are embryonically lethal, thus demonstrating that NEMO is clearly essential for NF-κB activation [229], [70]. Biochemical experiments showed that K63-linked polyubiquitination of Lys-399 was essential for NF-κB activation [165], and it was believed for some time that this ubiquitination would play an important role in animals. However, a knock-in mouse with a point mutation at this site was found to be largely normal with respect to NF-κB activation [166]. This shows that K63-linked polyubiquitination of Lys-399 is not important for NF-κB activation.

Traditionally, putative targets that have been proposed to modulate NF-κB activation by regulatory ubiquitination were identified by overexpression of proteins mutated at specific lysines. However, mutating a positively charged residue like lysine might change the protein conformation leading to incorrect protein folding, thereby altering other functional attributes of the protein. For example, the ubiquitination of RIP1 at K377 [163], [230] in response to TNFα was demonstrated by overexpression and knock-down studies to be essential in activating IKK.

The K377R mutant is not ubiquitinated; however it also does not get recruited to the activated TNF receptor complex [163]. This may be because of altered conformations in the mutated protein. In fact, a subsequent study by the same group has shown that cells which have endogenous ubiquitin genetically replaced with K63R ubiquitin do not show impaired IKK activation in response to TNFα [231]. One lesson to draw from this discrepancy is that knock-down and overexpression studies have limitations. The level of protein depletion affects experimental outcomes in knock-down studies, while overexpression of signaling components such as ubiquitin ligases or adaptor proteins (like the TRAFs and cIAPs) may lead to promiscuous oligomerization and signaling.

In order to evaluate the effect of regulatory ubiquitination on the NF-κB pathway, we examined various genes that are involved in regulatory ubiquitination based on previously published genetic and biochemical data. Specifically, we looked for two criteria:

1) The knock-out of this gene should result in a phenotype consistent with a profound dysregulation of NF-κB signaling, thus this gene would have to be non-redundant in terms of its effects on NF-κB signaling.

2) Biochemical and *in vitro* complementation studies of the gene in cell-lines (using different methodologies) would have to clearly point to an amino-acid that is involved in regulatory ubiquitination, such that mutating this residue would disrupt regulatory ubiquitination and NF-κB signaling.

Based on these criteria, we planned to generate a knock-in mouse with a point-mutation for the identified residue of interest. If regulatory ubiquitination is indeed important for NF-κB

signaling, then this knock-in mouse would be expected to resemble the knock-out (at least partially). If not, then some other property of the protein (apart from the ability to mediate regulatory ubiquitination) would be expected to be involved in NF-κB signaling.

The candidate genes that we considered included E2 enzymes (Ubc13), E3 enzymes (like TRAFs and cIAPs), adaptor proteins that are ubiquitinated by K63-linked ubiquitin chains (like RIP1) and deubiquitinases like A20.

1) E2 enzyme Ubc13: Two groups reported that Ubc13 deficiency causes embryonic lethality; however it is not clear if this lethality results from defective NF-κB signaling [232], [233].

2) E3 ligases like TRAF and cIAP proteins: TRAF proteins are highly redundant. There are a total of seven TRAF proteins, all of which have a common C-terminal coiled-coil TRAF domain. TRAFs2-7 also contains a N-terminal RING finger domain that is involved in ligating ubiquitin chains to substrates; thereby enabling these TRAFs to act as E3 ligases. Amongst them, TRAFs2, 5, and 6 have been shown to be important adaptors for IKK activation. TRAF6 plays a role in LPS-mediated signaling, and TRAF6 knockouts develop osteoporosis [161, 162]. TRAF2 and TRAF5 act downstream of the TNF receptor. TRAF2 knockouts are born normal and do not show any major defect in NF-κB signaling [234]. This might be expected as TRAF5 may compensate for TRAF2. Indeed, TRAF5 knockouts are also relatively normal because of compensation by TRAF2 [235]. However, TRAF2/5 double-knockouts are embryonic lethal [236]. It is not clear though if the RING domain of the TRAFs is essential for NF-κB activation ([237]. Additionally, along with the E3 ligase action, the RING domain of TRAF2 is also essential for recruiting IKK to the receptor complex. Hence, it would be difficult to ascertain if

any observed signaling defect would be because of ‘RING-mediated regulatory ubiquitination’ or ‘RING-mediated adaptor function’.

Similar to the TRAFs, cIAP1 and cIAP2 are also redundant and compensate for each other. None of the single knockouts show a phenotype [238], [239], because of compensation by the other isotype. Cells deficient in both the isotypes are sensitized to apoptosis resulting from decreased activation of NF-κB [193], [240].

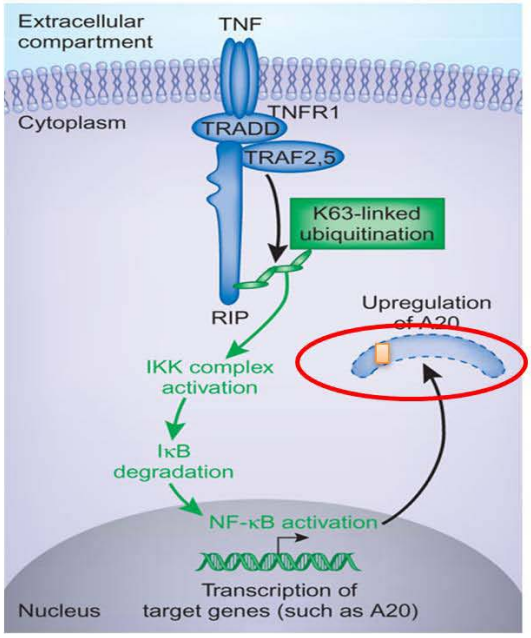
3) Adaptor proteins like RIP1: Mice deficient in RIP1 die within a couple of days after birth from tissue apoptosis as the anti-apoptotic NF-κB pathway cannot be activated in these animals [241]; thus RIP1 clearly satisfies the ‘genetic criteria’ above. Moreover, RIP1 has been shown to be polyubiquitinated by both K48 and K63 chains using linkage specific antibodies [197] and by mass-spectrometry [242], [243], [244] in response to TNF signaling (mass-spec analysis additionally shows evidence of K11 and linear ubiquitination). K377 of RIP1 was identified as the site modified with K63-linked ubiquitination; however as explained above, the mutated protein (K377R) is inactive as does not get recruited to the receptor complex. Other reports though have shown that RIP1 can also be ubiquitinated at Lys115, Lys570, Lys603 and Lys626 when overexpressed [245], and also at Lys115, Lys163–167 and Lys671 under endogenous conditions [246]. Moreover, there have been conflicting reports which suggest that RIP1 may not be needed for TNF-mediated NF-κB activation [247], [248]. Hence, we decided to forgo RIP1 as the ‘biochemical criteria’ is not clearly satisfied [249].

3) Deubiquitinase protein (A20): A20 is a deubiquitinase that downregulates NF-κB signaling and completely satisfies both the criteria outlined above. In fact, the genetic and biochemical work done on A20 suggests that K63-linked polyubiquitination may indeed play a

role in NF-κB signaling [170]. A20 knockouts die prematurely from rampant inflammation of multiple organs and cachexia as these animals are unable to downregulate persistent NF-κB signaling [134]. Hence, it is clear that A20 is non-redundant in terms of downregulating NF-κB. A20 has been extensively characterized as a protein that acts on both K48 and K63-linked polyubiquitin chains. Multiple biochemical evidences have shows that it may act by first removing K63 linked ubiquitin chains from adapter proteins such as RIP1, thereby attenuating NF-κB signaling. After this, A20 adds K48-linked polyubiquitin chains to RIP1 and targets it for proteasomal degradation, resulting in a complete block of NF-κB induced signals [196] (**Figure 1.13**). The initial deubiquitinase function is mediated by Cys103, and the subsequent E3-ligase function is mediated by Cys624/Cys627. Mutating any of these residues leads to persistent activation of NF-κB *in vitro*.

Hence, **to broadly ascertain the physiological significance of regulatory K63-linked polyubiquitination, our specific aim is to generate the C103A knock-in mouse and characterize the effects of the deubiquitinase activity of A20.** If Cys103 is indeed the catalytic residue responsible for the function of A20, then these mice would be expected to resemble the inflammatory phenotype of mice deficient in A20 (**Figure 1.14**).

A20	No deubiquitinase function



What happens in mice that have a C103A mutation?

Does it resemble the A20$^{-/-}$ mice?

Figure 1.14: Aim1: Determining physiological contribution of deubiquitinase function of A20 (modified from [250])

2nd broad aim: Examine the cause of functional differences between the structurally similar IκBα and IκBβ (specifically, determine the site of constitutive phosphorylation in IκBβ, the kinase that phosphorylates IκBβ, and the functional consequence of this phosphorylation)

While IκBα and IκBβ are structurally similar, they have different functions (**Table 1.2 and Figure 1.15**). This is partly because IκBα, but not IκBβ, is an NF-κB dependent gene. IκBα is the main inhibitor of NF-κB and suppresses inflammation by a negative feedback loop. Thus mice deficient in IκBα die within a week of their birth from hyperinflammation [73]. In contrast,

IκBβ knockouts are not only viable but are surprisingly resistant to LPS shock [186], [187]. This would not be expected in mice deficient for an inhibitor of NF-κB. It was determined from the study of the IκBβ knockouts that while IκBβ serves as a basal inhibitor of inflammation, it also enhances the inflammatory response by augmenting the expression of certain genes like TNFα [186] and IL-1β [187].

There are other differences between IκBα and IκBβ as well. IκBβ, but not IκBα, is constitutively phosphorylated in resting cells. It has been found that IκBα–NF-κB complexes shuttle between the nucleus and cytoplasm in quiescent cells while IκBβ–NF-κB complexes reside entirely in the cytoplasm [191], [251], [252]. Almost all NF-κB activating stimuli induce the rapid degradation of IκBα, which is then briskly resynthesized. Constitutively phosphorylated IκBβ on the other hand is degraded in a delayed fashion only by ligands (like LPS or IL-1β) that are known to cause persistent activation of NF-κB. IκBβ is then slowly resynthesized as an unphosphorylated protein in stimulated cells [35], [253]. The unphosphorylated IκBβ forms a complex with the p65:cRel heterodimer, and together they bind the κb2 promoter in the nucleus and enhance the transcription of genes like TNFα [186] and IL-1β [187]. This mechanism explains why knocking out the IκBβ gene makes the mice resistant to LPS-shock and collagen-induced arthritis.

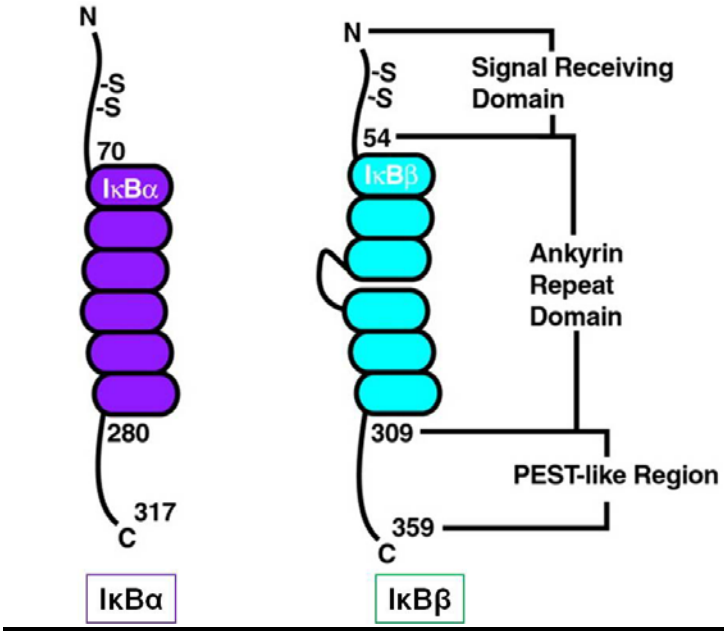


Figure 1.15: Primary structure of IκBα and IκBβ

	IκBα	**IκBβ**
Effect on inflammation	***Inhibits***	***Inhibits (and activates)***
Basal phosphorylation	***No phosphorylation***	***Constitutively phosphorylated***
Degradation Kinetics	***Rapid (15min)***	***Slow (1-2hr)***

Table 1.2: Differences between IκBα and IκBβ

We hypothesize that the functional differences between IκBα and IκBβ mainly arise from the constitutive phosphorylation of IκBβ. This allows the IκBβ to exist in two states: a phosphorylated, inhibitory form and an unphosphorylated, activating form. Hence, **to broadly understand the differences between IκBα and IκBβ, our specific aim is to discover the site**

of phosphorylation on IκBβ, the kinase that phosphorylates IκBβ, and the biological effects of phosphorylation (Figure 1.16).

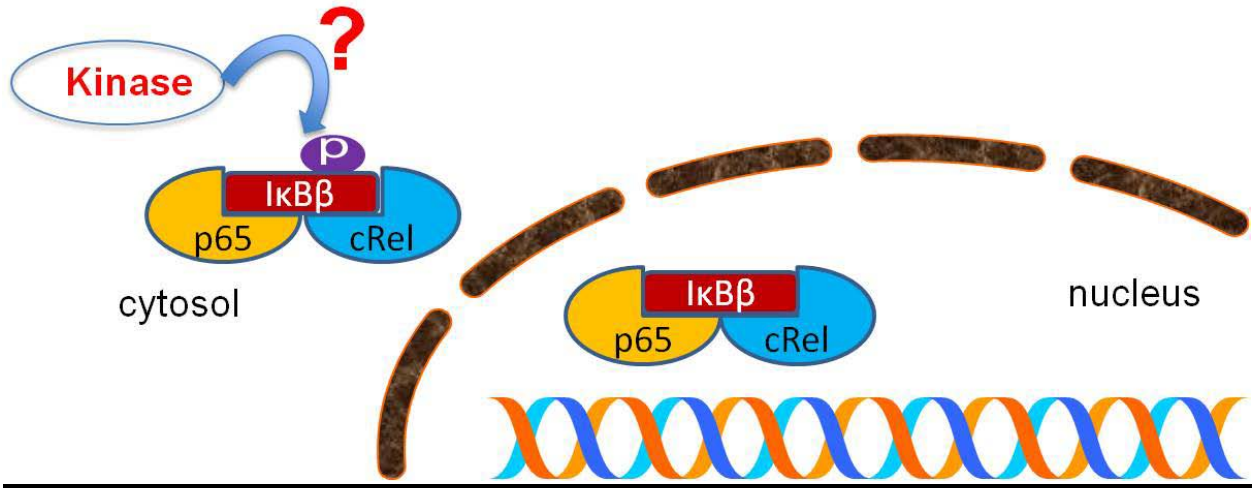


Figure 1.16: Aim2: Determining the kinase and site of phosphorylation in IκBβ

Chapter 2:

Regulation of NF-κB signaling by regulatory ubiquitination: specific pathways and A20

2.1: Regulatory ubiquitination and NF-κB

Given the importance of IKK in phosphorylating IκBs, it is imperative to understand how IKK is activated. There is no doubt that degradative ubiquitination of the IκBs (and other molecules) plays a profound role in NF-κB activation. One of the first clues that unconventional ubiquitin chains may also have a role to play in activating IKK came when it was found that IKK was activated by an E2 of the Ubc4/5 family [254], and that this activation was independent of proteasomal activity and the K48 residue of ubiquitin[76]. While this suggested the involvement of a proteasome-independent mechanism, the cellular relevance of this finding was unclear as the specific E3 ligase had not yet been identified. It was later determined that the ubiquitin ligase was TRAF6 [159], a protein that also functions as an adaptor molecule in the IL-1 and Toll-like receptor pathways [255], [256].

Subsequently though (and as detailed below), it has been shown that autoubiquitination of TRAF6 is not required for activation of NF-κB and MAPK pathways [237], [257]. While it is theoretically possible that the ubiquitin chains may provide a docking site for formation of the TAK-TAB signaling complex, it was shown using a TRAF6-RING mutant that ubiquitination was dispensable for the recruitment of the TAK-TAB complex. Thus, the activity ofTRAF6 as an adaptor protein (independent of ubiquitination) is enough to recruit the complex. The authors concluded that while TRAF6 ubiquitination may be a marker for activation, it is unlikely to play an essential role for downstream activation. Recent findings, mostly *in vitro* work, have advanced the proposition that K63-linked polyubiquitin chains may have a role to play in activating IKK in multiple pathways that activate NF-κB [123], [120].

The physiological targets of K63-linked polyubiquitination have not yet been identified [121]. The physiological context of regulatory ubiquitination can be clarified by generation of RING-mutant or deubiquitinating-mutant knock-in mice [120], something that has not been done for any of the enzymes involved in these pathways. In spite of the overwhelming biochemical evidence, genetic evidence that points to the specific role of regulatory ubiquitination is lacking. This is partly because all the experiments have been done either by knocking-down or overexpressing the relevant proteins. This has been ineffective as it could lead to oligomerization of TRAFs, and/or, induce promiscuity in these enzymes.

An example of this limitation is highlighted by knock-in studies in NEMO. Polyubiquitination of K399 in NEMO was shown to be essential for NF-κB activation by an experiment based on overexpression with BCL10. The K399R mutant was not ubiquitinated and NF-κB was only partially activated in NEMO deficient cell lines complemented with K399R [165]. However a murine knock-in model carrying this mutation was found to be largely normal with respect to NF-κB and MAPK activation [166]. While outlining the various signaling pathways below, I will be highlighting such discrepancies in order to have an accurate understanding of the present status of the field.

2.2: IL-1R/TLR signaling pathways

TLR (Toll like receptors) are PRRs that recognize various PAMPs that are present in different microbes (bacteria and viruses) including bacterial LPS and viral nucleic acids. Interluekin-1 (IL-1) is a family of cytokines secreted by cells during the inflammatory process and this serves as a defensive response during pathogenic invasion [258]. Upon ligand binding, both pathways activate subsets of pro-inflammatory genes. Both TLR and interleukin-1 receptor

(IL1R) are transmembrane proteins that have a common cytosolic domain called Toll-IL-1R (TIR) domain. Following stimulation, this domain helps recruit TIR-domain adaptor proteins that can bind to the TIR domain of TLR and IL1R. The most common example of such an adaptor is the myeloid differentiation primary gene 88 (MyD88). MyD88 then recruits two kinases to the IL1R, IRAK4 and IRAK1. IRAK1 binds TRAF6, which together with Ubc13/Uev1A catalyzes the formation of K63-linked polyubiquitin chains. Both IRAK1 and TRAF6 have been reported to be modified by K63-linked chains. The polyubiquitin chains on TRAF6 recruits the TAK1-TAB2-TAB3 complex (a protein complex of TGF-β activated kinase 1 with adaptor proteins TAB1 and TAB2) [259], [167]. The complex binds the ubiquitin chains with the UBD of the TAB proteins. This recruitment of the TAK-TAB complex leads to the activation of TAK1 [260]. Subsequently, TAK1 phosphorylates other downstream kinases like IKK (leading to NF-κB activation) or MKKs (leading to the JNK and p38 activation).

Similar pathways are also activated in binding of TLR ligands. As an example, let us consider the binding of LPS to TLR4 as the mechanistic details have been confirmed by multiple studies. LPS binding activates two branches downstream of TLR4, both of which lead to activation of TAK1, which subsequently phosphorylates and activates IKK. In one branch, receptor binding leads to the recruitment of TRAM and TRIF proteins to TLR4, which then recruits both TRAF6 and RIP1 to the signaling complex. In the other branch, receptor binding leads to Myd88 recruitment which then promotes the assembly of IRAK proteins, TRAF6 and TAK-TAB complexes as described above. It has been proposed almost entirely on the basis of *in vitro* evidence that K63-linked polyubiquitination of TRAF6 and RIP1 play an essential role in downstream activation of NF-κB [120].

However the genetic evidence is unsatisfactory as TAB proteins have not been found to be physiologically essential, as would be critical if the activation of TAK were indeed to depend on TAB binding to ubiquitin chains [261]. Additionally, it has been showed that Ubc13 knockouts have impaired activation of JNK and p38 kinase, but normal activation of IKK [233]. Thus NF-κB signaling is normal in UBC13 deficient B cells, bone marrow-derived macrophages, or MEFs when stimulated with IL1-β or multiple TLR ligands including LPS, bacterial lipopeptide and CpG DNA. Subsequently though, in another study, UBC13 was shown to be important in IKK activation [231, 262-264]. The reason for this inconsistency has been speculated to be incomplete deletion of Ubc13 by Cre recombinase in the first study. Indeed, titrating the Cre retroviruses and studying the effects of variable Ubc13 depletion, the authors concluded [263] that a minor amount of residual Ubc13 was enough to activate IKK. This however raises doubts on whether the K63 chains indeed play an actual role in IKK activation or if the ubiquitin chains results from aggregation of residual Ubc13 with TRAF6 during NF-κB activation. In this regard, multiple proteins that associate with TRAF6 have been showed to be ubiquitinated by K63 chains [167], [265], [266], [172], [214]. It is not clear if their ubiquitination is actually intrinsic for the act of signaling.

Along with a lack of genetic evidence, certain biochemical studies have also suggested that ubiquitination of TRAF6 may not be essential. For example, a study found that a ligase-mutant of TRAF6 (i.e, TRAF6 with RING domain deleted) [237] did not block IKK activation by IL-1β. However, this mutant failed to activate JNK and TAK1. In addition to demonstrating that the ubiquitin ligase activity of TRAF6 is not required for IKK activation, this study also suggested that TAK1 may not be the kinase for IKK, and that IKK could activate itself by autophosphorylation. A second independent study confirmed that NF-κB (and MAPK) pathways

could be activated by IL-1 and that this does not require TRAF6 autoubiquitination. Thus, complementation of TRAF6-knockout MEFs with a TRAF6 mutant that lacks lysines, and hence cannot be ubiquitinated, shows normal IKK activation. This mutant TRAF6 is physiologically active and rescues osteoclastogenesis when retrovirally transduced in TRAF6-knockout bone marrow macrophages. This may be because the lysine-deficient TRAF6 mutant could serve as an adaptor protein and interact with the TAK1-TAB1-TAB2 complex [257]. Subsequently, other conflicting studies have demonstrated the importance of the RING domain of TRAF6 in IKK activation via the IL-1 mediated pathway [263], [267], [268], [269], [265].

Additional evidence for the proposed mechanism of IKK activation was obtained by an elegant system which replaced endogenous ubiquitin with K63R mutant ubiquitin following tetracycline treatment. The authors show that in cells having the K63R ubiquitin, IKK cannot be activated by IL-1β. This demonstrates the importance of K63-linked polyubiquitin chains in mediating IKK activation. Intriguingly though, the same study also shows that IKK can still be activated normally in response to TNFα even when only K63R ubiquitin is present, suggesting that IKK may be activated by TNFα by a K63-polyubiquitin independent mechanism [231].

Recently it was demonstrated that free, unconjugated K63-polyuniquitin chains (not bound to any substrate) could also activate TAK1 [168]. The authors synthesized the K63-linked polyubiquitin chains by treatment with TRAF6 and Ubc13/Uev1A. As expected, the UBD domain of TAB2 associated with these chains, bringing the TAK1 in close proximity. Subsequently, TAK1 was activated by phosphorylation at Thr-187. Activated TAK1 then phosphorylates IKKβ, leading to activation of IKK complex in a NEMO dependent manner. It is not clear how the TAK1 would distinguish between polyubiquitin chains freely floating within

the cell and polyubiquitin chains that are attached to TRAF6 (or for that matter, any other protein). This concern for specificity seems to be a recurring issue in the case of K63-linked polyubiquitin chain mediated activation of IKK.

NF-κB activation is reversible; and the case for positive regulation by K63-linked regulatory ubiquitin chains is supported by the activity of deubiquitinases like CYLD and A20 that disassembles these chains, thereby negatively regulating NF-κB. There is much biochemical evidence, often based on the overexpression of the DUB, that shows that deubiquitinases may block the activation of IKK by cleaving K63 chains. An example is the familial cylindromatosis tumor suppressor (CYLD), which specifically removes K63 chains from TRAF6 [215, 270], [140], [214]. Another example of a deubiquitinase is A20, which prevents NF-κB activation mediated by IL-1β or LPS by preventing the interaction between TRAF6 and Ubc13 [271], [272], [273]. While knockouts of A20 and CYLD show dysregulated NF-κB activation, it has not been demonstrated by making knock-ins that their deubiquitinase function is actually responsible for NF-κB regulation [134], [221], [218].

Figure 2.1 shows how regulatory ubiquitination could potentially regulate the NF-κB pathway downstream of IL-1R/TLR.

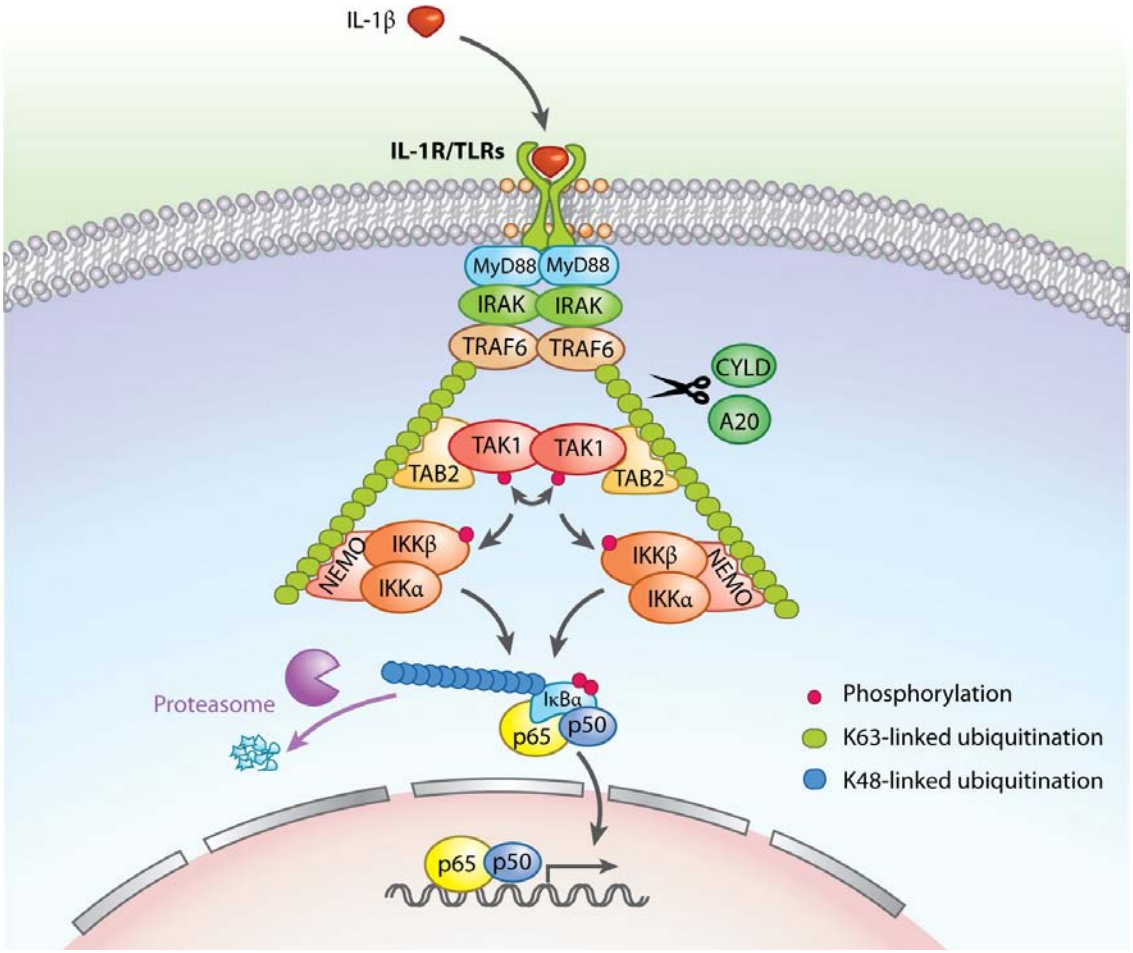


Figure 2.1: Ubiquitin-mediated activation of TAK1 and IKK in IL-1R/TLR pathways [132]

2.3: TNF receptor (TNFR) signaling pathway

The pathway is demonstrated in Figure 2.2. Discovered in 1975, tumor necrosis factor-α (TNF-α) was so named as it caused necrosis of murine tumors [274]. TNF-α also has potentially protective roles as a proinflammatory cytokine that stimulates multiple responses and activates NF-κB signaling. Dysregulation of these responses results in a whole host of inflammatory and autoimmune diseases [275].

TNF-α binds to two receptors that are responsible for mediating its functions. The receptors are TNFR1 and TNFR2. While TNFR1 is expressed ubiquitously in almost all mammalian cells, TNFR2 is expressed mostly in lymphocytes [276]. For the purposes of this discussion, we will only focus on signals emanating from the TNFR1 pathway. TNF-α exists in two forms: a membrane bound form and a soluble form. The membrane bound form is converted to the soluble form by TNFα converting enzyme (TACE). Both forms are capable of binding to the receptor. Binding of TNF-α causes trimerization of TNFR1 and consequent recruitment of TNFR1-associated death domain protein (TRADD). Remarkably, TRADD can assemble two complexes that mediate potentially opposing functions downstream. Complex1 mediates the activation of NF-κB, which has an antiapoptotic and proinflammatory role, while Complex 2 initiates apoptosis [277]. However, TNF-α normally does not cause apoptosis as NF-κB is rapidly activated with the consequent production of multiple anti-apoptotic proteins like cIAPs and c-FLIP. Promotion of the degradation of cIAPs leads to apoptosis [278], [279].

Complex I is composed of TRADD, RIP1 (or receptor-interacting protein kinase 1) and multiple RING domain containing E3 ligases like TRAF2, TRAF5, cIAP1 and cIAP2. cIAP1 and cIAP2 catalyze K63 polyubiquitination on RIP1 [231], [193, 280]. However, cIAPs are promiscuous ubiquitin ligases and ubiquitin chains added by cIAPs are not confined to K63 alone [280], [193]. Consequently, ubiquitin chains with other linkages have been found on RIP1 (detected using antibodies specific for K48 and K63 chains) [281], and this could potentially affect the specificity of the downstream pathway.

RIP1 has indeed been shown to be essential for TNF mediated NF-κB activation and RIP1 knockouts die early (1-3 days) from extensive apoptosis of lymphoid and adipose tissue

because of their inability to access the NF-κB pathway [241]. While these animals do survive longer than mice deficient in IKKβ or p65, this difference may be because of the involvement of RIP1 in NF-κB independent death-pathways like necroptosis [282], [248]. However, there are conflicting studies that have reported that RIP1 may not be universally essential in all cases of TNF-induced activation of NF-κB [247], [248], and that NF-κB can still be activated in the absence of RIP1. It is possible, though, that this residual NF-κB activity is a result of the activation of the noncanonical pathway [283].

Experiments in cell lines showed RIP1 is associated with NEMO and this association is essential for TNFα-induced IKK activation [284], [285], [248], [241]. RIP1 is a kinase and given the phenotype of RIP1 knockouts, it would be a satisfactory candidate for a kinase that could activate IKK. However, the kinase activity has been shown to be dispensable for NF-κB activation [286], [285], [241]. This provided an impetus to uncover the mechanism by which RIP1 mediates the TNFα pathway.

Since RIP1 mediates the TNF-pathway in a kinase independent manner, it is tempting to speculate that it serves as an essential adaptor molecule. NEMO can bind directly to both positive and negative regulators of the NF-κB pathway. Indeed, it has been known for some time now that RIP1 can recruit IKK to the TNFR signaling complex by directly binding with NEMO [287]. NEMO can also bind with A20, which is known to negatively regulate the TNF signaling pathway [287]. There are multiple reports that the function of RIP may be to assemble a scaffold for recruitment and activation of IKK [284], [285], [288].

Ubiquitin chains may also serve as a scaffold for recruitment of receptor complexes. RIP1 ubiquitination has been proposed to be important for downstream activation of TAK1 and

IKK. The TAK-TAB complex and IKK complex is recruited to Complex1 by the binding of TAB2 and NEMO to the polyubiquitin chains respectively [163]. It has been shown that RIP1 gets ubiquitinated at K377 and the K377R-RIP1 mutant fails to activate NF-κB [163]. However, the K377R-NEMO also does not get recruited to Complex1 upon receptor stimulation. Recruitment to the receptor is a pre-requisite for participation in signaling. Hence, while it is clear that the K377R mutant is functionally compromised, this may not be because it cannot be ubiquitinated. It could also be because the mutant cannot serve as an effective adaptor protein. RIP1 is indeed decorated by K63-linked polyubiquitin chains upon stimulation with TNFα, and this has been directly [197] verified by using a K63-specific antibody. However, it is not known if this ubiquitination is essential for the NF-κB pathway.

There are other examples in the pathway where it is unclear as to whether the adaptor function or regulatory ubiquitination is actually required for downstream singling. Both the TNFα and IL-1 mediated pathways seem to activate TAK1 and IKK. They also both use TRAF family adaptor proteins (TRAF2 and 5 for TNFα mediated pathway and TRAF6 for IL-1β mediated pathway). Given that TRAF2 and TRAF6 are structurally similar, it might be speculated that the TNF pathway could also use TRAF2 or/and TRAF5 to catalyze the ubiquitin chains, just like TRAF6 in the IL-1β pathway. Indeed TRAF2 is essential in the context of TNF-α-stimulated NF-κB activation [234], [289]. Absence of TRAF2 decreases RIP1 ubiquitination. This was seen using TRAF2 RNAi [196] and by gene ablation [290]. Genetic evidence showed that TRAF2 and TRAF5 double-knockouts have impaired TNFα signaling (but not IL-1β signaling). Additionally, the MEFs of the double knockouts are more susceptible to TNF induced cytotoxicity [236]. However, TRAF2 cannot bind to E2 enzymes like Ubc13, as the structure of its RING domain is different from the TRAF6 RING domain [291] and it cannot catalyze

synthesis of K63-linked ubiquitin chains. Hence, it can be interpreted that the important role of TRAF2/5 in the TNFα mediated pathway is a consequence of its adaptor function. Of course, it is possible that the adaptor function of TRAF2 acts to recruit another ligase (like cIAP) to RIP1.

In order to ligate ubiquitin chains on to RIP1, both the TRAFs and cIAPs would need to collaborate with the E2 enzyme UBC13. It has been reported that a dominant–negative mutant of UBC13 blocks TNFα and TRAF2 mediated NF-κB activation [159]. Ubc13 knockouts are embryonic lethal; however it is not entirely clear if this is because of dysregulation of NF-κB signaling [232], [233]. There is conflicting genetic data on this topic: while one group showed reduced activation of NF-κB in $UBC13^{+/-}$ macrophages and splenocytes [232], another group demonstrated that $UBC13^{-/-}$ MEFs have normal NF-κB signaling in response to TNFα [233]. These discrepancies may be attributed to the different cell lines used by the researchers or to redundancy with another E2 enzyme. In this regard, it has been shown that another E2 enzyme (UBC4/5) can also activate IKK (the ligase is as yet unidentified) [76]. It is safe to conclude that much more work needs to be done to identify the physiologically relevant E2 enzyme and E3 ligase, if K63-linked regulatory ubiquitination indeed plays a central role in the NF-κB pathway.

In a more recent finding, another E3 ligase which catalyzes the formation of linear ubiquitin chains [292] has been shown to be recruited to the TNFR1 upon receptor stimulation [244]. This E3 ligase complex is known as LUBAC and is composed of HOIP, HOIL-1 and Sharpin [293]. The E3 ligase activity has been verified *in vitro* as LUBAC and Ubc5 have been shown to add linear polyubiquitin chains to NEMO [293]. This linear polyubiquitination of NEMO has been proposed to be important for NF-κB activation. However, the recruitment of LUBAC to TNFR requires only the TRADD, TRAF2 and cIAP1/2 proteins. It does not require

NEMO or RIP1, thus it is difficult to understand how linear ubiquitination of NEMO might activate NF-κB in a cellular setting. Indeed, there is no evidence of linearly ubiquitinated NEMO in cells.

Additionally, RNAi of HOIP only partially inhibits TNFα induced activation of IKK. A naturally occurring mutant mouse (called cpdm) that has a stop codon in the sharpin gene and is deficient for sharpin expression shows chronic proliferative dermatitis and reduced IKK activation [242, 294, 295]. Surprisingly though, others reported that these mice have normal IKK or NF-κB activation (the data even shows a somewhat enhanced IKK activity) [296]. Even if these mice were to show an inflammatory phenotype, it clearly does not resemble the lethality that is associated with knockouts of IKK components [297]. Crossing the cpdm mice with the IL-1 receptor accessory protein (IL-1RAcP)-deficient mice rescues the inflammatory phenotype; thus the inflammation is caused by enhanced IL-1β signaling [298]. Treatment of cpdm mice with a proteasome inhibitor like bortezomib also ameliorates the inflammation, which is further evidence that the inflammation in these mice results from excessive NF-κB activation (and not decreased NF-κB activation as the model would suggest). Other factors might also be involved in the cpdm mice as Sharpin is also known to inhibit activation of integrins [299].

While the knockouts of the various components that mediate regulatory ubiquitination do not all exhibit phenotypes expected to result from knocking out essential member of the NF-κB pathway, genetic ablation of A20 indeed resulted in a drastic phenotype and mice that die from multi-organ inflammation and cachexia [134]. This is because A20 knockouts show persistent and hyperactivated NF-κB signaling. A20 has a unique mechanism of action; it has been shown to first remove K63-linked polyubiquitin chains from RIP1 to attenuate NF-κB signaling, and

then ligate K48-linked polyubiquitin chains on RIP1 to completely inhibit NF-κB [196]. As I will show in my thesis, the deubiquitinase activity of A20 is actually dispensable in the physiological context. This is quite remarkable given the wealth of biochemical data stating otherwise. A number of other deubiquitinases of RIP1 have also come to light. A prominent example is CYLD which deubiquitinates RIP1 and inhibits NF-κB in response to TNF [140]. Another DUB is Cezanne which resembles A20, has an OTU domain, and deubiquitinates RIP1 upon TNFα stimulation [212]. **Figure 2.2** shows how regulatory ubiquitination could potentially regulate the NF-κB pathway downstream of TNFR.

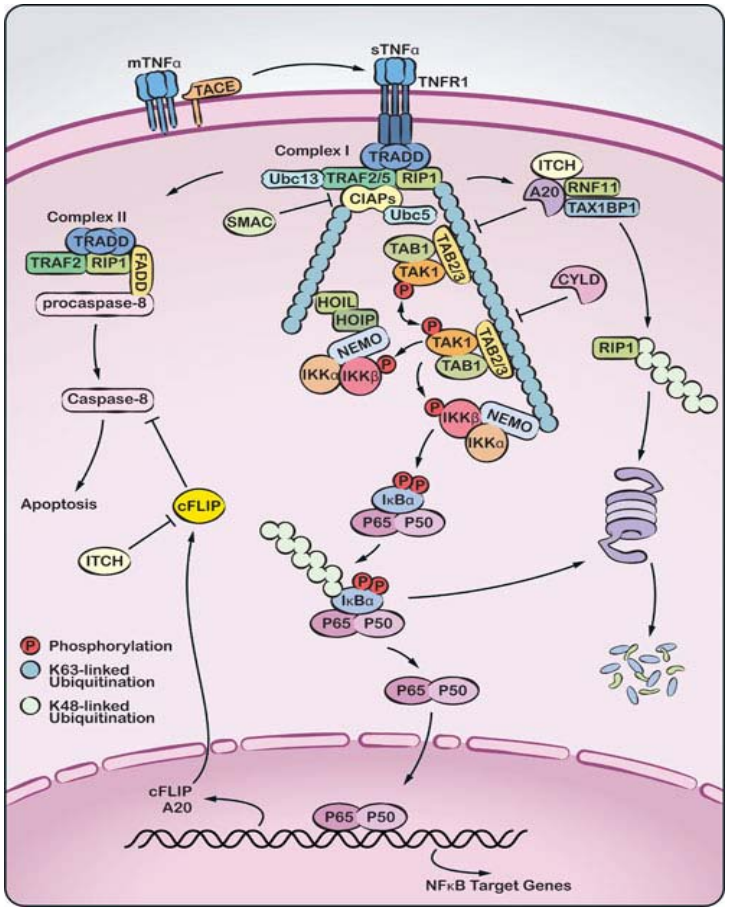


Figure 2.2: Ubiquitin-mediated activation of TAK1 and IKK in TNFα-mediated pathway [123]

2.4: Deubiquitinases and NF-κB

The process of ubiquitination can be reversed by the action of deubiquitinases (DUBs) just like phosphorylation can be reversed by phosphatases. Thus, deubiquitinases can reverse the effect of E3 ligases. Bioinformatic estimates predict around 100 deubiquitinases in the genome [300], [301]. While some DUBs are metalloproteases, most are cysteine proteases. The cysteine proteases may be classified by the presence of distinct protease domains. They may be ovarian tumor (OTU) proteases, ubiquitin-specific proteases (USP), Machado-Joseph disease proteases (MJD) or ubiquitin C-terminal hydrolases (UCH).

A20 and CYLD are the common deubiquitinases in the NF-κB pathway. While A20 is a OTU deubiquitinase, CYLD is a USP deubiquitinase. Nevertheless, they share many of their substrates. Since A20-knockouts die from multi-organ inflammation and cachexia, it is clear there is no functional redundancy with CYLD. This is probably because of temporal reasons, as they target the substrates at two different times during the inflammatory process [211]. CYLD prevents spontaneous activation of NF-κB in quiescent cells while A20 is essential to terminate NF-κB signaling in activated cells.

CYLD is constitutively active and is inactivated in stimulated cells by IKK mediated phosphorylation (in a NEMO dependent manner) [224]. However, it is unclear from the data if the transient phosphorylation happens before TRAF2 ubiquitination and NF-κB activation, as would be expected if CYLD was regulating the process [224]. A20 on the other hand is basally expressed at very low levels but rapidly induced by proinflammatory stimuli to downregulate NF-κB after an initial inflammatory phase [222]. Unlike CYLD, A20 is a NF-κB dependent gene. While expression of A20 is mainly controlled by NF-κB mediated transcription, it is also

post-translationally modified by IKKβ induced phosphorylation at Ser381 [302]. Phosphorylation of A20 increases its ability to downregulate NF-κB. Considered together, phosphorylation and inactivation of CYLD by IKK provides a window for NF-κB activation before it is inducibly turned off by A20 by a negative feedback mechanism. Hence A20 and CYLD serve precise and temporally distinct roles in downregulating NF-κB [222].

It is worth noting that while the proposed deubiquitinase activity of both A20 and CYLD is predicated on K63-linked polyubiquitin chains, they both promiscuously cleave other chains as well. CYLD has been shown to deubiquitinate linear polyubiquitin chains (besides K63-linked polyubiquitin chains) [220]. For specificity, CYLD has been proposed to require various adaptor molecules like p62 in osteoclasts (selectively binds to and recruits CYLD to deubiquitinate TRAF6 downstream of RANK) [225]. *In vitro*, A20 has been shown to have a greater reactivity towards K48-linked polyubiquitin chains and deubiquitinates K48-linked chains better than K63-linked polyubiquitin chains [219], [210]. To achieve this preference for K63-linked chains *in vivo*, A20 forms a complex with other proteins like TAX1BP1 [303].

Just as there is strong biochemical evidence implicating E3 ligases in NF-κB activation, there is also similar evidence that implicates deubiquitinating enzymes in downregulation of NF-κB. Additionally, complete genetic knockouts show that deletion of these DUBs indeed lead to rampant inflammation. Thus, both CYLD and A20 are very important in negatively regulating NF-κB activation. However, to conclusively prove the physiological requirement of the deubiquitinase function, mice containing knock-in mutations which destroy the deubiquitinase function would be necessary. Given the clear physiological importance of A20 and CYLD, it is essential to decipher the mechanism of action for these two proteins.

Since this thesis studies the mechanism of action of A20, this deubiquitinase is discussed in detail below.

2.5: A20 (TNFAIP3 or TNFα Induced Protein 3 gene)

2.5.1: Structure and proposed mechanism of action

A20 was discovered in human umbilical vein endothelial cells (HUVEC) as a zinc finger protein that is rapidly induced upon stimulation with TNFα, hence it is also called 'TNFα Induced Protein 3 [202], [304]. The function of A20 was to provide protection from TNF mediated cytotoxicity [304]. It was shown that A20 is virtually absent at basal levels, but rapidly induced by a plethora of PAMPS, mitogens or proinflammatory cytokines [202] in a NF-κB dependent manner. While cells of the innate immune system (dendritic cells, macrophages) show inducible expression of A20 [202], thymocytes have constitutive expression of high levels of A20 that is downregulated upon TCR stimulation [305], [211].

An analysis of the structure of A20 **(Figure 2.3)** shows a N-terminal OTU domain followed by seven C-terminal zinc finger domains. A cysteine residue at position 103 (in the OTU domain) has been shown to be important for the deubiquitinase activity [196, 204]. Multiple studies based on overexpression have demonstrated that A20 downregulates NF-κB signaling downstream of the TNFR and TLRs [208], [306], [272]. Upon TNFR stimulation, A20 is inducibly expressed and targets ubiquitinated RIP1 in an enigmatic two-step sequential process to downregulate NF-κB [196]. A20 acts as a dual ubiquitin-editing enzyme that can act both as a deubiquitinase and an E3 ligase. The Cys103 residue in the OTU domain first deubiquitinates K63-linked polyubiquitin chains from RIP1, thereby attenuating NF-κB activation. After this, the

Cys624/Cys627 in the fourth zinc finger domain (ZNF4) ligates K48-linked polyubiquitin chains to RIP1, thereby triggering proteasomal degradation of RIP1 and completely inactivating NF-κB [196]. E3 ligases are typically characterized by the presence of a RING or HECT domain; however, the E3 ligase function of A20 is uniquely mediated by a zinc finger domain. A considerable fraction of cellular A20 has been found to localize to lysosomes. This localization is mediated by zinc finger domains and has been found to lead to degradation of TRAF2 [307, 308]. Surprisingly though, a deubiquitinase mutant of A20 (C103A) has been shown to inhibit IKK both *in vitro* and *in vivo*, leading to normal activation of NF-κB [309].

A20 can also inhibit NF-κB in response to LPS stimulation. However, it does so by a different mechanism. Once induced, A20 attenuates NF-κB activation by preventing further K63-linked ubiquitination by disrupting interactions between Ubc13 (E2 enzyme) and TRAF6 (E3 ligase) [271]. At later time points, A20 abrogates NF-κB signaling by adding K48-linked polyubiquitin chains to Ubc13 targeting it for proteasomal degradation. This process is also dependent on ZNF4 (C624/C627) and Cys103. Another publication showed that A20 directly inhibited LPS-mediated NF-κB signaling by deubiquitinating TRAF6 [200].

The mechanisms are elegant and C624/C627 and C103 have been shown to be important in downregulating NF-κB both in response to TNF and LPS stimulation. However the experiments are based on overexpression and knock-down of the protein. This thesis will characterize knock-in mice in which the Cys103 residue is mutated to test the physiological importance of the deubiquitinase function of A20.

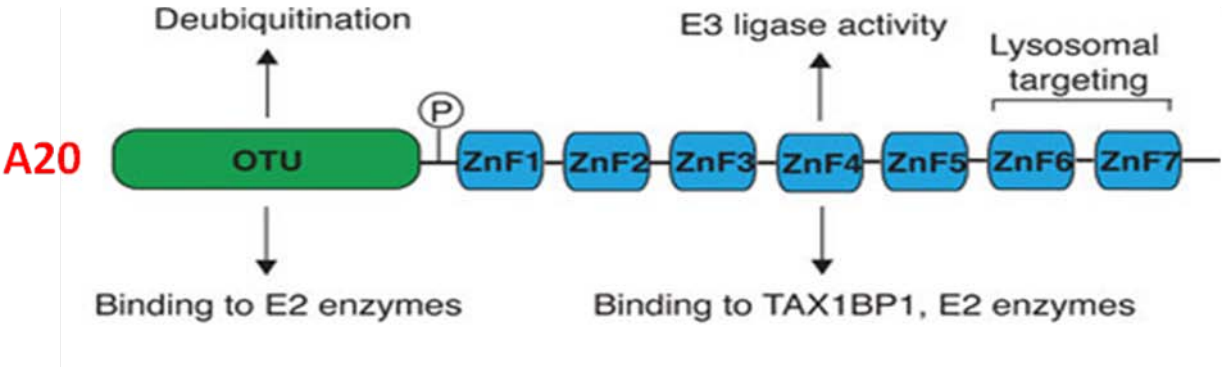


Figure 2.3: Structure of A20 [211]

2.5.2: Function of A20

The anti-apoptotic and NF-κB inhibitory role of A20 was elucidated after the generation of A20-knockout mice. A20-knockouts are perinatally lethal. They die because of spontaneous, extensive multi-organ inflammation and cachexia, resulting from persistent, unabated NF-κB signaling [134]. As would be expected, they also succumb to different pro-inflammatory stimuli including sub-lethal doses of TNF-α or LPS [134]. A20-knockout MEFs show persistent activation of NF-κB in response to TNF-α, LPS and IL-1 stimulation and are more sensitive to apoptosis as compared to wild type MEFs [134].

The inflammation in A20-knockouts is mediated by myeloid cells and is independent of the adaptive response. This was found by deleting A20 in mice lacking B and T cells (*Rag1*$^{-/-}$ mouse). Thus, inflammation is not rescued in a *TNFAIP3*$^{-/-}$*Rag1*$^{-/-}$ double deficient mice [134]. Surprisingly, even though A20 was considered a feed-back inhibitor of TNF mediated signaling, the inflammation in A20-knockouts is independent of TNFR signaling. Thus the inflammation also continues unabated in *TNFAIP3*$^{-/-}$ *TNFα*$^{-/-}$ and *TNFAIP3*$^{-/-}$ *TNFR1*$^{-/-}$ double-knockouts

[200]. While the deubiquitinating property of A20 is certainly important *in vitro*, this observation casts serious doubts on the physiological relevance of this function. This is because the deubiquitinase role has been implicated in downregulating TNF signaling, and the physiological relevance of A20 is clearly not dependent on TNF signaling.

The inflammation in A20 knockouts is recued by crossing with *Myd88*$^{-/-}$ mice. Thus, the spontaneous inflammation in A20 knockouts is triggered by TLR-mediated signaling, and the *TNFAIP3*$^{-/-}$ *Myd88*$^{-/-}$ double-knockouts are normal [200], [310]. Consistently, A20-deficient mice can be successfully treated with broad-spectrum antibiotics and cured of the severe inflammation [310]. Hence, it can be inferred that A20-knockouts have constitutive TLR signaling driven by commensal intestinal flora. Thus, A20 is essential to maintain intestinal immune homeostasis.

A20 has also been specifically deleted from various organs and these conditional-knockouts have been used to determine the role of A20 in these organs **(Table 2.1)**. Thus, A20 was specifically deleted from intestinal epithelial cells (IECs) by crossing with Villin-Cre mice [311]. The A20 IEC conditional knockout (A20$^{\text{IEC-KO}}$) mice were found to be healthy. However, they have a greater propensity of developing colitis when induced with dextran sulphate sodium (DSS). This colitis is rescued by crossing with TNFR-knockouts [311]. Thus, TNFR signaling is responsible for the systemic, intestinal pathophysiology.

A20 plays a profound role in the innate immune system. Specific ablation of A20 in myeloid cells causes spontaneous development of rheumatoid arthritis accompanied by osteoclastogenesis [312]. This is caused by a higher level of proinflammatory serum cytokines, as would be expected from protracted activation of NF-κB [312]. The pathology was shown to be

dependent on TLR4-MyD88 and IL-6 signaling but independent of TNF [312]. Upon stimulation with LPS, both macrophages [200] and dendritic cells (DCs) [313] deficient in A20 produced more proinflammatory cytokines (IL-6 and TNF-α) and showed an enhanced expression of co-stimulatory molecules [313]. siRNA based silencing of A20 in DCs triggered constitutive NF-κB activation and antigen presentation [313]. Thus, A20 seemed to be important in not only determining the activation threshold of DCs, but also in regulating antigen presentation. Indeed when A20 is selectively deleted from DCs by crossing with CD11-Cre transgenic mice, the mice develop autoimmunity and show spontaneous proliferation of T cells and expansion of plasma cells. They have symptoms of systemic lupus erythematosus (SLE) [314]. The DCs were resistant to apoptosis probably because of upregulation of Bcl-2 and Bcl-x [314]. In this regard, patients of SLE [315] have multiple SNPs associated with A20.

The importance of A20 in mediating antigen presentation of DCs might imply that A20 may also be important for adaptive immunity. In fact, A20 has been determined to be essential for terminating NF-κB signaling in both T and B lymphocytes. The expression pattern of A20 in these lymphoid cells is different from myeloid cells. In lymphoid cells, A20 is constitutively expressed. The basal levels of A20 are particularly high in T cells [305]. Experiments based on overexpression reveal that A20, but not the deubiquitinase mutant, can downregulate NF-κB signaling in response to CD3 and CD28 receptor stimulation [316]. A20 acts by removing K63-linked polyubiquitin chains from MALT1 to downregulate NF-κB [207]. Conversely MALT1 has paracaspase activity to cleave A20 and facilitate NF-κB activation [317]. This balance between ‘MALT1 mediated A20 cleavage’ and ‘A20 mediated MALT1 deubiquitination’ fine-tunes NF-κB activity in T cells.

A20 is constitutively expressed in B lymphocytes as well. Intriguingly though, CD40 signaling further increases the expression of A20 [318]. This would suggest that A20 plays an important role in downregulating NF-κB in these cells. Indeed, selectively deleting A20 in B cells (by crossing with CD19-Cre mice) results in autoimmunity mediated by production of autoantibodies [319]. The ($A20^{CD19\text{-}KO}$) mice show normal development and growth. However, they have more immature and germinal center (GC) B cells as compared to their littermates. Their splenic B cells also proliferate more upon stimulation with α-CD40, LPS or CpG [319]. They show enhanced non-canonical and canonical NF-κB signaling as evidenced by phosphorylation of p100 and degradation of IκBα respectively [319]. Thus, A20 maintains B cell homeostasis by inhibiting both canonical and non-canonical signaling in B lymphocytes. However, one observation in these mice ($A20^{CD19\text{-}KO}$) does not correlate with the well documented anti-apoptotic effects of A20. The B cells in these mice are surprisingly resistant to Fas-mediated apoptosis. This is because of the higher expression of antiapoptotic molecules like Bcl-x in these mice.

The cross-regulation of A20 and MALT1 is not unique to T cells alone, but is present in B cells as well. Dysregulation of this cross-regulation may result in B-cell lymphoma. In B-cell lymphomas, the paracaspase activity of MALT1 is constitutively active. This deactivates A20, and leads to chronic, persistent activation of NF-κB resulting in lymphoma [320].

Cell type	Genetic modification	Mouse phenotype	Related human disease
B cells	Cre-mediated deletion of *TNFαip3* in cells that express CD19	Germinal centre and plasma cell dysplasia; production of autoantibodies; renal immunoglobulin deposition; B cell resistance to FAS-mediated cell death	Systemic lupus erythematosus
DCs	Cre-mediated deletion of *TNFαip3* in cells that express CD11c	DC activation; expansion and activation of T cell and myeloid cell populations; colitis; spondyloarthritis	Inflammatory bowel disease
DCs	Cre-mediated deletion of *TNFαip3* in cells that express CD11c	DC activation; expansion of T cell and plasma cell populations; increased uptake of apoptotic cells by DCs; autoantibody production; nephritis	Systemic lupus erythematosus
Macrophages and granulocytes	Cre-mediated deletion of *TNFαip3* in cells that express lysozyme M	Increased IL-6 production; production of collagen-specific autoantibodies; protection against influenza A virus infection	Rheumatoid arthritis
Intestinal epithelial cells	Cre-mediated deletion of *TNFαip3* in cells that express villin	Hypersensitivity to experimental colitis	Inflammatory bowel disease
Intestinal epithelial cells	Villin-driven expression of a *TNFαip3* transgene	Protection against DSS-induced colitis	Inflammatory bowel disease
Keratinocytes	Cre-mediated deletion of *TNFαip3* in cells that express keratin 14	Epidermal hyperproliferation; hair and skin defects; sebaceous gland hyperplasia	?

Table 2.1: Phenotypes of cell type-specific deletion of A20 [20]

2.5.3: Regulation of A20 by ubiquitin-binding proteins and adaptors

A20 exists in a complex with many other proteins (like Tax1 binding protein 1 (TAX1BP1), Itch, Ring finger protein (RNF) 11, and possibly ABIN-1 and YMER, and this

"A20 ubiquitin-editing complex" has been proposed to be important for inhibiting NF-κB signaling [211]. Thus, the interaction of A20 with RIP1 or TRAF6 is mediated by TAX1BP1 [321] while another E3 ligase (Itch) is essential for A20 promoting K48-linked polyubiquitination of RIP1 [250]. ABIN-1 has been shown to be important in terminating the NF-κB response by deubiquitinating NEMO [322], [205].

TAX1BP1 was discovered using the HTLV-I Tax oncoprotein as bait in a yeast two-hybrid screen [323]. Later, TAX1BP1 was shown to bind A20 and cooperatively promote cell survival [303]. TAX1BP1 knockouts have been generated using two methods, by gene trapping and by a conventional gene targeting method. The resulting phenotypes are different for unknown reasons. The knockout generated using gene trapping is embryonically lethal because of cardiac defects at E13.5 [324]. The knockout generated using gene targeting [321] developed normally till four months. After this, they succumbed to inflammatory cardiac valvulitis. While the first phenotype resembles the A20-knockout and suggests that TAX1BP1 could be an essential 'adaptor protein for A20', the second phenotype does not readily comply with the mechanism of cooperative-action.

However both the A20 and the TAX1BP1 knockouts are hypersensitive to proinflammatory cytokines because of enhanced activation of NF-κB [321], [324]. They also succumb to sub-lethal doses of IL-1β and TNF-α. RIP1 is persistently ubiquitinated in *Tax1bp1*$^{-/-}$ MEFs upon TNF stimulation, similar to the A20-knockout MEFs [324]. This suggests that A20 and TAX1BP1 act in a complex to reduce RIP1 ubiquitination. Since TAX1BP1 does not have deubiquitinating activity, it probably inhibits RIP1 ubiquitinating by acting through A20 [324].

TAX1BP1 has two zinc finger (ZnF) domains in the C-terminus and one of them is known to harbor a UBD. This UBD recognizes the K63-linked polyubiquitin chains of RIP1, serving as the adaptor domain for TAX1BP1 to recruit A20 to the receptor complex [324], [321]. The ZnF domains of a TAX1BP1 have a 'PPXY' motif (P-Proline, X-variable amino acid, Y-tyrosine'). Such motifs preferably interact with 'WW' (W-tryptophan) motifs in other proteins [325]. This motif is important for a functional TAX1BP1 molecule as TAX1BP1 mutants lacking this motif have persistent NF-κB activation [250]. It was later discovered that Itch contains the 'WW' motif and interacts with TAX1BP1 [250]. The TAX1BP1/Itch complex is inducibly formed upon TNF stimulation and is responsible for recruiting A20 to the receptor complex and deubiquitinating RIP1 [250].

Itch-knockouts also show hyperactive NF-κB signaling, however the inflammation in these mice is confined to the lungs and skin alone [326] as compared to A20-knockouts, which show inflammation throughout the body. Additionally, unlike A20—knockouts, the inflammation in Itch-deficient mice depends on the adaptive immune system and is recued by crossing with *Rag1*$^{-/-}$ mice [250]. Thus, it is probable that Itch has functions independent of A20.

RNF11 also interacts with A20, TAX1BP1, Itch and NEMO as identified in a high-throughput yeast two-hybrid screen [327]. RNF11 is an E3 ligase and is overexpressed in cancers of different tissues (breast, pancreas, etc) [328], [329]. This suggested a possible link with the NF-κB pathway and it was subsequently demonstrated that RFN11-TAX1BL1-Itch complex is inducibly formed upon stimulation with TNF-α or IL-1 [330]. Overexpression of RNF11 negatively regulate NF-κB signaling, while knockdown of RNF11 using siRNA

increases activation of NF-κB [330]. Furthermore, cells deficient in RFN11 showed increased ubiquitination of RIP1 and TRAF6 [330]. Hence, it has been suggested that A20 acts in a complex with RFN11, TAX1BL1 and Itch. RNF11 is also known to participate in TGF-β signaling [331], [332]. Hence, RNF11 clearly plays a complex role and delineation of its physiological function will have to await the generation of knockout mice.

ABIN-1 is another A20-interacting protein identified by a yeast two-hybrid screen [306], whose overexpression inhibits NF-κB signaling in response to a wide range of stimuli [306], [333]. ABIN1 has been proposed to be an adaptor molecule essential for the interaction of A20 and NEMO, leading to deubiquitination of NEMO by A20 [205]. Interestingly, a pathogenic E3 ligase IpaH9.8 found in Shigella cooperates with ABIN-1 to add K48-linked polyubiquitin chains to NEMO, thereby targeting it for proteasomal degradation and inactivation NF-κB in the process [334]. However, a major discrepancy is that MEFs deficient in ABIN-1 show normal NF-κB activation [335].

Similarly, the yeast two-hybrid screen identified another A20 interaction protein named YMER [336]. YMER was also identified in a protein array using a polyubiquitin bait [337], and this polyubiquitin binding domain of YMER is essential for inhibition of NF-κB signaling [336]. Overexpressing YMER downregulates NF-κB, while YMER knockdown cells shows augmented NF-κB activation [336]. YMER interacts with RIP1 and hence has been proposed to act in a complex with A20 and aid in RIP1 deubiquitination [336].

2.5.4: The role of A20 in autoimmune diseases and cancer

Polymorphisms of the TNFAIP3 gene **(Figure 2.4)** has been implicated in a number of autoimmune diseases (rheumatoid arthritis, type 1 diabetes, inflammatory bowel disease, psoriasis, SLE, coronary artery disease, celiac disease, and SLE) [338], [135], [136], [139], [138],[201], [339], [340], [341], [342]. Based on murine studies, it could be rationalized that these polymorphisms affect the expression and/or function of the expressed A20 protein [211]. A mutation in the coding region of the OTU domain (A125V) has been found in the African population [343]. The data tentatively suggests that this affects A20-mediated deubiquitination of TRAF2. Two other mutations (V377M and P656L) are associated with lung cancers. The mechanistic effect of these two mutations on A20 is not clear.

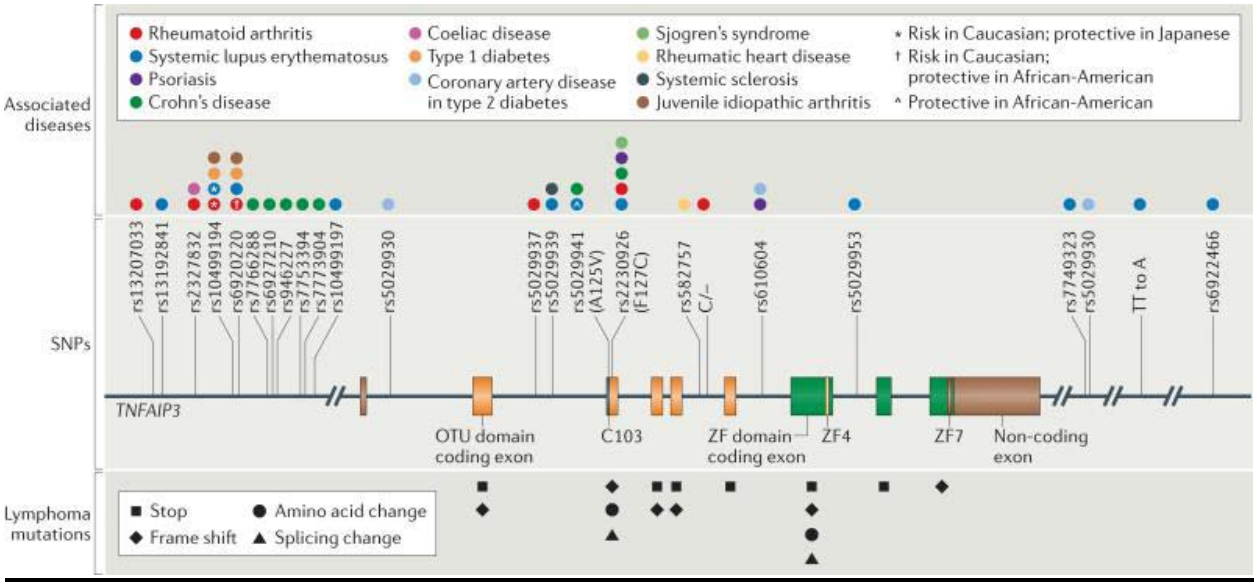


Figure 2.4: Polymorphisms in A20 associated with human diseases [20]

A20 acts as a tumor suppressor for many different B-cell lymphomas. A20 is on chromosome 6, and deletion of chromosome 6q is often associated with non-Hodgkin lymphomas [344]. Several point mutations that could inactivate A20 lead to marginal zone lymphoma, Hodgkin lymphoma, diffuse large B-cell lymphoma (DLBCL) or MALT lymphoma [345], [136], [135], [138]. A reduction in the levels of the A20 protein (by methylation of promoter) could lead to MALT lymphoma [346]. The tumor suppressor function of A20 has been conclusively proven by demonstrating that B-cell lymphomas can be rescued by expressing A20 in lymphoma cell lines that lacks both A20 alleles [136], [135].

It is interesting to note that oncogenic transformation of MEFs has been found to be associated with reduction of expression of A20 protein. For example, Ras-mediated transformation of primary MEFs leads to the upregulation of most NF-κB-controlled genes. It, however, reduces levels of A20 [347].

A20 inhibits TNF mediated cytotoxicity [304] and has prosurvival activity in this context. Hence, it is conceivable that A20 may be an oncogene in certain circumstances. This is indeed the case in breast cancer and glioma. A20 is estrogen-regulated in breast cancer and protects against the apoptotic effects of tamoxifen [348]. A20 is needed for glioma stem cell survival [349]. Indeed overexpression of A20 is a hallmark of breast tumor and glioma and may serve as a therapeutic marker for these diseases [348], [349].

2.5.5: Pathogens modulating A20 activity

Since A20 is an essential regulator of NF-κB, oncogenic viruses often inactivate it, thus promoting chronic activation of NF-κB [211]. A few examples are outlined below. They highlight the critical role that A20 plays in controlling excessive immune responses.

The genome of Human T-cell leukemia virus type I (HTLV-I) encodes an oncogenic protein called Tax which mediates activation of NF-κB and has been hypothesized to be associated with adult T-cell leukemia (ATL) [350]. Tax binds to TAX1BP1 [323], [351], and hinders the association of A20 and Itch with TAX1BP1 adaptor protein [323], [324]. As a result the "A20 ubiquitin editing complex" is not formed and A20 cannot be recruited to RIP1 [323]. By a similar mechanism, upon IL-1 stimulation, Tax does not allow recruitment of A20 to TRAF6 receptor complex [271]. In response to TNFα, Tax prevents association of Ubc13 with A20, thereby preventing the consequent degradation of Ubc13 [271]. In this way, Tax inhibits A20 (by binding to TAX1BP1) and activates NF-κB [211].

The human papillomavirus virus (HPV) E2 protein also interacts with TAX1BP1. It is currently unclear if this interaction affects NF-κB signaling during infection with HPV [352]. Epstein Barr Virus (EBV) encodes an oncogenic protein called LMP1. This protein interacts with A20 to promote constitutive activation of NF-κB [353], [354]. A20 also deubiquitinates IRF7 in Raji cells infected with EBV virus [355]. Thus, the infection is not mediated solely through the inactivation of the NF-κB pathway.

In addition to encoding for proteins that hijack the cellular role of A20, some viruses and bacteria have also evolved to directly encode for a DUB that resembles A20. This could in theory allow the pathogen to inhibit NF-κB [211] and reduce the proinflammatory response by the host. In effect, this would provide the pathogen a strategy for 'immune evasion'. The Crimean Congo hemorrhagic fever virus (CCHFV) causes hemorrhages and 30% of infections are lethal [356]. It encodes a protein named L protein which has a N-terminal OTU domain (just like A20). This is not unique to CCHFV, but also found in Nairobi sheep disease virus and nairoviruses Dugbe virus (DUGV). This protein deubiquitinates NF-κB activators, thereby attenuating TNF-α-mediated activation of NF-κB. The bacteria Yersinia (causes plague) has evolved to encode a gene called YopJ. This virulence factor could potentially deubiquitinate TRAF2 or TRAF6, leading to inactivation of NF-κB and reduced expression of proinflammatory cytokines [357]. Much like mammalian DUBs, pathogenic DUBs also seem to lack specificity. Thus, YopJ has been found to cleave both K63-linked and K48-linked polyubiquitin chains [357]. In this way, by mimicking the DUBs, pathogenic bacteria and viruses can find a way to escape the host immune response.

2.5.6: Regulation of A20 deubiquitinase activity

Recently, the crystal structure of the OTU domain was solved independently by two groups [210], [209]. The structure is similar to other cysteine proteases, and the architecture of catalytic cysteine (C103) and accompanying histidines is conserved [209]. The C103 (responsible for deubiquitinase function) is located in an alpha helix, which may provide the

platform for binding of ubiquitin chains [210]. However biochemical evidences by other groups have suggested that the seventh zinc finger motif is important for ubiquitin binding [309].

A20 cleaves K48-linked polyubiquitin chains rather than K63-linked polyubiquitin chains *in vitro*, and this is at odds with the proposed mechanism of disassembling K63-linjed chains from RIP1 and TRAF6 [210], [219]. Additionally, unlike most of cysteine proteases of OTU family, A20 deubiquitinates K63-linked chains from the TRAF6/ubiquitin interface [210]. While some groups have confirmed the importance of the deubiquitinase activity of A20 [196], [200], [324], others have found that that this property of A20 is dispensable for downregulating NF-κB [204], [307]. Moreover, inhibition of antiviral signaling requires the ZF domains [358] but not the DUB domain of A20 [359], [360], [358]. These discrepancies may be attributed to distinct experimental designs employed by different groups (cell lines, varying levels of overexpression, etc).

2.6: Conclusion and Perspectives

Despite the large body of literature implicating A20 in inflammatory responses, the mechanism by which A20 downregulates inflammation has remained elusive. Multiple biochemical and knockdown approaches in cell culture have suggested that A20 functions as a deubiquitinase by disassembling regulatory K63-ubiquitin chains on upstream signaling molecules such as RIP1. In the next chapter, we report the creation and characterization of a knock-in mouse that expresses a mutated form of A20 that lacks the deubiquitinase activity. The knock-in mouse that we have generated abrogates the deubiquitinase function of A20 *in vivo*, and is the ideal tool to study the physiological role of the deubiquitinase function of A20.

Chapter 3:

The deubiquitinase activity of A20 is dispensable for its role in NF-κB signaling

3.1: Abstract

Ubiquitination of multiple signaling adaptor molecules by K63 linked ubiquitin chains have been proposed to be a key regulatory mechanism in NF-κB activation. Deubiquitinase enzymes such as A20 have been suggested to limit the persistence of NF-κB activation by removing regulatory ubiquitin chains from ubiquitinated substrates. A20 has garnered significant interest as mice lacking A20 die prematurely from multi-organ inflammation and cachexia, as a result of increased NF-κB signaling. Thus, it is evident that A20 is non-redundant in its ability to limit the persistence of NF-κB signaling. A20 is believed to function by first removing K63 linked ubiquitin chains from adapter proteins such as RIP1, and then polyubiquitinating the same substrates with K48 linked ubiquitin chains that trigger proteasomal degradation. However the exact role of the deubiquitinase function of A20 in its ability to downregulate NF-κB signaling, had not been examined in a physiological setting. To understand the physiological relevance of A20-mediated deubiquitination, we generated a knock-in mice that lacks the deubiquitinating function of A20 (A20-OTU mice). We hypothesized that these mice would have an inflammatory phenotype because of increased, persistent NF-κB signaling. However our results show that A20 OUT mice display normal NF-κB activation and no inflammatory phenotype, thereby demonstrating that the deubiquitinase activity of A20 is dispensable for normal NF-κB signaling.

3.2: Introduction

NF-κB is an ubiquitously expressed, inducible transcription factor that regulates the expression of numerous target genes, particularly in the immune system. In unstimulated cells, NF-κB is sequestered in the cytoplasm through its binding to inhibitory IκB proteins that mask the nuclear localizing signals on the NF-κB proteins. NF-κB can be activated upon stimulation of cells with a wide variety of inducers including pro-inflammatory cytokines such as TNFα and IL-1β, or components of pathogenic microbes (PAMPs) such as bacterial lipopolysaccharide (LPS). Upon engagement of cognate receptors e.g. TNF-receptor or Toll-like receptor 4, signaling pathways are triggered that lead to the activation of a protein kinase complex known as the IκB kinase (IKK). The activated IKK phosphorylates the IκB proteins on specific serine residues that lead to their polyubiquitination with K48-linked ubiquitin chains, and consequent degradation by the proteasome. The released NF-κB then migrates to the nucleus where it binds to promoters of target genes and activates transcription [108].

Besides the well-characterized K48-linked polyubiquitination that triggers proteasome-mediated degradation of substrate proteins such as IκBs, polyubiquitination of signaling adapter proteins with K63-linked chains have been proposed to have critical regulatory function in NF-κB activation pathways. RING-finger proteins such as TRAFs are believed to act as ubiquitinating enzymes and molecules such as RIP1 are well-characterized substrates that undergo K63-linked ubiquitination. Similar to kinases and phosphatases, deubiquitinating enzymes have been proposed to act to limit the consequence of regulatory ubiquitination by removing the polyubiquitin chains. Amongst the various deubiquitinases that have been suggested to act on K63-linked chains in the NF-κB pathway, the best characterized is A20 (TNFAIP3) [131].

A20 was initially identified as a TNFα-inducible zinc-finger protein that protects cells from TNFα induced cytotoxicity [304]. A20 is expressed at very low levels in most cell types but is rapidly induced in response to various PAMPs or proinflammatory cytokines [304]. Subsequently, many studies, mainly in overexpression systems, have demonstrated that A20 downregulates NF-κB signaling in multiple pathways including the tumor necrosis factor (TNFα) and Toll-like receptor (TLR) pathways[208], [306], [272]. Mice deficient in A20 die prematurely from multi-organ inflammation and cachexia as a result of increased NF-κB signaling as would be predicted from the *in vitro* studies. These mice are also sensitive to proinflammatory stimuli and cannot withstand even sub-lethal doses of TNFα or LPS. Additionally, the A20 deficient MEFs show persistent NF-κB signaling as evidenced by increased IκBα degradation [134].

Intriguingly, although A20 was initially identified as a TNFα-inducible gene, the spontaneous inflammation in A20 deficient mice continues unabated even in A20(TNFAIP3)$^{-/-}$ TNFα$^{-/-}$ and A20(TNFAIP3)$^{-/-}$TNFR1$^{-/-}$ double mutant mice. Thus, the spontaneous inflammation in A20 deficient mice results from TNFα-independent signaling. Instead the inflammation in these mice in largely eliminated by removal of MyD88, and the TNFAIP3$^{-/-}$Myd88$^{-/-}$ mice do not show the severe inflammation characteristic of A20 deficient mice, indicating that TLR signaling drives the spontaneous inflammation in these mice [200]. Consistently, treatment of A20 deficient mice with broad-spectrum antibiotics rescues the inflammatory phenotype, thereby indicating that a dysregulated intestinal flora contributes to the constitutive TLR signaling which results in perinatal lethality in these mice [310]. Thus, it has been clearly demonstrated that A20 is essential and non-redundant in restricting persistent TLR mediated activation of NF-κB and subsequent lethality. Therefore while many studies have focused on the role of A20 in response to TNFα, it is imperative to understand the role of A20 in the context of TLR and LPS signaling [281], [196].

It has been shown by various biochemical assays that A20 can function both as a deubiquitinase (DUB) and as an ubiquitin ligase. A20 has a N-terminal OTU domain followed by seven C-terminal zinc finger domains. Cys-103 in the OTU domain has been showed to be essential for the deubiquitinating function of A20 [204], [196] . Previous biochemical studies using recombinant A20 has demonstrated that A20 is a unique ubiquitin modifying enzyme regulating both the activity (by removing K63 linked chains) and stability (by adding K48 linked chains) of signaling molecules such as RIP and TRAF6 (Nature, 2004, 430, 694-699) [196]. These studies showed that in response to TNFα stimulation, A20 acts by first deubiquitinating the regulatory K63-linked polyubiquitin chains from RIP1, thereby initially attenuating NF-κB signaling. Subsequently, A20 acts as an E3 ubiquitin ligase adding K48-linked polyubiquitin chains to RIP1 leading to degradation of RIP1 and terminating any residual NF-κB signaling. In the TLR and the IL-1R pathway, A20 inhibits NF-κB signaling by disrupting the binding of the E3 ligase, TRAF6, with E2 ubiquitin conjugating enzymes like Ubc13 or UbcH5c. The deubiquitinase activity mediated by C103 residue has been shown to be essential in mediating this interaction as well [271].

Despite the A20 C103A mutants being completely deficient in DUB activity, some studies have shown that overexpression of A20 C103A can also inhibit NF-κB signaling [204, 208, 307]. We were intrigued by these observations because they suggested that the ability of A20 to inhibit NF-κB activation might not be due to the ability of A20 to deubiquitinate K63-ubiquitinated substrates. Therefore, to accurately delineate the physiological ramifications of A20's deubiquitinating function, we generated a gene-targeted mouse with a C103A point mutation to eliminate the DUB activity of A20. Given the severe multi-organ inflammation in $A20^{-/-}$ mice that led to perinatal lethality [134], we hypothesized that these A20-C103A knock-in deubiquitinase mutant mice would show a phenotype similar to the A20 knock-out mice. Furthermore since dysregulation of A20 has been implicated in a host of diseases [20], these knock-in mice could prove to be a potential model for studying these disorders in a

physiologically relevant setting. However, as described in this report, the A20 C103A KI mice do not show any aberrant pro-inflammatory mice and demonstrate normal lifespan without any observable phenotype. Analysis of NF-κB signaling in cells isolated from these mice show no discernible difference from normal, wild-type cells, including the degree of ubiquitination of signaling adapter proteins. Therefore our studies reveal that the deubiquitinase function of A20 is not important for the well-described role of A20 in NF-κB signaling.

3.3: Results

3.3.1: Generation of A20-OTU knock-in mice.

In order to evaluate the physiological contribution of A20's deubiquitinating function, we decided to create a mouse with a mutation in the OTU domain to abolish the deubiquitinase activity. We used a BAC based approach, as described in the methods section, to generate a knock-in mouse that harbors a point mutation at position 103, namely a change from cysteine to alanine **(Figure 3.1A)**. Heterozygous A20 knock-in mice (C103A or C/A) were intercrossed to generate the homozygous A20-OTU knock-in mice (or A/A mice). Sequencing of genomic DNA from both C/A and A/A mice confirmed the presence of the correct C103A point mutation **(Figure 3.1B)**. The sequencing chromatogram of the genomic DNA isolated from heterozygous mice shows both cysteine and alanine at position 103, while the homozygous mice encodes for only alanine. The genotyping strategy and the photographs of the respective littermates are shown in **Figure 3.1C**. In this thesis, we refer to the wild type littermates as C/C meaning they have a cysteine in both alleles at position 103; the heterozygous mice as C/A as they have a cysteine and alanine in the two alleles; and the homozygous knock-in mice as A/A as they have only the mutated alanines on both alleles.

We next isolated BMDMs from both WT and A/A littermates and found roughly equal levels of A20 mRNA and protein **(Figure 3.1D)** following LPS and TNFα stimulation. A20 was initially found at relatively low levels but is rapidly induced upon LPS stimulation in both the C/C and A/A littermates.

3.3.2: Characterization of A20 OTU knock-in mice

Although $A20^{-/-}$ mice were runted by 1 week of age and die perinatally of multi-organ inflammation and cachexia, the homozygous A/A knock-in mice were normal **(Figure 3.1E)** and did not display any external signs of inflammation (for the observed period of 10 months after birth). They were born in Mendelian ratios and both the C/C and A/A littermate adults weighed the same **(Figure 3.1E).** Histological examination of 6 week old $A20^{-/-}$ mice had revealed severe tissue damage in multiple organs. However histology of major organs (kidney, liver, spleen, thymus, lung and heart) of WT (C/C), heterozygous knock-in (C/A) and homozygous knock-in (A/A) littermates did not reveal any differences (**Figure 3.2A**). Hence, the deubiquitinating function of A20 is not important for maintaining basal tissue homeostasis.

3.3.3: Characterizing the cells of immune system of C103A knock-in mice in the steady state

Unlike $A20^{-/-}$ mice that have increased numbers of myeloid lineage cells in the bone marrow and spleen, the C/C, C/A and A/A littermates had comparable frequencies of myeloid lineage cells (CD11b+). Within the myeloid compartment, the frequencies of monocytes (CD11b+Ly6c+Ly6G-) and granulocytes (CD11b+Ly6c+Ly6G+) were similar across all indicated genotypes (**Figure 3.2B**). Similarly, analysis of B cell development indicated that the differentiation pattern of CD19+B220+ and CD19-B220+ cells in heterozygous and homozygous knock-in mice was comparable to the WT littermates. In addition, analysis of surface IgM and IgD expression in CD19+ B cells of the bone marrow indicated similar expression patterns

amongst wild type, heterozygous and homozygous knock-in mice (**Figure 3.2C**). Consistently, analysis of erythroid (Ter119+) and megakaryocyte (CD41+) lineage cells suggested comparable frequencies in the bone marrow (**Figure 3.2D**).

In mice, T cell development occurs in the thymus and undergoes distinct stages of differentiation including the, earliest, double negative (DN) stage (CD4-CD8-), double positive (DP) stage (CD4+CD8+) and subsequently into CD4+CD8- and CD4-CD8+ single positive cells [361]. Analysis of thymus of the three genotypes indicated a roughly equal differentiation of CD4 and CD8 cells between the genotypes as shown in **Figure 3.2E**. Consistent with the normal differentiation of myeloid, lymphoid and erythroid lineages in the BM and thymus, the spleen also showed normal proportions of B cells (**Figure 3.2F)**, T cells (**Figure 3.2G)**, dendritic cells (**Figure 3.2H)** and myeloid cells (**Figure 3.2I)**. Therefore these results suggest that the deubiquitinase function of A20 does not play a role in differentiation and maintenance of immune cell types.

As mice age to 6 months, the A/A deubiquitinase mutant mice develop splenomegaly and their spleens weigh around 145 mg as compared to their age and sex-matched wild-type C/C littermates whose spleen weight around 65 mg. Both the wild-type and mutant mice weight around the same **(Figure 3.2J)**. Analysis of the bone marrow and spleen of older mice showed that the homozygous mutant mice showed an increase in myeloid cell population **(Figure 3.2K)**.

3.3.4: Activation of wild type and mutant BMDM and BMDCs in response to LPS and TNFα *in vitro*

A20 is negligibly expressed in the basal state but is rapidly induced upon stimulation by LPS and TNFα, and the newly synthesized A20 helps down-regulate NF-κB activity. Hence, we

wanted to test the contribution of the deubiquitinating function of A20 in inhibiting NF-κB activation following stimulation with LPS and TNFα.

We differentiated bone marrow cells of wild type (C/C), heterozygous (C/A) and homozygous (A/A) mice into dendritic cells and macrophages in the presence of either GMCSF or MCSF respectively. After seven days of *in vitro* culture, we stimulated them with either TNFα or LPS. ELISA analysis revealed that LPS stimulation produced roughly equal amounts of TNFα, IL-6 and IL-12 by BMDM and BMDCs of both C/C and A/A mice (**Figure 3.3A**). Similar results were obtained following TNFα stimulation (IL-6 and IL-12 were roughly similar for both C/C and A/A littermates) as shown in **Figure 3.3B**.

In addition, we also analyzed the DCs and macrophages by flow cytometry for their activation status (as determined by upregulation of CD40, CD80, CD86 and MHC-Class II) after 48 hours of stimulation with LPS and TNFα. While both DCs and macrophages showed upregulation (of CD40, CD80, CD86 and MHC-Class II) upon stimulation, the levels of expression were comparable amongst C/C, C/A and A/A littermates (**Figure 3.3C** and **Figure 3.3D**).

Taken together, these data indicated that the C103A deubiquitinase domain is not involved in limiting inducible NF-κB activation.

3.3.5: Response of A20 OTU knock-in mice to LPS shock

The perinatal lethality manifested by lack of A20 is largely mediated by TLR based signaling. Thus, A20 is important for restricting LPS induced inflammation *in vivo* and we wanted to determine the physiological contribution of the deubiquitinase activity of A20 in LPS dependent responses. We therefore challenged the C/C and A/A knock-in mice with LPS using

established protocols and observed that both WT and the A/A knock-in mice succumbed to LPS shock (**Figure 3.3E**). We also analyzed the serum levels of the key acute phase cytokines (TNFα, IL-6 and IL-12) after LPS injection and found that while TNFα peaked after 1 hour, IL-6 and IL-12 peaked later in agreement with previous studies. However, there was no significant difference in the levels of the cytokines between WT and the A/A mice after LPS shock (**Figure 3.3F**). Hence, the deubiquitinating function of A20 does not play an important role in limiting inflammatory responses *in vivo* following LPS administration.

3.3.6: Similar activation of NF-κB in BMDMs from both wild type and homozygous mice

Before biochemically testing the activation of NF-κB in wild-type and homozygous mice , we first showed that the C103A mutation eliminates deubiquitinase activity in A20 **(Figure 3.4A)**. A20 analogs (wild type and C103A-A20) were immunoprecipitated from LPS-stimulated BMDMs of wild-type and homozygous A/A mice and an in vitro deubiquitinase assay was carried out with recombinant K48 or K63-linked polyubiquitin chains **(Figure 3.4A).** Our results clearly demonstrate that the C103A mutation eliminates DUB activity of A20, as C103A-A20 is unable to deubiquitinate either K48-linked or K63-linked ubiquitin chains in the in vitro DUB assay.

Next, we then wanted to test the role of the A20 deubiquitinase activity in NF-κB activation in cells stimulated with TNFα. We isolated and cultured BMDMs from C/C and A/A knock-in mice, stimulated them with TNFα, and prepared nuclear fractions to test NF-κB binding to DNA by EMSA (**Figure 3.4B**). Both the WT and the A/A BMDMs showed elevated (but similar) binding of p65-p50 heterodimers to the DNA following stimulation. The nuclear extract isolated after stimulating the WT BMDMs for 30 minutes was used for the super-shift assay and analysis with the unlabeled probe. Western blotting showed similar activation of NF-κB as tracked by degradation of IκBα. pJNK, p38 and pERK signaling were also found to be similar between wild-type and A/A littermates. As RIP1 in stimulated cells has been suggested to be

deubiquitinated by A20, we immunoprecipitated RIP1 from stimulated cells and tested it for ubiquitination. Both the WT and the C103A-A20 was recruited to RIP1 in roughly equal amounts and similar kinetics following TNFα stimulation (**Figure 3.4C**). Intriguingly, we also found roughly equal amounts of ubiquitinated RIP1 in the BMDMs of both genotypes following TNFα stimulation. These results suggest that the deubiquitinase activity of A20 does not affect RIP1 ubiquitination in cells.

Similar results were also obtained upon stimulation of BMDMs with LPS. Both the wild type and the homozygous mutant showed similar activation of NF-κB as analyzed by EMSA (**Figure 3.4D**). The result is consistent with the kinetics of disappearance of IκBα as analyzed by western blotting (**Figure 3.4E**). Since TRAF6 has been shown to be deubiquitinated by A20 in response to stimulation with LPS, we immunoprecipitated TRAF6 from stimulated cells and found that the levels of ubiquitination (and K63-linked ubiquitination) of TRAF6 is more in BMDMs derived from A/A mice as compared to their wild-type littermates after stimulation with LPS. The kinetics and amounts of WT and the C103A-A20 recruited to TRAF6 was similar after stimulation with LPS (**Figure 3.4E**), showing the structural integrity of C103-A20 protein.

3.4: Discussion

Ubiquitination is a reversible post-translational modification that involves the attachment of one or more ubiquitin monomers to the substrate. This reversible post-translational modification involves the activity of ubiquitinating enzymes (like kinases) that add ubiquitin chains to the substrate, and deubiquitinases (like phosphatases) that disassembles these ubiquitin chains. While ubiquitination is best known to target the substrate protein for proteasome-mediated degradation, recent studies have suggested various nonproteolytic consequences of ubiquitination. K48-linked ubiquitination of substrates targets them for degradation, while

regulatory K63-linked ubiquitination has been shown to activate various kinases including IKK *in vitro* leading to activation of NF-κB. Thus ubiquitination has emerged as a mechanism whereby covalent attachment of diverse polyubiquitin chains lead to different biological outcomes. However while it is clear that K48-linked polyubiquitination leads to proteasomal degradation, the physiological consequences of regulatory K63-linked ubiquitination remains to be established in animal models [123].

A20 is one of the best characterized of all enzymes that acts on K63-linked ubiquitin chains. It is believed to deubiquitinate K63-linked ubiquitin chains and this deubiquitinase activity has been proposed to be essential in the downregulation of NF-κB signaling for both TNFα and LPS mediated pathways. However, this is difficult to reconcile with the observation that the OTU domain of A20 disassembles K48-linked polyubiquitin chains much more potently as compared to K63- linked polyubiquitin chains in an *in vitro* setting [209, 210]. This is an intriguing discrepancy as the deubiquitinating function of A20 would be expected to be specific to K63-linked ubiquitin chains for it to play such a profound role in a tightly regulated process such as NF-κB activation.

While the physiological role of the deubiquitinating property of A20 requires further study, there is no doubt that A20 is essential in limiting inflammation, very likely through NF-κB activation. Dysregulation of A20 has now been implicated in various autoimmune diseases and cancer. Polymorphisms in the A20 locus increases disease susceptibility in multiple autoimmune diseases including type I diabetes, psoriasis, rheumatoid, arthritis, systemic lupus erythematosus, etc. [20] Recently, an A20 polymorphisms (A125V and F127C) were discovered in the DUB domain which increases susceptibility to autoimmunity by impairing A20 mediated deubiquitination [343]. It has been speculated that the Cys103, the residue primary important for deubiquitinating property could be important in the physiological mechanism of A20.

To directly ascertain the contribution of the deubiquitinating role of A20 in its biological function, we replaced the wild-type A20 gene with a mutant form that lacks deubiquitinating activity. Our studies of these knock-in mice show that abolishing the deubiquitinase activity of A20 does not affect its ability to be recruited to RIP1 or TRAF6, and hence the mutation does not affect the protein structure. Furthermore, it is important to appreciate that the phenotypes observed in the knock-in mice are the result of the C103A mutation regulated by endogenous mechanisms as compared to previous *in vitro* experiments that relied on overexpression systems and exogenous gene regulatory mechanisms.

The mutant protein in the homozygous mouse and the wild type protein in the wild type littermate are induced in equal amounts following inflammatory stimuli. This is what is typically expected in a gene targeted knock-in system. However, as A20 expression is directly induced by NF-κB signaling, A20's capacity to downregulate NF-κB signals exemplifies a negative feedback mechanism. Moreover, previous studies of hypomorphic A20+/- cells suggest that the ability of A20 to negatively regulate NF-κB signaling is directly dependent on the amount of A20 in the cells [319, 362]. Thus if Cys103 was indeed important for restricting NF-κB signaling, it would be expected that the levels of the mutant protein would be increased in the homozygous mice. This was the first hint that Cys103 may not be a playing an important role in downregulating NF-κB signaling in a physiological context. This is not surprising as the deubiquitinase activity mediated by Cys103 has been shown to promiscuously cleave unanchored K11-, K48- and K63-linked polyubiquitin chains in addition to disassembling K63-linked chains from *in vitro* substrates [196, 200, 209, 210, 363].

A20's Cys103 residue has been proposed to be crucial for down regulating NF-κB signaling *in vitro* [196, 271]. Thus, it was expected that the A20-C103A mice would at least partially resemble the $A20^{-/-}$mice [134]. However, while $A20^{-/-}$mice develop spontaneous multi-organ inflammation and perinatal lethality, the A20-C103A mice are grossly normal in the basal

state. While A20 $^{-/-}$ mice have an increased number of myeloid lineage cells in the bone marrow and spleen, we found an overall normal population of both myeloid and lymphoid cells in the bone marrow, spleen and thymus in the C/C, C/A and A/A littermates. Thus, the deubiquitinating activity of A20 does not play a major role in the maintenance of basal immune homeostasis. Moreover even following LPS stimulation, the response of the AA knock-ins were similar to wild type littermates.

Our results are in agreement with a recent study that also found that mice lacking the deubiquitinating activity of A20 are grossly normal for at least four months and contained normal number of lymphocytes, however older mice (6 months) develop splenomegaly and show increased number of myeloid cells [281]. It is therefore possible that Cys103 plays a role in older mice. However as our data shows, it does not play a role in directly inhibiting NF-κB. In this regard, defects in the NF-κB pathway would manifest itself in much younger mice. For example, it is worth pointing out that complete knockouts of A20 are perinatally lethal from persistent inflammation resulting from NF-κB activation. In agreement with the previous study, we also find that in addition to NF-κB signaling, other pathways like pJNK, p38 and pERK signaling were also comparable in BMDMs derived from wild-type and A/A littermates. Thus, the deubiquitinase function of A20 is not responsible for inhibiting TNF-induced cell death.

The study also found RIP1 to be slightly more ubiquitinated in A/A cells as compared to our study [281]. The discrepancy in the levels of RIP1 ubiquitination might be explained by the different cell lines and experimental conditions used by them and us. While they used MEFs, we used BMDMs for our study. Experimentally, we checked ubiquitin levels of total cellular RIP1 while they examined ubiquitin levels of RIP1 recruited to the TNFR complex. The slightly different levels of ubiquitination of RIP1 in the two studies might be attributed to these causes.

We found that the levels of ubiquitination (and K63-linked ubiquitination) of TRAF6 is more in the A/A mice following LPS stimulation. Importantly, both the wild-type and the

C103A-A20 was recruited to TRAF6 in roughly equal amounts and with similar kinetics following stimulation with LPS, showing that the C103A-A20 is not structurally altered. However, the kinetics of degradation of IκBα as determined by western-blotting is not affected in the A/A mice. This shows that while the Cys103 might play a role in deubiquitinating TRAF6, it does not play a role in inhibiting NF-κB. It is also possible to argue that regulatory ubiquitination of TRAF6 is not intrinsic to the activation of NF-κB following LPS stimulation.

These investigators also concluded, similar to our observations, that the deubiquitinase activity of A20 was not required for prevention of spontaneous cachexia and premature death. Surprisingly however, this study focused exclusively on the role of Cys103 in limiting TNFα induced NF-κB activation, despite previous reports from the same group that A20 functioned *in vivo* in limiting TLR/MyD88-dependent pathways [200, 310]. Hence, our study focusing on the role of A20 C103 on LPS/TLR-mediated responses is vital for understanding the physiological role of the deubiquitinating property of A20 in restricting persistent TLR mediated activation of NF-κB.

As our studies clearly demonstrate, the C103A mutation eliminates DUB activity of A20. Inspite of that, NF-κB activation is not altered in A/A mice. Hence, it is safe to conclude that the deubiquitinase activity of A20 is dispensable for its well-documented role in NF-κB signalling. However, the E3 ligase function of the ZnF4 domain could partially compensate for the lack of deubiquitinating function *in vivo*, even though A20 is only supposed to ubiquitinate substrates such as RIP1 after first deubiquitinating them. Interesting, a recent study with mice lacking the E3 ligase activity demonstrated that neither of the C103 or the ZnF4 motif were singly responsible for all of A20's functions in restricting TNFα signaling, and in the ZnF4 motif appeared more important than the deubiquitinase activity in restricting TNFα signaling in embryonic fibroblasts (MEFs). The mechanism underlying such an observation remains unclear, but also casts doubt on the importance of A20 as a deubiquitinating enzyme. As we know that

the A20 protein is important in inflammatory signaling, these studies suggest that A20 plays a more important role that does not involve ubiquitination/deubiquitination in exerting its regulatory function in inflammatory signaling. In this respect, the seventh zinc-finger motif (ZnF7) of A20 was recently proposed to be involved in direct inhibition of IKK by a non-catalytic mechanism [309]. Thus, it is probably fair to say that significant additional studies will be needed to determine the actual role that A20 plays in regulating inflammatory/NF-κB signaling.

3.5: Materials and Methods

3.5.1: Generation of A20C103A knock-in mice

The A20C103A mice were generated using conventional gene-targeting approaches. In brief, exon 3 harboring Cys103 was PCRed out using a bacterial artificial chromosome (BAC) bearing the A20 gene and cloned into a PL452 plasmid having a Lox-Neo-Lox cassette. Standard site-directed mutagenesis using Stratagene kits was used to mutate Cys103 to Alanine. The relevant part of the A20 construct was then transferred from PL452 plasmid to the BAC using homologous recombination. This BAC construct which now harbors the Lox-Ne0-Lox cassette along with a C103A mutation was retrieved into a pMCS_DTA plasmid (with diphtheria toxin selection gene) which was linearized and electroporated into CSL2J2, albino C57BL/6J ES cells. Correctly screened ES cell clones were injected into blastocysts derived from C57BL/6 mice to give rise to chimaeras (in the Columbia University Transgenic Core facility). Genetic transmission of the allele was confirmed by PCR and subsequent sequencing to confirm the presence of the C103A mutation. The LoxP flanked neomycin sequences were deleted by crossing the transgenic mice with the EIIA-Cre deleter mice. The C/A heterozygous mice were bred to generate age-matched C/C wild type and A/A homozygous mice for various experiments.

3.5.2: Cells

Bone marrow cells of 6-8 weeks aged matched wild type (C/C), heterozygous (C/A) and homozygous (A/A) mice were differentiated into dendritic cells and macrophages in the presence of either GMCSF or MCSF respectively. In the *in vitro* experiments, BMDMs were stimulated with 10ng/ml TNFα or 1ug/ml LPS

3.5.3: Biochemical experiments

Immunoprecipitations and western blotting were performed as has been described previously [196]. For the in vitro DUB assay, immunoprecipitated A20 was incubated 37 °C with recombinant K48 or K63-linked polyubiquitin chains in 20µl of DUB buffer (25 mM Hepes pH 7.4, 1 mM DTT, and 5 mM MgCl2) for 1 hour. Samples were then subject to western-blot analysis with the indicated antibodies. Gel shift assay was done using the Li-cor EMSA kit as per the user manual. The κB probe was purchased from Li-cor while the Oct1 probe was custom-made. Antibodies used in this study include anti-murine RIP monoclonals (BD Bioscience, clone 610458), ubiquitin (Santa Cruz, sc-8017), (IκBα (Santa Cruz, sc-371), A20 (Imgenex, 161A), βtubulin (Abcam), .

3.5.4: LPS-induced shock

LPS was injected intraperitonially at a concentration of 50 mgkg-1 of mice body weight. The mice were monitored for survival every eight hours. In a separate experiment, the mice were bled 1 h, 2 h and 6h after LPS treatment and the serum cytokine levels measured by ELISA [186].

3.5.5: Flow cytometry, ELlSA and qRT–PCR

Cell preparations, flow cytometric and ELISA analyses were performed as previously described [186], [319]. ELISA was performed with kits from BD Biosciences. Cells were analyzed by flow cytometry using LSRII and Flowjo software (Tree Star).

BMDMs were stimulated and RNA was isolated using the RNA Easy kit (Qiagen). RNA was reverse transcribed (SuperScriptIII reverse transcriptase; Invitrogen-Life Technologies), and SYBR Green master mix (QuantiTect SYBR green; Invotrogen) was used to quantify relative gene expression of the corresponding mRNA with normalization to β-actin (by using the formula 2^-(CT gene of interest – CT actin)) [186] .

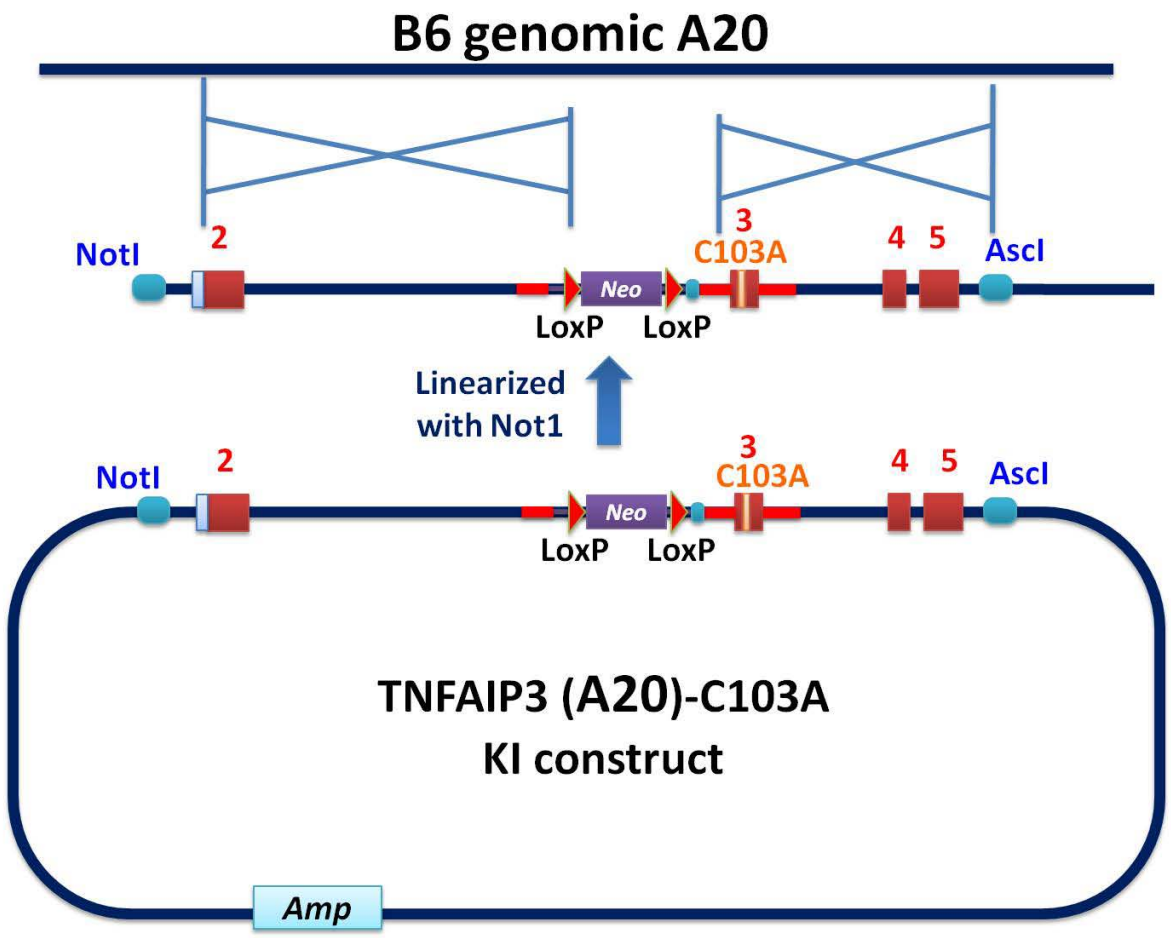


Figure 3.1A: Gene targeting strategy for generating the A20OTU/OTU mice: The plasmid construct was linearized using Not1 and chimeras were obtained after homologous recombination with the B6-genomic A20 in the ES cells.

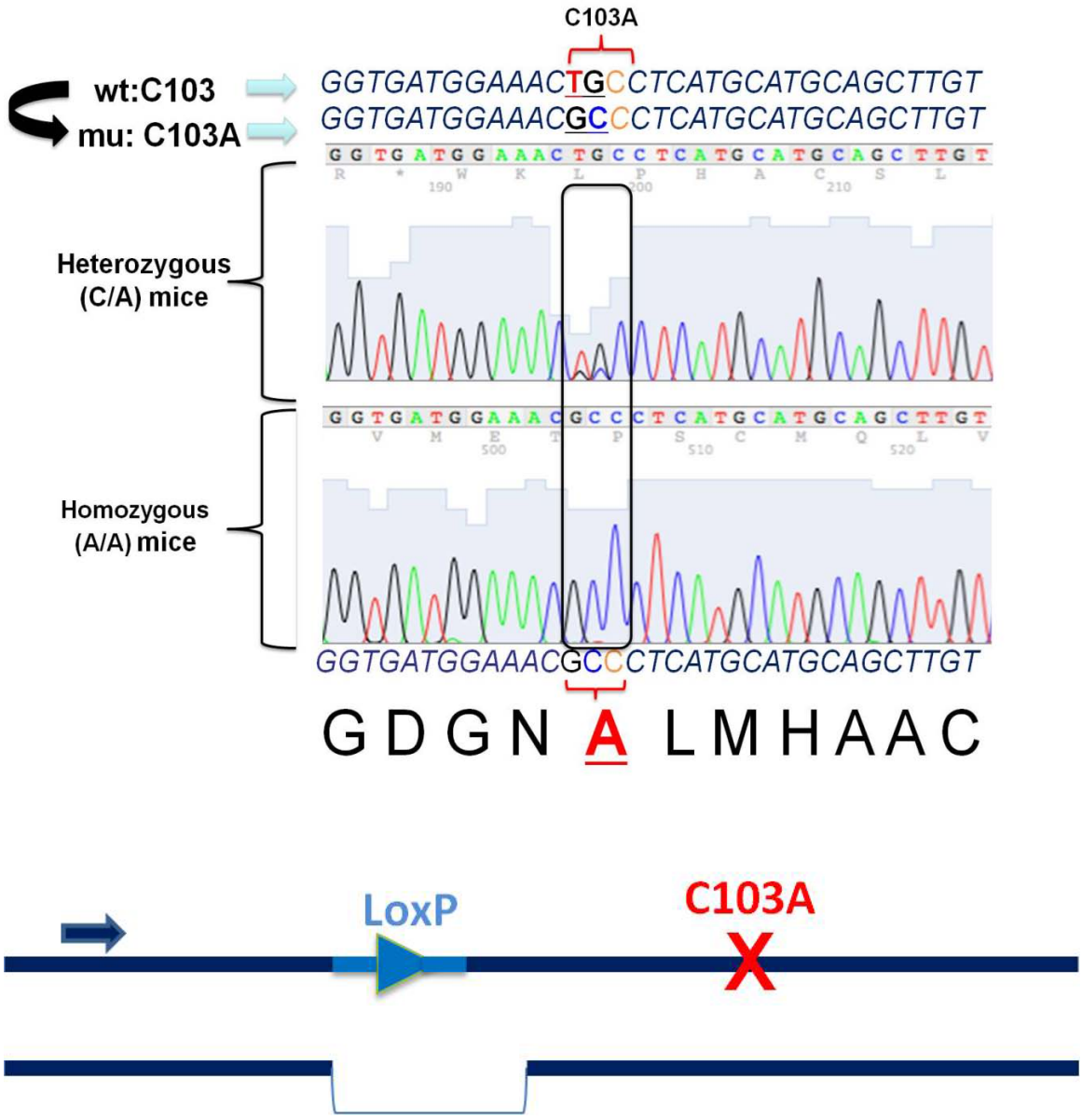


Figure 3.1B: Sequencing of genomic DNA from homozygous (A/A) and heterozygous littermates (C/A). (Schematic of sequencing strategy: Blue denotes the LoxP sequence and the plasmid sequences of the targeted locus; purple denotes the genomic sequence; red 'X' demotes the C103A mutation in the targeted locus. The sequencing primer used has a genomic sequence. Hence, sequences of the DNA from the homozygous A/A mice show only the 'alanine'; while sequences of the DNA from the heterozygous C/A mice show both 'cysteine' and the 'alanine').

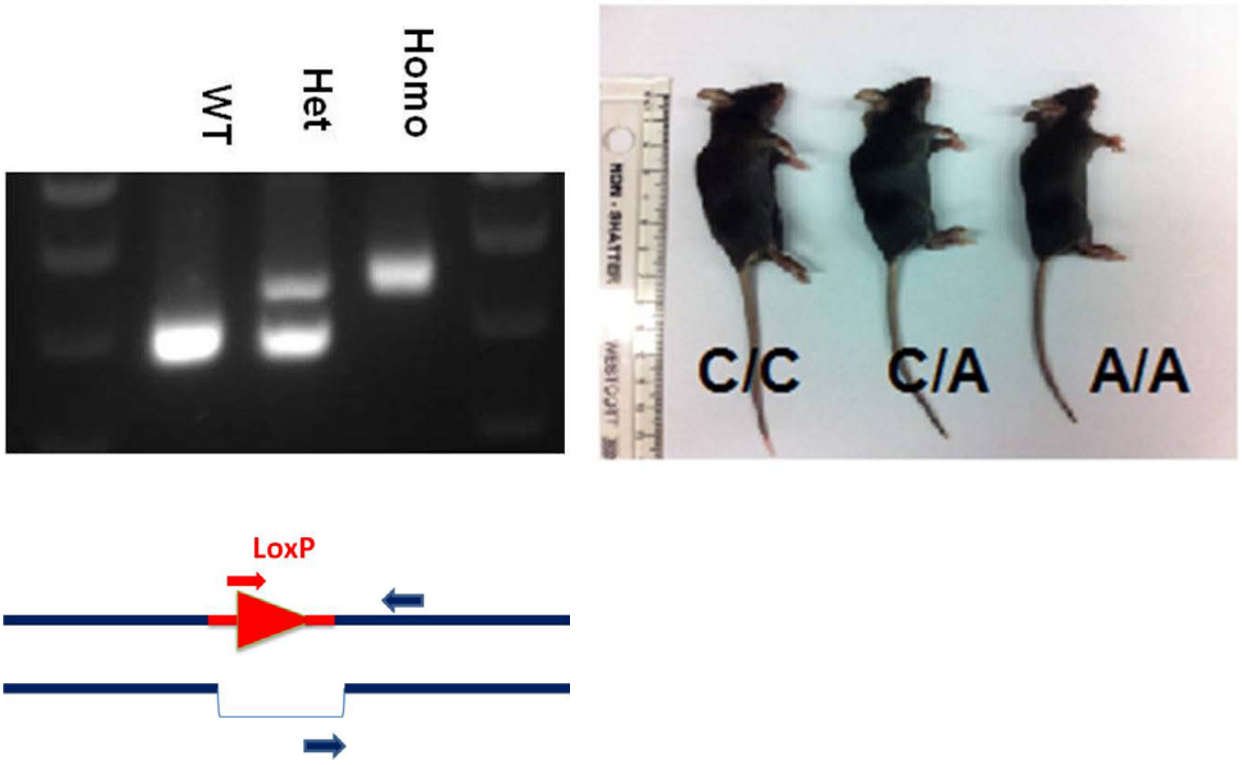


Figure 3.1C: Genotyping strategy and photograph of wild type, heterozygous and homozygous littermates (Schematic of genotyping strategy: Red denotes the LoxP sequence and the plasmid sequences of the targeted locus; purple denotes the genomic sequence. Forward primers used are Lox (red) and wild-type primer (purple); hence the homozygous gives a slow migrating upper band while the wild type yields the lower band)

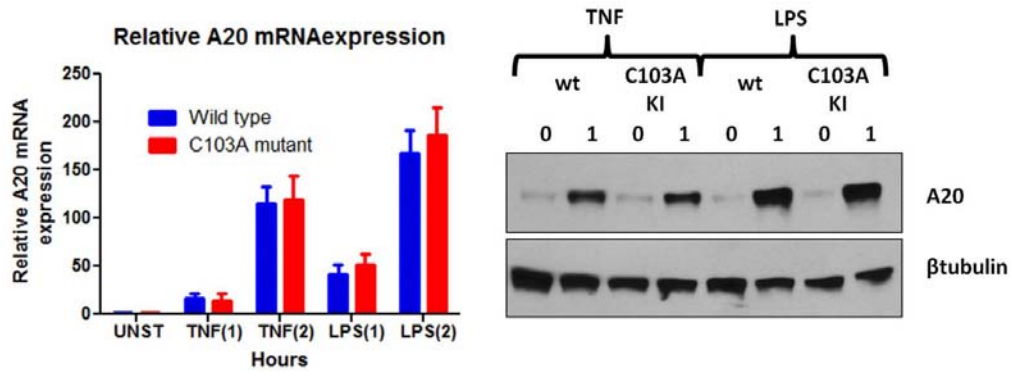


Figure 3.1D: Relative A20 mRNA and protein expression in macrophages from littermates. Error bars represent standard deviation. Data represents 3 mice for each genotype.

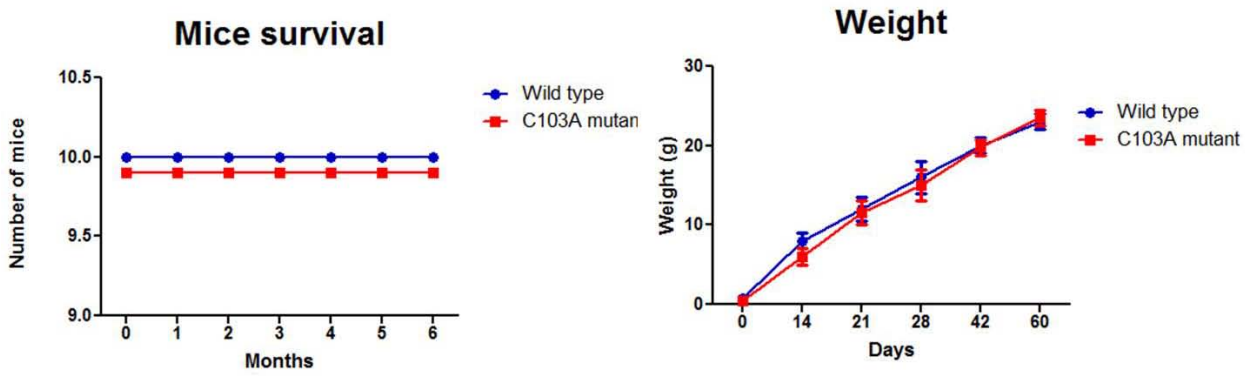


Figure 3.1E: Normal survival curves and weight of littermates

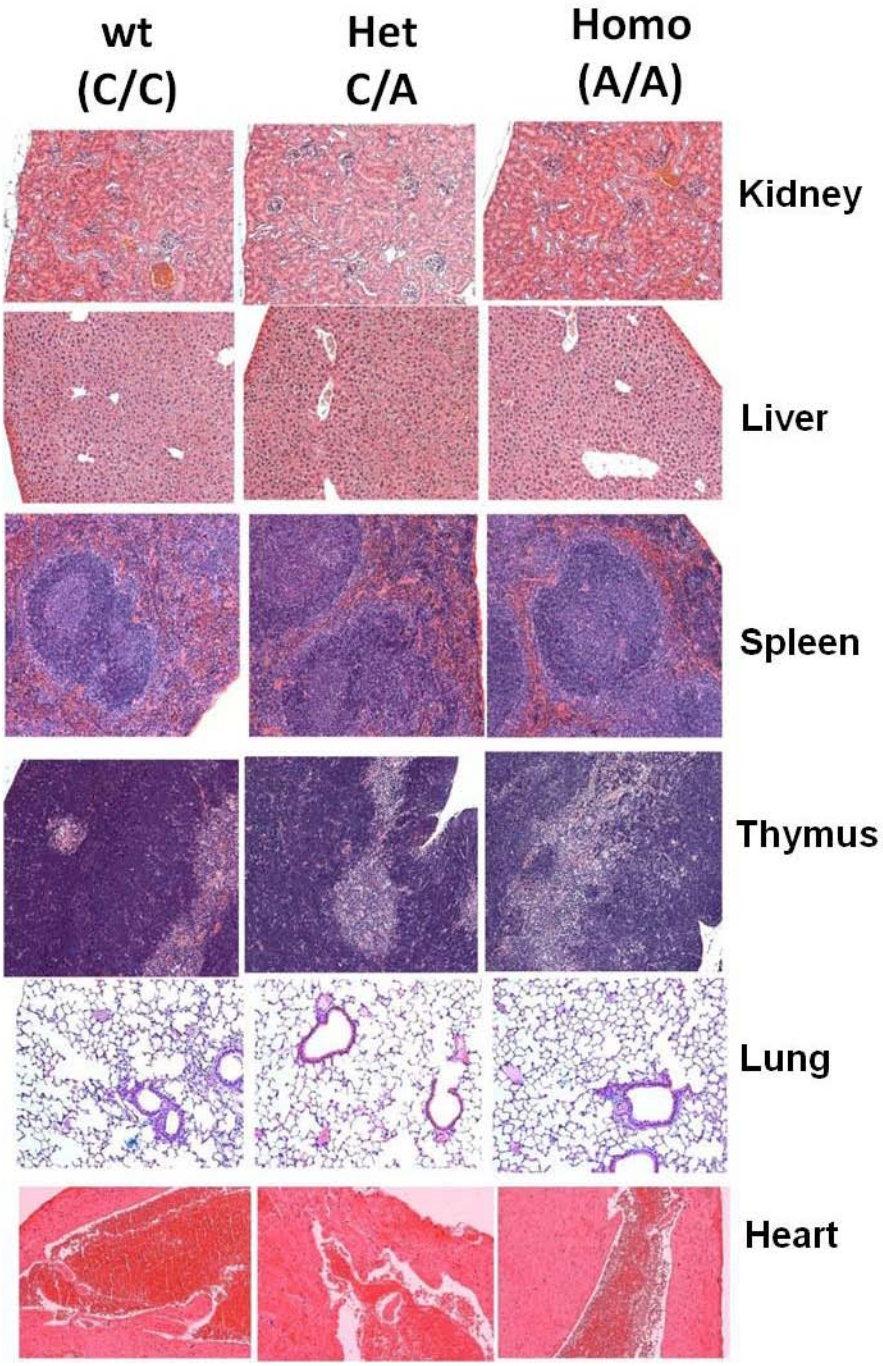


Figure 3.2A: Histology of kidney, liver, spleen, thymus, lung and heart of littermates

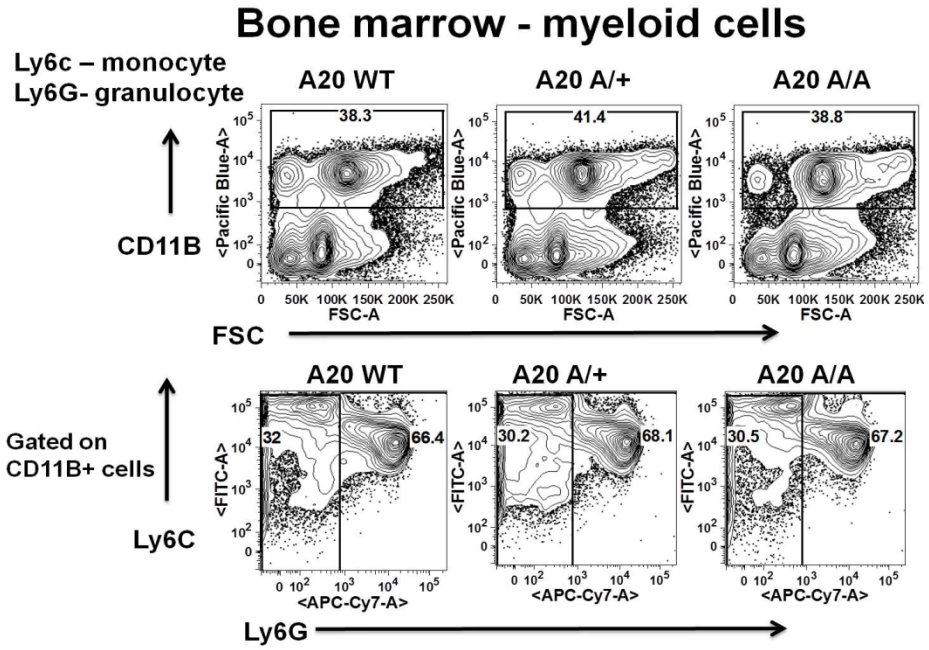


Figure 3.2B: Characterizing the myeloid cells of the bone marrow

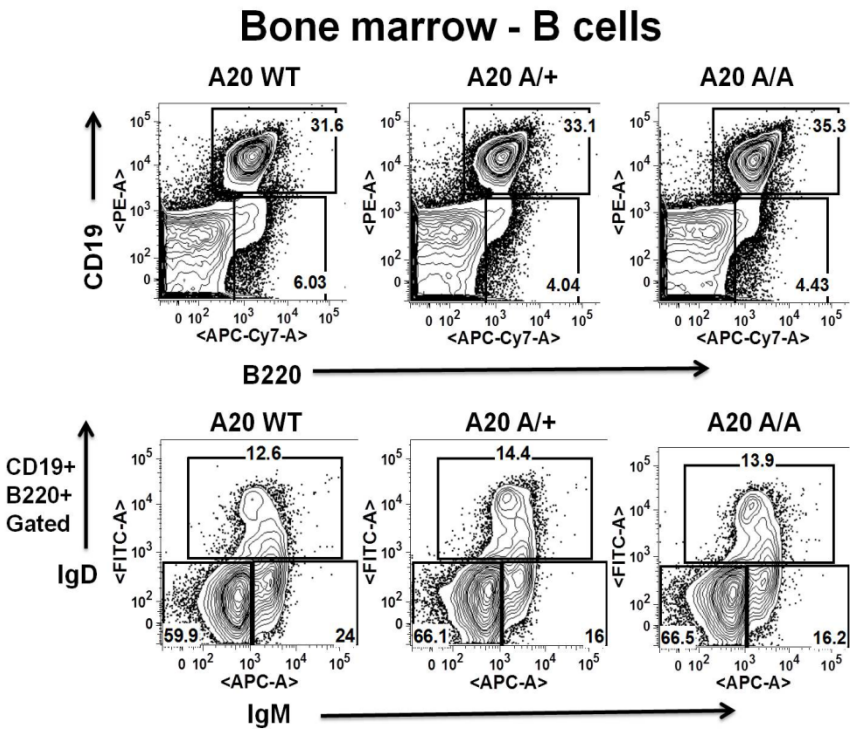


Figure 3.2C: Characterizing the B cells of the bone marrow

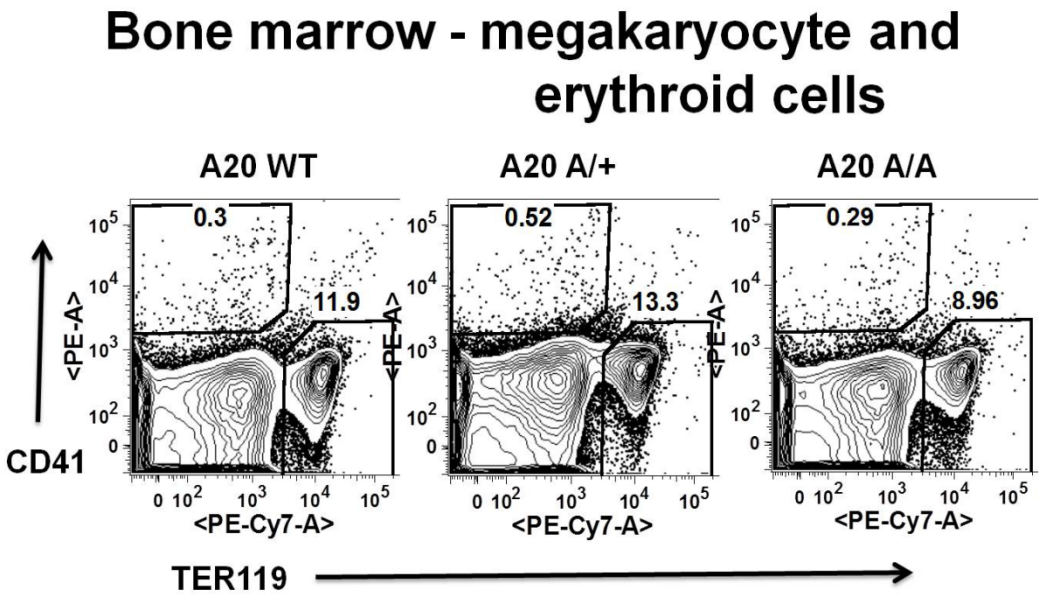


Figure 3.2D: Characterizing the megakaryocytes and erythroid cells of the bone marrow

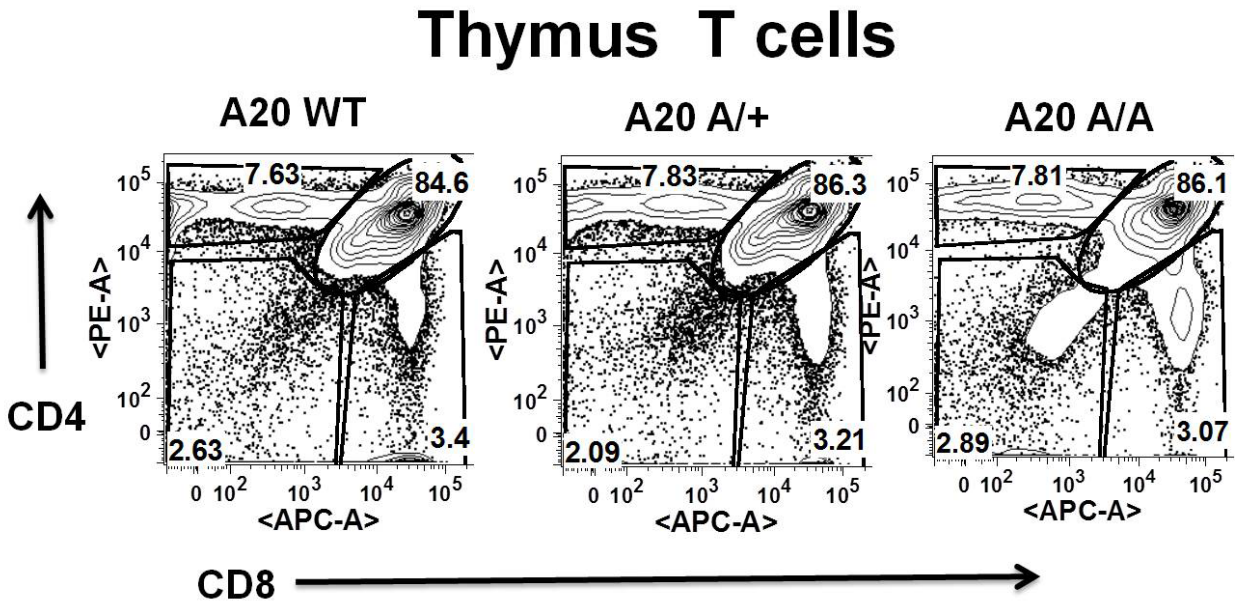


Figure 3.2E: Characterizing the CD4 and CD8 cell lineage of thymus

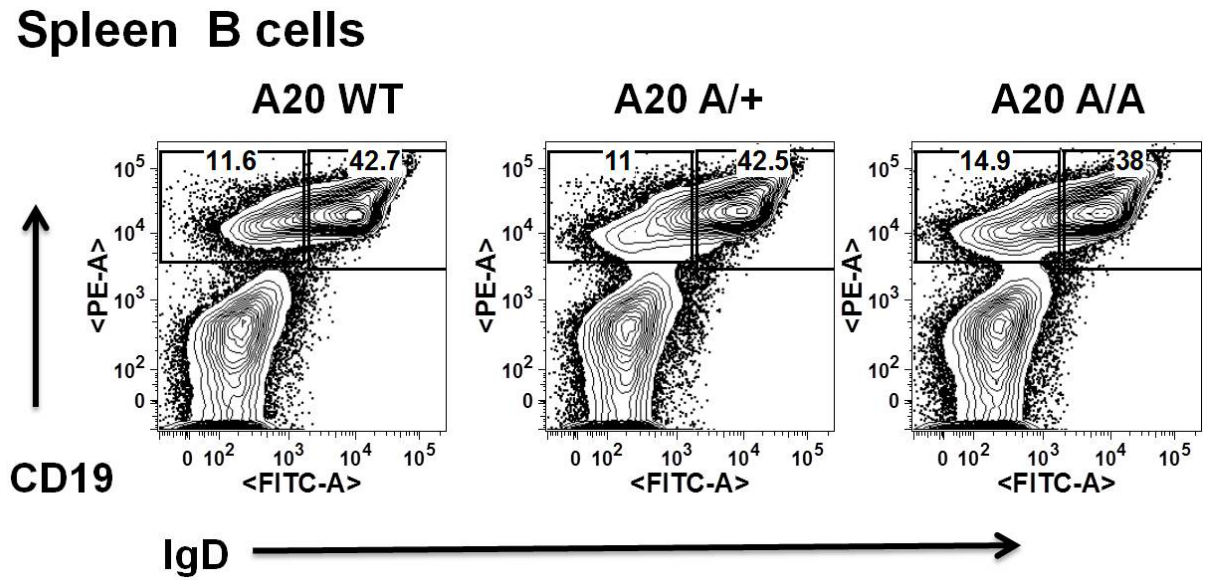


Figure 3.2F: Characterizing the B cells of the spleen

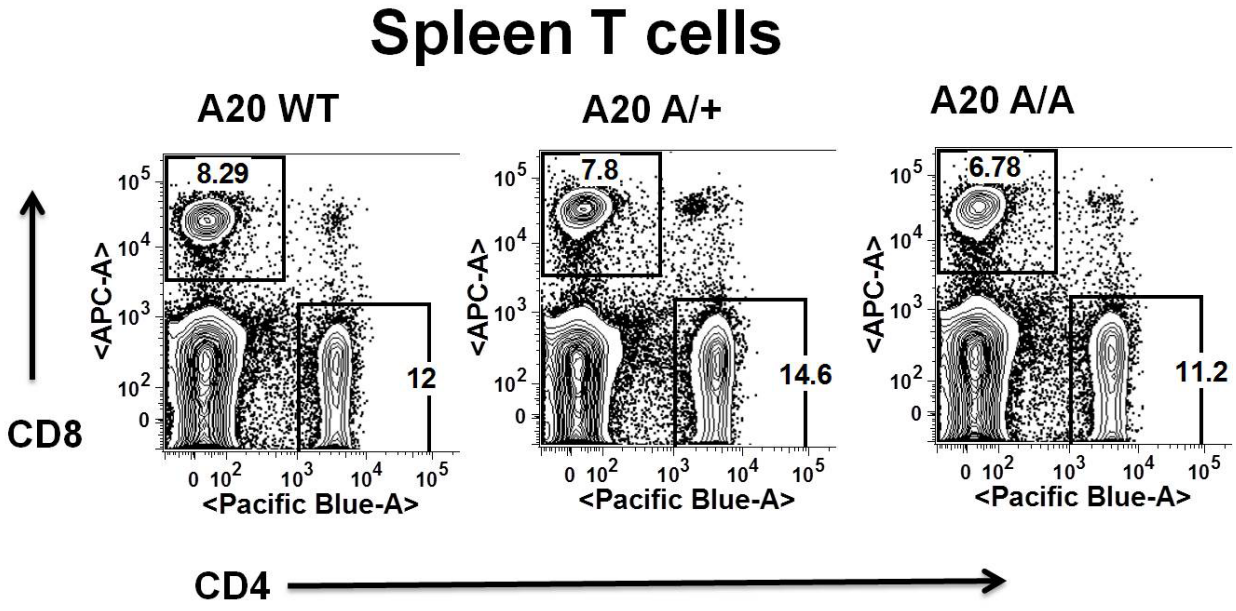


Figure 3.2G: Characterizing the T cells of the spleen

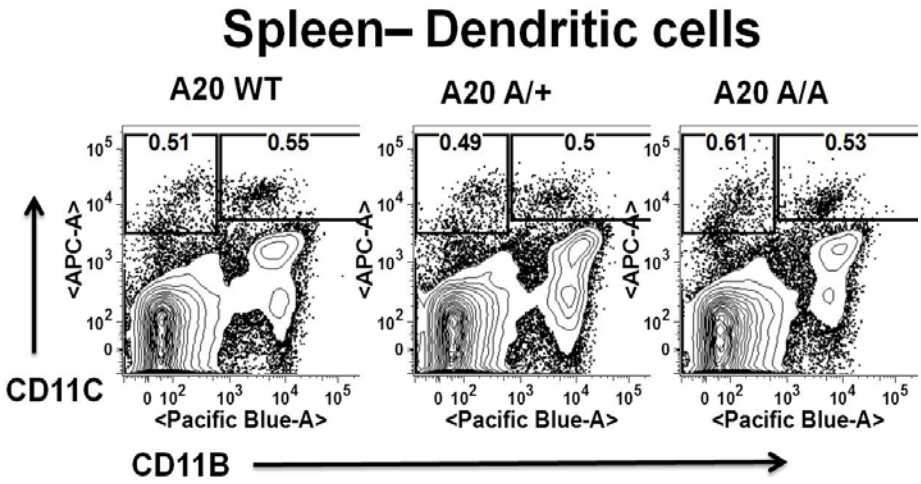


Figure 3.2H: Characterizing the dendritic cells of the spleen

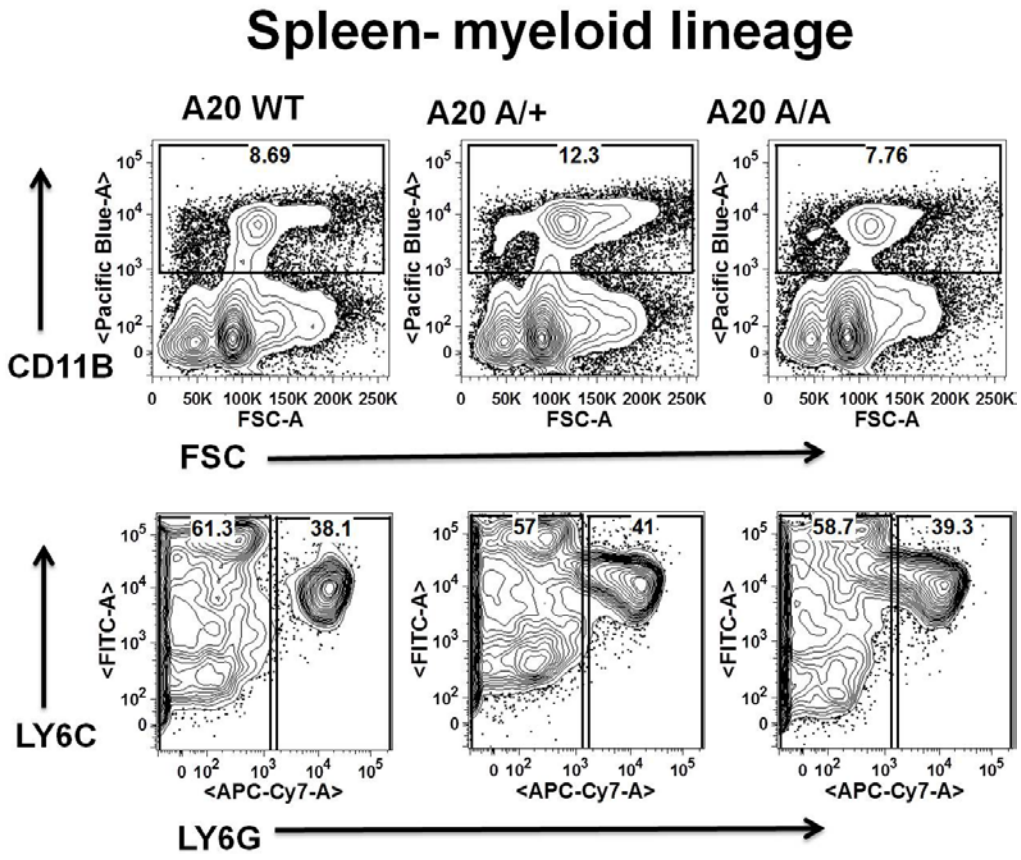


Figure 3.2I: Characterizing the myeloid cells of the spleen

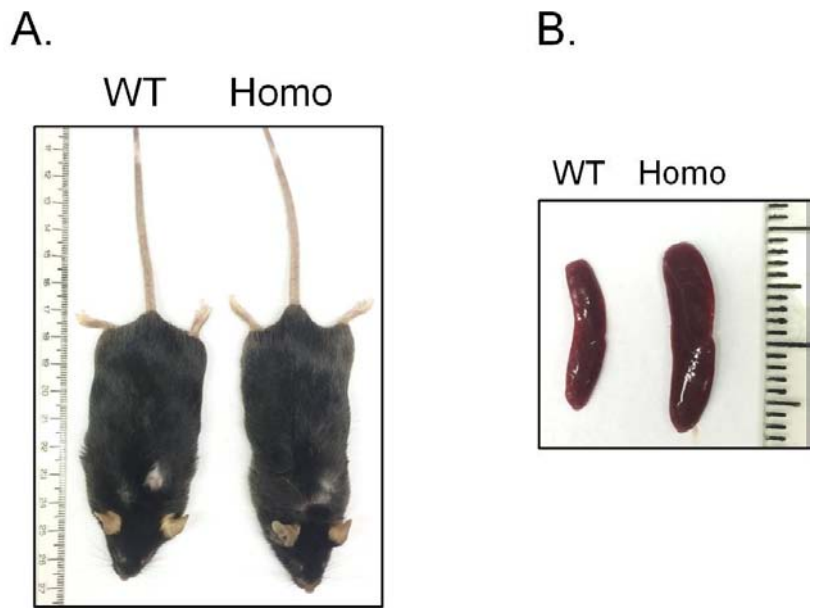


Figure 3.2J: A) Photograph of 6-month old C/C (WT) and A/A (Homo KI) mice B) Photograph of spleen of 6-month old C/C (WT) and A/A (Homo KI) mice

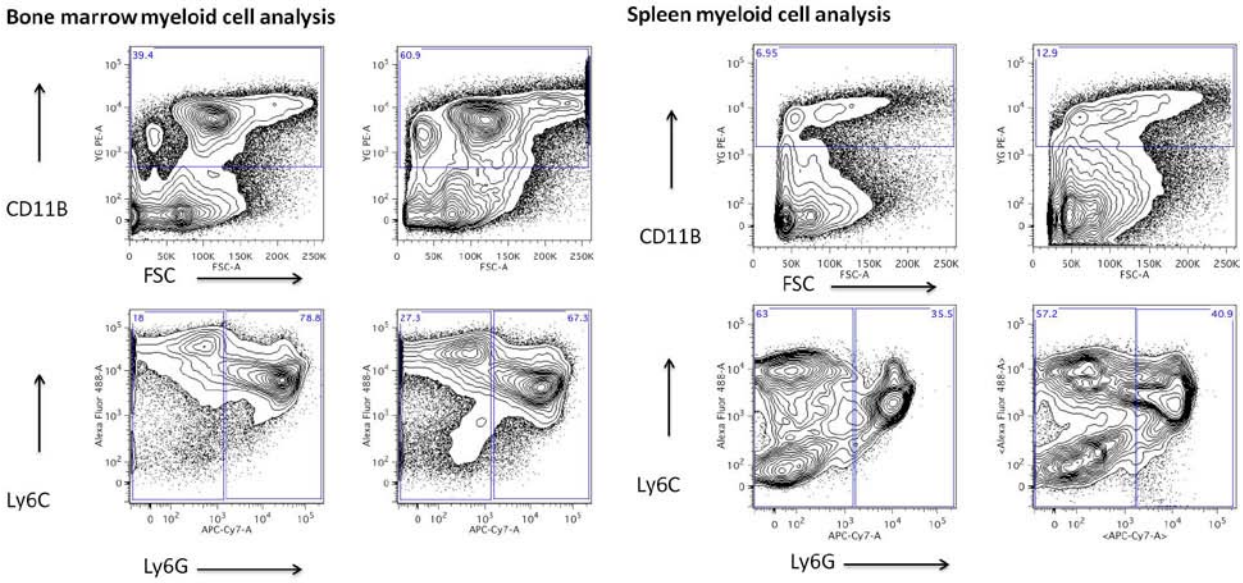


Figure 3.2K: 6-month old A/A (Homo KI) mice show increased numbers of myeloid cells as compared to sex-matched C/C (wild type) littermates

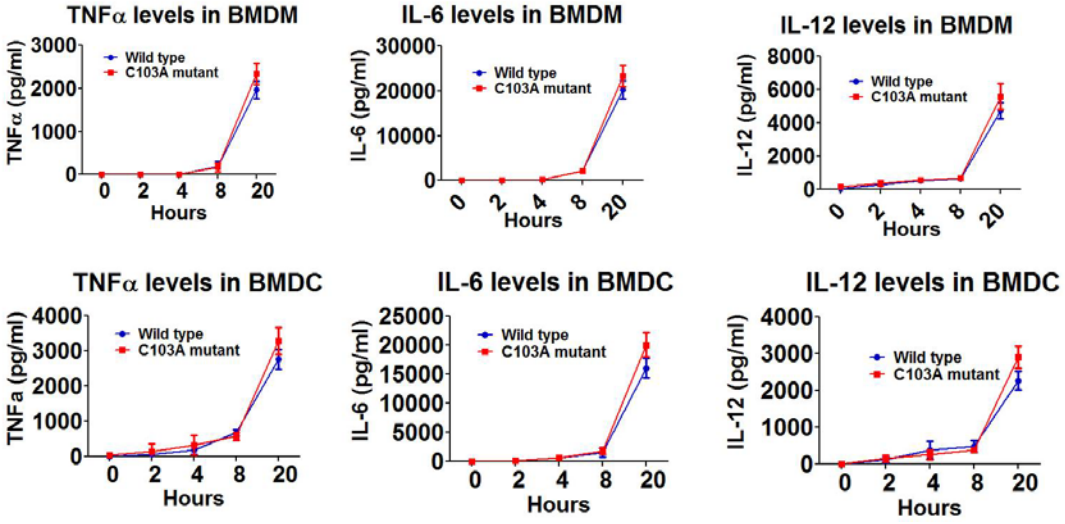


Figure 3.3A: TNFα, IL-6 and IL-12 produced by BMDM and BMDC in response to 1ug/ml LPS was measured by ELISA. Error bars represent standard deviation

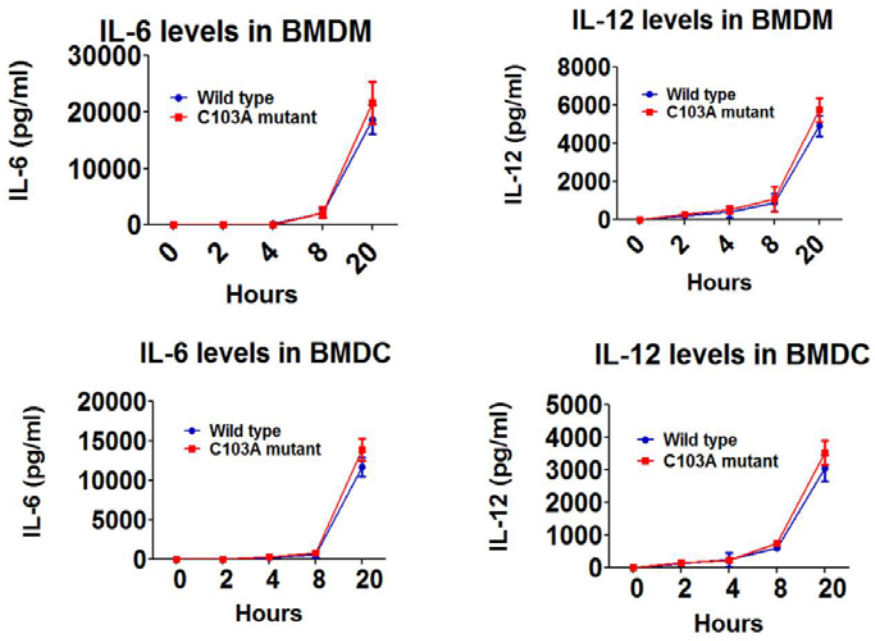


Figure 3.3B: IL-6 and IL-12 produced by BMDM and BMDC in response to 10ng/ml TNFα was measured by ELISA. Error bars represent standard deviation.

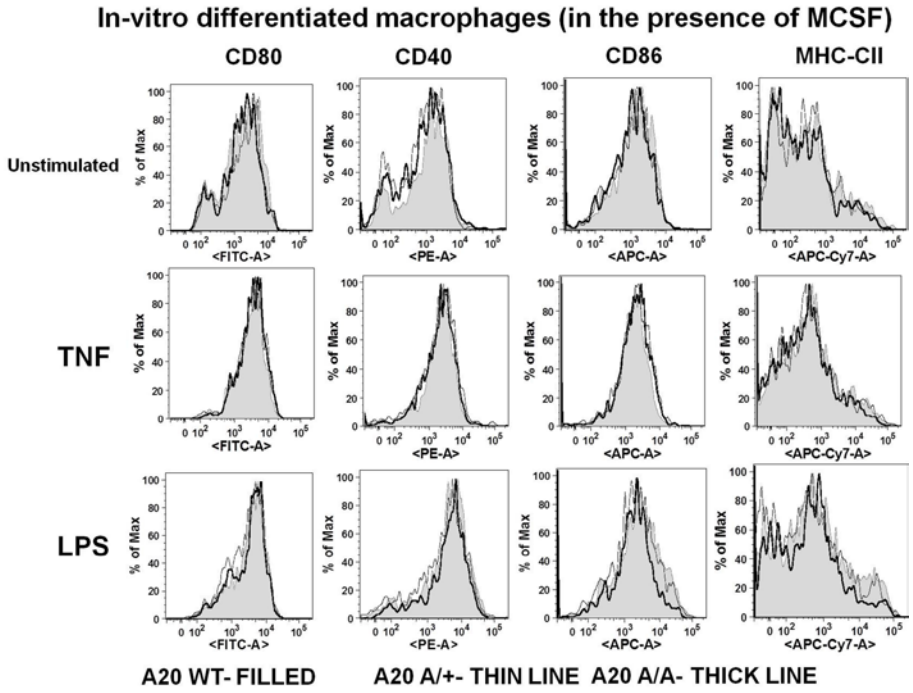


Figure 3.3C: Analysis of activation status of BMDM by flow cytometry

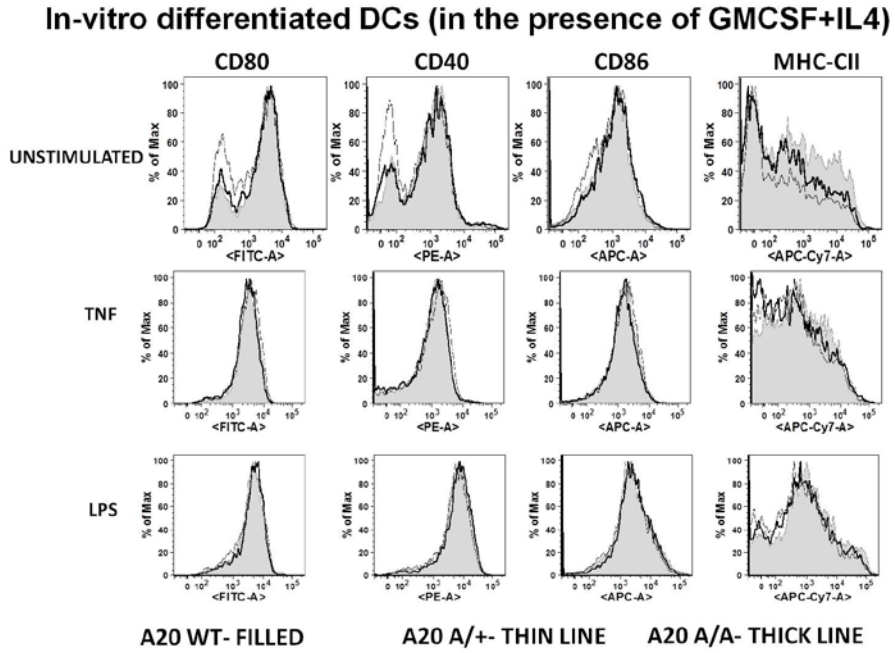


Figure 3.3D: Analysis of activation status of BMDC by flow cytometry

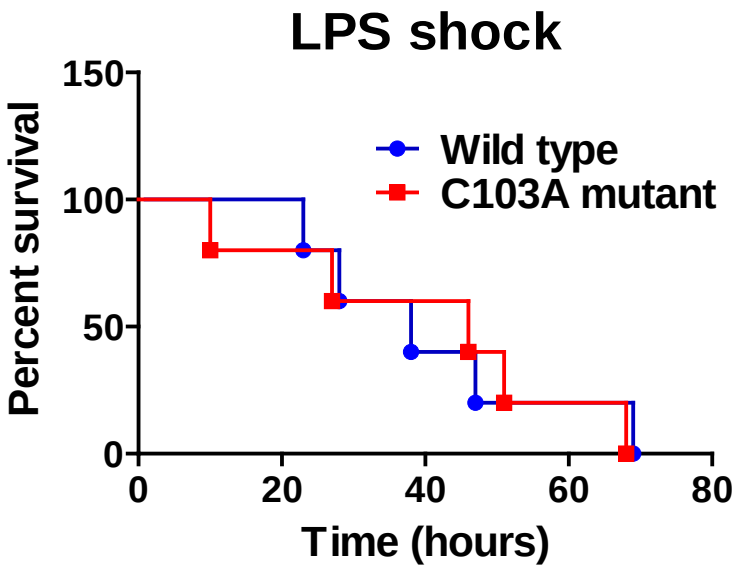


Figure 3.3E: Age and sex matched mice (n=5; experiment repeated thrice) were given intraperitonial injections of 50ug/ml LPS and survival was scored every 6 hours for 72 hours.

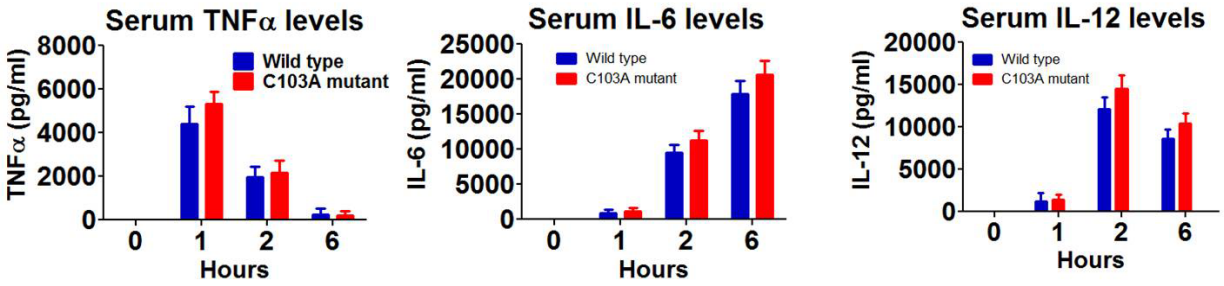


Figure 3.3F: Serum cytokine levels of TNFα, IL-6 and IL-12 in mice injected with 50ug/ml LPS was measured by ELISA for indicated time points. Error bars represent standard deviation

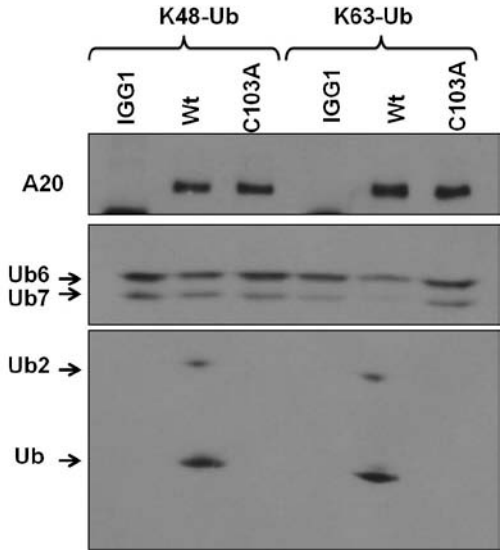


Figure 3.4A: C103A mutation eliminates deubiquitinase activity of A20 as determined by in vitro deubiquitinase assay

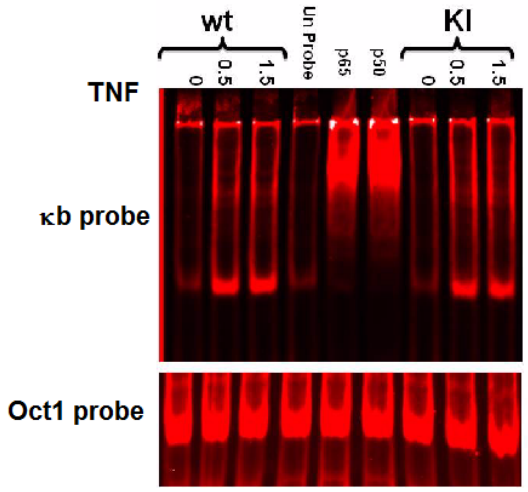


Figure 3.4B: NF-κB binding to DNA in response to TNFα stimulation was analyzed by Electrophoretic mobility shift assay. EMSA was performed with nuclear extracts after stimulating BMDMs isolated from wild type and homozygous littermates with 10ng/ml of TNFα for the indicated time points. The nuclear extract isolated at 30 minutes from wild type BMDMs was used for the super-shift assay (with p65 and p50 antibodies) and analysis with the unlabelled κB probe.

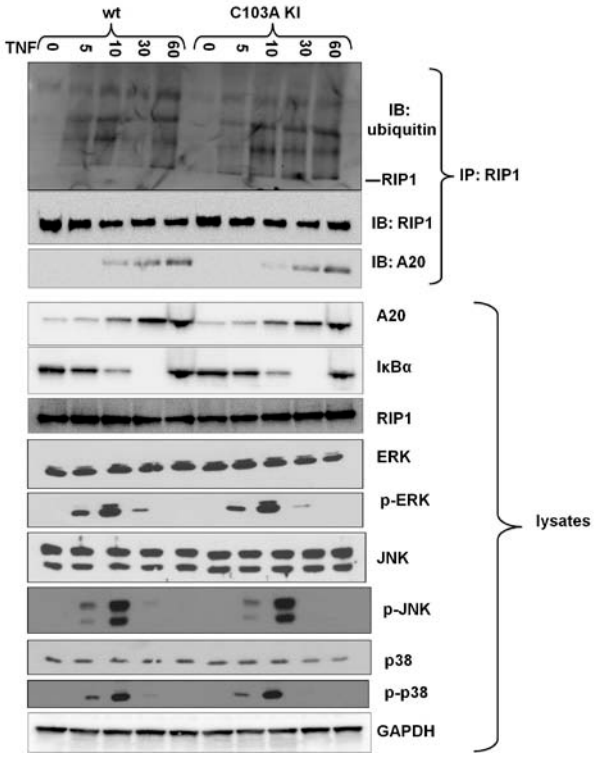


Figure 3.4C: BMDMs isolated from wild type and homozygous littermates were stimulated with 10ng/ml TNFα for the indicated time points. The cells were lysed and immunoprecipitated with RIP1 antibody and immunoblotted with indicated antibodies.

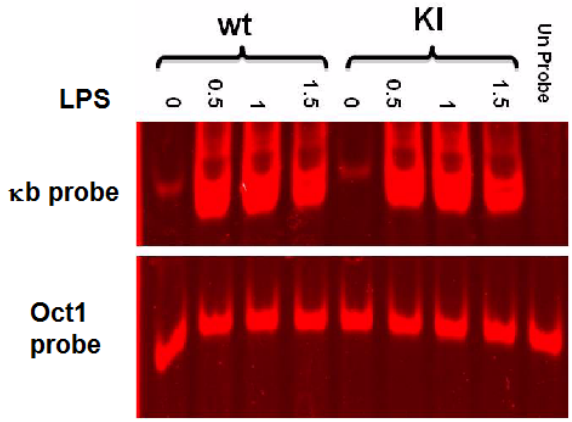


Figure 3.4D: EMSA was performed as in (A); stimulation was with 1ug/ml of LPS.

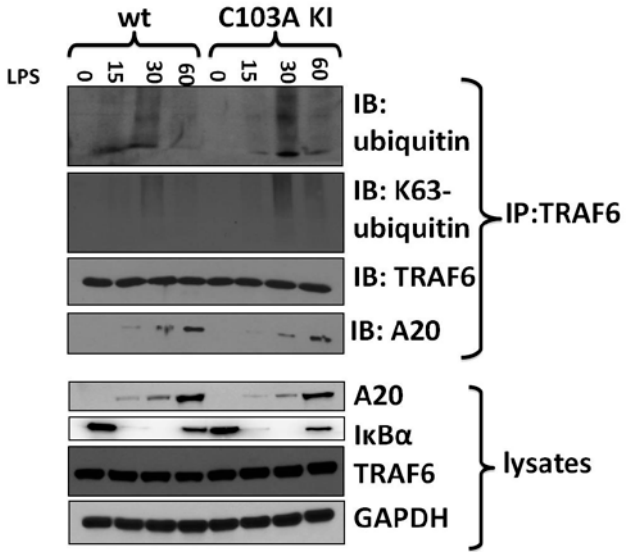


Figure 3.4E: BMDMs isolated from wild type and homozygous littermates were stimulated with 1ug/ml of LPS for the indicated time points. The cells were lysed and immunoprecipitated with TRAF6 antibody and immunoblotted with indicated antibodies.

Chapter 4:

Inhibitors of NF-κB (IκB)

4.1: Inhibitors of NF-κB (IκB)

The IκBs are the master-regulators of NF-κB activity, and they can inhibit NF-κB activity via a negative feedback mechanism [62]. An intriguing historical observation was that the activation of NF-κB was transient in nature and that the inhibition of NF-κB required ongoing protein synthesis [364]. This correlates with observations that the IκBα protein is rapidly regenerated after initial degradation [47] [48, 50, 365]. Additionally, IκBα is transcriptionally regulated by NF-κB [48, 50, 178, 365] and indeed, one of the earliest NF-κB target genes actually encodes for IκBα [50]. Around this time, it was reported that although IκBα has a strong Nuclear Export Signal, it was still found in the nucleus [366] thereby indicating that it is possible that IκBα enters the nucleus and dissociates from the DNA-bound p65:p50 heterodimers. This leads to the export of the heterodimers from the nucleus to the cytoplasm, an example of a classic negative feedback loop [48, 50, 178, 366, 367]. This kind of negative-feedback regulation was also supported by genetic data demonstrating that IκBα-deficient mice were dead within a week of birth. This was as a result of persistent NF-κB activation upon treatment with TNFα or LPS [47, 368]. While IκBα is the most obvious inhibitor of NF-κB activation, it is by no means the only inhibitor.

4.2: Why so many IκBs?

The accepted paradigm is that the IκB proteins are fundamentally regulatory inhibitors of NF-κB. They function by forming an inhibitory complex with NF-κB and keeping NF-κB sequestered in the cytoplasm, where it is inactive in the basal state. The generally accepted model is that these IκBs are inducibly phosphorylated by the IκB kinase complex, and that this

leads to their stimulus-dependent destruction. This is the key regulatory event required for the activation of the inducible transcription factor NF-κB.

The basic question then is: Why are there so many IκBs? It may be expected that the simple answer to this question would be 'biological redundancy', but the answer turns out to be much more elaborate than that as the different counterparts have unique functions **(Table 4.1)**.

Indeed, intense research in the last fifteen years has shown that the family of IκBs does not act solely as reversible cytoplasmic inhibitors of NF-κB. The different IκBs are functionally heterogeneous and the specific IκB-NF-κB complex is a regulatory module that may act to inhibit, or even promote transcriptional activity in response to various stimuli [186]. In this light, it is best to modify the traditional idea of IκBs acting as cytoplasmic inhibitors of NF-κB with the notion of IκBs acting as chaperones or cofactors of NF-κB. The activity of the IκB cofactor is necessary for stabilization of the NF-κB heterodimers not only in the cytoplasm, but in the nucleus as well. The binding of IκB also provides an interface to interact with other molecules and alter the transcriptional response. It also might facilitate cross talk with other heterologous pathways [108].

The phenotypes of mice deficient in the different IκB proteins are shown in **Table 4.2**. Genetic evidence has also suggested the need to modify the traditionally held model of IκB-mediated cytoplasmic sequestration of NF-κB [369]. Thus, cells lacking all three IκBs (IκBα, **IκBβ** and IκBe) show increased basal activation of NF-κB, in spite of having a normal subcellular distribution of p65 [369]. In brief, there is an intricate relationship between the IκB and NF-κB family of proteins which modulates NF-κB-dependent transcription in response to the specific physiological environment.

Table 1. **Characteristics and functions of IκB family proteins**					
Proteins	**Induction/NF-κB target**	**Signal-mediated degradation**	**Binding preferences**	**Promoter recruitment**	**Function**
1. NF-κB, nuclear factor-κB; n.d., not determined. 2. *IκBβ can occur as phosphorylated (P) or as hypophosphorylated (HP) species. 3. †Human splice variant. ↓ and ↑ denote respectively decrease or increase in transcription.					
IκBα	++/yes	+++	Heterodimeric NF-κB DNA-bound NF-κB	*In vitro* (NMR)	Sequesters NF-κB in the cytoplasm; removes DNA-bound NF-κB, cytoplasmic-nuclear shuttle
IκBβ1 (P)*	+ (delayed)/no	++	Heterodimeric NF-κB	–	Sequesters NF-κB in the cytoplasm
IκBβ1 (HP)*		n.d.	p65:c-Rel	ChIP	Coactivator of p65:c-Rel, TNFα↑, IL-1β↑
IκBβ2†	+ (delayed)/no	–/+	Heterodimeric NF-κB	–	Sequesters NF-κB in the cytoplasm
IκBε	+ (delayed)/yes	++	Heterodimeric NF-κB	–	Sequesters NF-κB in the cytoplasm
Bcl-3	++/yes	n.d.	p50:p50, p52: p52	EMSA, DNA-pull-down, ChIP	Cyclin D1 ↑, MDM2 ↑, cytokine expression ↓, gene expression profile
IκBζ	++/yes	n.d.	p50:p50	ChIP	IL-6 ↑, TNFα↓ gene expression profile
IκBNS	++/yes	n.d.	p50:p50	DNA-pull-down ChIP	IL-2 ↑ IL-6 ↓ gene expression profile
IκBη	– or +/no	n.d.	p50:p50	n.d.	Cytokines, e.g. IL-1β, IL-6 ↑
p105	++/yes	++	NF-κB monomers and dimers	–	Sequesters NF-κB in the cytoplasm
p100	++/yes	–	RelB	–	Sequesters NF-κB in the cytoplasm

Table 4.1: Functions of different IκB proteins [19]

Table 2. **Knockouts of IκB family proteins**		
Knockout	**Lethality**	**Phenotype**
IκBα$^{-/-}$	7–10 days after birth	Severe widespread dermatitis and extensive granulopoiesis; persistent NF-κB activation after TNFα or LPS treatment
IκBβ$^{-/-}$	No	Resistant to LPS-induced septic shock and collagen-induced arthritis; increased and prolonged expression of cytokines, e.g. TNF and IL-1β
IκBε$^{-/-}$	No	Increased expression of individual Ig isotypes and cytokines
IκBα$^{-/-}$ IκBε$^{-/-}$	Neonatal	Severe malfunction of lymphopoiesis; nearly complete absence of B and T cells; NK cell number reduced
Bcl-3$^{-/-}$	No	Defects in splenic microarchitecture and T-cell differentiation; severe defects in protective humoral immune responses
IκBζ$^{-/-}$	No	Severe atopic dermatitis-like disease with inflammatory cell infiltration; impaired expression of specific genes activated by TLR/IL-1R signal transduction, e.g. IL-6
IκBNS$^{-/-}$	No	High sensitivity to LPS-induced endotoxin shock and intestinal inflammation; reduced T-cell proliferation; deregulated cytokine production
p105$^{-/-}$	No	Defects in stress response and various immune functions
p100$^{-/-}$	No	Defects in secondary lymphoid organ development; impaired B-cell maturation

Table 4.2: Phenotypes of murine knockouts of different IκB proteins [19]

IκBs can be broadly subdivided into three categories **(Figure 4.1)**:

a) **proto-typical IκBs,** that are expressed in cytoplasm and are known to undergo phosphorylation, degradation, and resynthesis upon stimulation. The first member of the IκB protein family, IκBα, was cloned in 1991 [370], and is characterized by the conserved ankyrin repeat domains. After this, the second member to be identified and cloned was IκBβ [34, 371, 372]. Subsequently, three independent groups discovered IκBε [36] [37, 373]. These are the three proto-typical IκBs.

b) **atypical nuclear IκBs,** barely expressed at basal levels, but induced upon stimulation. Bcl-3, is the first member of the family of atypical IκBs. It was initially cloned and discovered as a proto-oncogene in chronic lymphocytic leukemia [374]. Later, it was also identified as an IκB family member [375] [371, 372]. Typical of many atypical IκBs, it has been demonstrated that Bcl-3 can act as a transcriptional coactivator [376, 377]. Subsequently, many other members of this groups have been discovered like IκBζ/MAIL [378-380], IκBNS [381] and, recently, IκBη [382]. In contrast to the typical IκBs, all atypical IκBs with the significant exception of IκBη have a low level of expression under resting conditions, but are rapidly induced by NF-κB-stimulators [374, 381-383]. Initially, IκB proteins were assumed to function only as inhibitors of NF-κB activity. However, the discovery of these atypical IκB-like family members has demonstrated that the functions of IκB are much more complicated than previously imagined. The atypical IκBs are located predominantly in the nucleus and may interact with other nuclear factors. Such interaction may prevent degradation of DNA-bound NF-κB dimers, which may increase or decrease transcription [384]. It is also possible that the atypical IκBs might fine-tine NF-κB-dependent gene regulation by competing with the cytoplasmic IκBs for binding DNA-bound NF-κBs in the latter part of the activation process.

c) **Precursor proteins p105 and p100**: they have an N-terminal Rel homology domain and C-terminal ankyrin repeats [385-391]. p100 and p105 are known to form stable complexes with other NF-κB family members via the N-terminal Rel homology domain and/or the ankyrin repeats in the C-terminal end. Thus, they function like IκB in sequestering NF-κB, blocking nuclear translocation and eventual DNA binding. [371] [74] [392] [393] [394] [395] [396]. Following proteolytic processing, these precursor proteins release NF-κB proteins p50 (from p100) and p52 (from p105) using distinct mechanisms [397] [398]. Specifically, p105 is

proteolytically degraded under multiple IKK-activating conditions [399] [400] [401]. Like any typical IκB, p105 is also phosphorylated by IKKβ at serines 927 and 932, followed by β-TrCP mediated degradation [402] [403] [404]. In this way, p50 homodimers may be released in a signal-dependent manner. It has been recently shown that noncanonical signaling can also liberate p50 from cytoplasmic p100 [405]. Both p105 and p100 knockout mice exhibit defects in the immune system [98]. These phenotypes are probably a result of the knockouts lacking the encoded precursor protein as well as the IκB-like function of these proteins. There may be a feedback regulation for these precursors as they are under the transcriptional regulation of NF-κB (like IκBα and IκBε) [406] [407].

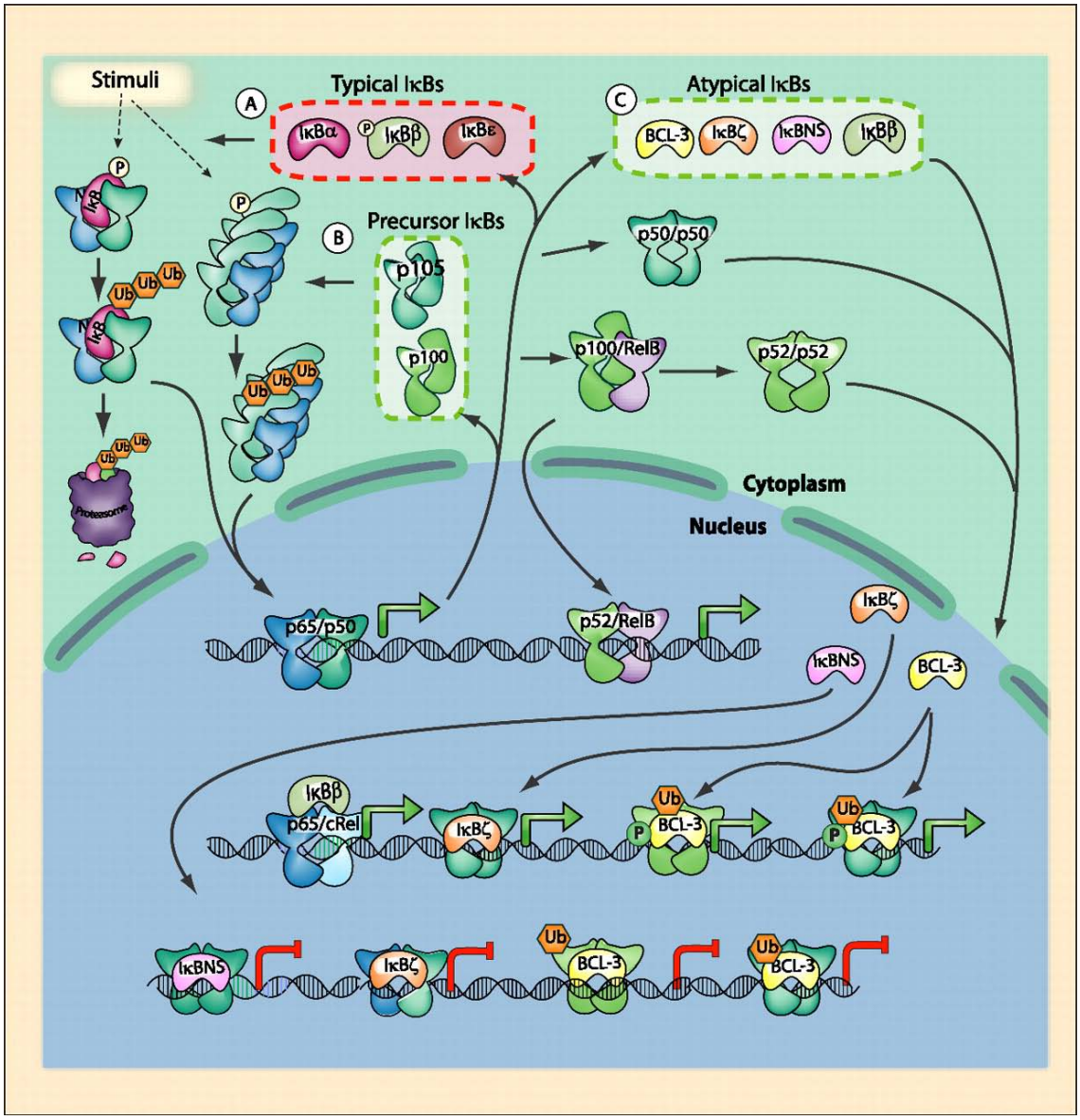


Figure 4.1: Typical and atypical IκB proteins. [108]

4.3: Ankyrin repeats: signature of IκBs

The single most important structural feature of IκB proteins is their conserved 'ankyrin repeat domain (ARD)' [19] **(Figure 4.2)**. The ARD motif is responsible for binding to NF-κB

proteins, thereby enabling the IκBs to perform their most important functions. ARDs frequently mediate protein–protein interactions in a wide range of proteins with different functions. An ankyrin repeat consists of 3 amino acids, and has a broad phylogenetic distribution. They fold to a helix-loop-helix conformation [408], [409]. While the typical IκBs (IκBα, IκBβ and IκBε) have six ankyrin repeats, the atypical IκB molecules have seven or eight. Structural studies suggest the binding of one NF-κB dimer with one IκB molecule. This confirms the 1:1 stoichiometry of binding as observed in previous biochemical studies involving cross-linking experiments and native gel analysis [410], [411], [412], [413].

It has been seen that ARD mediated interaction stabilizes the IκB protein. Interestingly, the closer the sequence of the ankyrin repeat in a particular protein is to the bioinformatically derived consensus sequence, the more stable is the protein. This is obviously an empirically derived axiom but has been found to be able to explain the low stability of free IκBα [414]. This is because only half the primary sequence of the ARD of IκBα matches the theoretical sequence. Besides, it has been documented by a number of studies that the 5^{th} and 6^{th} ankyrin repeats is not fully folded in free IκBα. They fold fully only in a complex with NF-κB. This explains the stability of IκBα-NF-κB complex as compared to free IκBα [415], [416], [417], [418].

The N-terminal part of IκBs is unfolded and contains the serines that are phosphorylated by IKK. These serines are in a ‘signal response domain’, also known as the ‘degron’ as phosphorylation leads to degradation of the IκB molecule. Upon phosphorylation, lysine residues located about ten amino acids upstream of the site of phosphorylation undergoes polyubiquitination with K48-linked polyubiquitin chains. This ‘signal response domain’ is followed by the ARD. The third domain in the C-terminus of IκB is known as the PEST domain.

This region is abundant in proline (P), glutamic acid (E), serine (S) and threonine (T) [419], [412]. They are arranged in short stretches of more than ten negatively charged amino acids. The PEST domain is also found in NF-κB precursor proteins (p105 and p100) [411] and may be involved in facilitating protein turnover. The ankyrin and PEST domains of IκBα, Bcl-3, or p105 have been found to be sufficient in binding the dimerized Rel domain [411], [372]. The N-terminal 'signal response domain' is not required for binding but for being able to respond to upstream stimulus.

While the binding of ARD to the NF-κB dimer imparts stability to the complex [410], [413], it is not immediately clear how the 'de novo' synthesized IκB molecules displace the NF-κB dimers from the DNA. The 'partially folded' 5^{th} and 6^{th} ankyrin repeats along with the PEST domain seem to be playing an important role in dissociating the dimer from the κB site [190], [417]. This is because mutations facilitating the stable folding of these ankyrin repeats (without affecting the IκB-NF-κB binding affinity) reduce the ability of IκBα to remove the dimers from the κB site [190]. A ternary complex comprising of a NF-κB dimer, IκBα, and DNA has been found in solution, as determined by NMR based experiments [420]. These experiments also provide a mechanism by which IκBα dislodges the p65:p50 dimer from κB site. The first contact with the NLS of p65 is made by the 'well-folded' ankyrin repeats putatively to obscure the NLS. After this, the 'unfolded' ankyrin repeats associates with the dimerization domain in p65 and p50. This results in the complete folding of all ankyrin repeats and configures the negatively charged PEST domain to effectively dislodge the IκBα from the κB binding site [420].

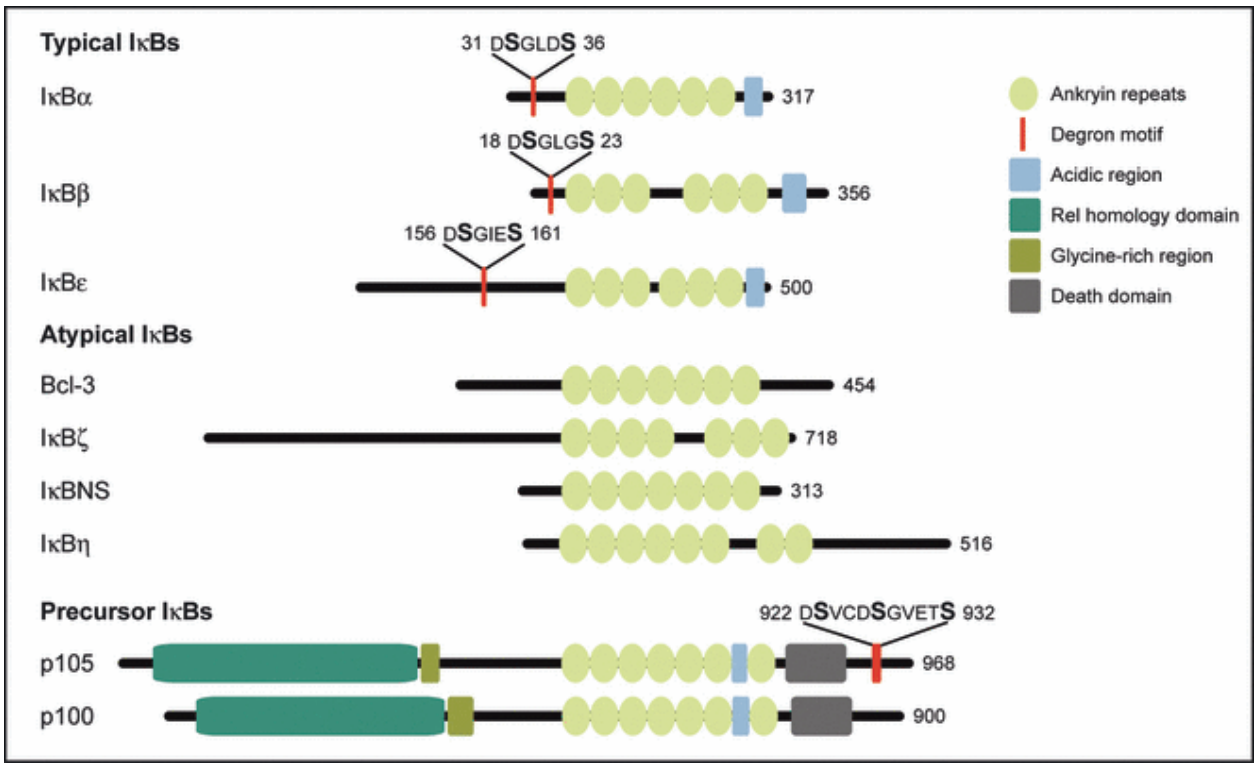


Figure 4.2: Structure and domains of the IκB protein family. [19]

A few of the important IκBs are described below in further detail:

4.4: IκBα (the prototypical inhibitor of NF-κB)

IκBα is by far the most well characterized member of the IκB family and has a molecular weight of 37-kDa. It is the prototypic IκB, which sequesters the main NF-κB (p65/p50) heterodimer in the cytoplasm [421], [370], [422], [423]. It is degraded in response to a range of ligands including the various Toll-like receptor (TLR) ligands and the various cytokines (TNFα, interleukin-1 (IL-1) etc). Upon stimulation, IκBα undergoes phosphorylation by IKK, and rapid subsequent degradation [52], [51], [424] via the proteasome [54], [55], [53]. This results in the

release of the heterodimers [38], [370], translocation of active NF-κB dimers (p65-p50) from the cytoplasm to the nucleus, and expression of NF-κB dependent genes [25].

Since the IκBα gene is transcriptionally regulated by NF-κB, it forms an autoregulatory, negative feedback loop [50], [367], [178], [48]. Thus, in mice deficient in IκBα, NF-κB is persistently unregulated [73], [368] leading to embryonic lethality. Lethality can be rescued by knocking-in an IκBβ gene downstream of the IκBα promoter [425].

The discovery of the negative-feedback mechanism prompted investigations to discover the underlying mechanisms. The p65:p50:IκBα complex has been found to undergo cytoplasmic-nuclear shuttling [61], [180]) and the reason was initially unclear as it was expected that IκBα would sequester the heterodimer only in the cytoplasm. However it was soon found that while IκBα interacts with p65:p50 in the resting state, it does not completely mask the NLS of p50 [252], [410]. Hence in the basal state, IκBα is found (in a complex with the heterodimer) both in the nucleus and the cytoplasm. It is degraded upon stimulation and then rapidly resynthesized. The current model is that the resynthesized IκBα goes to the nucleus. There, it binds the deacetylated p65-p50 heterodimers and the trimeric inactive complex moves back to the cytoplasm.

It is not clear why the *de novo* synthesized IκBα translocates to the nucleus, especially as IκBα does not have a NLS (and instead has a nuclear export sequence). One suggestion has been that IκBα has a non-classical NLS in its second ankyrin repeat. Another study has proposed a 'piggy-back' mechanism, by which IκBα uses the NLS of another unknown protein [426], [427]. IκBα is unique (unlike IκBβ) and can remove heterodimers bound to the DNA. This is because the binding of NF-κB with IκBα (K_D =40 pM) is much stronger than the binding of NF-κB with

DNA (K_D =3-10nM). This has been shown using different analytical techniques using NMR, stopped flow-fluorescence and surface plasmon resonance [428], [420], [190].

The nuclear export sequence (NES) of IκBα, present between residues 45-54, has thus been proposed to be very important for translocating the IκBα:p65:p50 complex to the cytoplasm [74], [75], [429], and subsequent termination of NF-κB activation [251]. But mice carrying a gene that encodes IκBα with a mutant NES are perfectly viable [430].

4.5: IκBε (inhibitor of chronic NF-κB signaling)

IκBε is a 45 kDa protein that also undergoes IKK dependent phosphorylation, degradation and resynthesis just like IκBα. Moreover, the IκBe is also a NF-κB dependent gene [179], [36]. However, the kinetics of degradation and resynthesis is much more rapid for IκBα as compared to IκBe [37]. It is conceivable that that the both IκBα and IκBe inhibit NF-κB at distinct time points by a negative feedback mechanism. This may have significant impact in the regulation of post-stimulus NF-κB inhibition, especially in response to TNFα [179].

IκBε is predominantly associated with Rel:p65 heterodimers in the basal state [37], [431]. It is phosphorylated by IKK at Ser 157 and Ser 161. After this, it is proteasomally degraded by β-TrCP [37], [432]. The rapid phosphorylation of IκBα as compared to IκBe may be in part because of the higher binding affinity of IKK to IκBα. Additionally, IκBε has also been found to be associated with the PP6 phosphatase in unstimulated cells. This may also delay the phosphorylation [433]. This is supported by the observation that knocking down PP6R1 (essential subunit of PP6) has been found to increase the rate of IκBε degradation [434]. IκBe is predominantly cytoplasmic and does not undergo as much nuclear-cytoplasmic shuttling as

IκBα. This may be because IκBe has a noncanonical NES between residues 343 and 352 [431], [429], [435].

IκBε is less ubiquitous than IκBα and it is predominantly found in hematopoietic cells. IκBe knockout have minor defects in components of the hematopoietic lineages. This may be because IκBα compensates for the loss of IκBe [183], [436]. IκBε is expressed at various stages of developing B cells and may regulate p65 and cRel containing complexes [437], [438]. Thus, B cells deficient in IκBε have been found to have increased levels of basal and induced cRel [439]. It is clear though that more *in vivo* work is necessary to elucidate the temporal behavior of IκBε.

4.6: IκBβ (inhibitor and activator of NF-κB signaling)

Humans have two splice variants of the protein, the longer isoform IκBβ1 (43 kDa) and shorter, C-terminally truncated IκBβ2. The mouse orthologue IκBβ corresponds to human IκBβ1 and is degraded is response to LPS or IL-1β. There have been reports that in human, IκBβ2 is more abundant as it is resistant to stimulus-dependent degradation [440]. Though the promoter of IκBβ has a NF-κB-binding site, transcription of IIκBβ does not seem to be regulated by NF-κB [34], [441], [442].

Unlike any of the other IκBs, IκBβ is constitutively phosphorylated. This phosphorylation may be important for inhibition of NF-κB complexes in the cytoplasm [443]. Similar to the other IκBs, upon stimulation, IKK phosphorylates IκBβ at Ser 19 and Ser 23 [444] leading to β-TrCP-mediated proteasomal degradation [445]. However, the kinetics of NF-κB activation is not significantly altered in IκBβ deficient cells as compared to the IκBα or IκBe deficient cells [60], [179]. Interestingly, the stimuli that degrade IκBβ (LPS or IL-1) are known to cause persistent

activation of NF-κB [34]. Multiple groups have reported that there is a significant difference in the overall pattern and kinetics of IκBβ degradation [423], [34], [191], [189] as compared to the other IκBs.

After degradation, IκBβ is resynthesized in a hypophosphorylated form. Hypophosphorylated IκBβ does not mask the NLS of p65 and can be found in the nucleus in a complex with p65:cRel dimers bound to DNA [34], [35], [188], [189], [191]. Crystallographic data has confirmed that IκBβ-bound p65 homodimers can indeed bind the DNA [192]. The DNA-bound "hypophosphorylated IκBβ-NF-κB complex" cannot be removed by IκBα. The presence of this complex in the nucleus led our group to hypothesize about 15 years ago that hypophosphorylated IκBβ might actually be augmenting the expression of certain genes [35], though it was unclear at that time if the complex was increasing or inhibiting transcription [191], [192], [253]. The idea emerged that IκBβ could act as a chaperone, protecting the DNA-bound, functional p65:cRel heterodimer in the nucleus from IκBα.

The phosphophorylated IκBβ on the other hand masks the NLS of p65 and is found in the cytoplasm of unstimulated cells (IκBβ does encode a NES). Acting as a bona fide inhibitor, the phosphorylated form inhibits DNA binding *in vitro* [35], Phillips, 1997 #408}, [191]. Indeed, *in vitro* data suggests that IκBβ might have greater affinity for NF-κB as compared to IκBα, and hence completely masks the NLS of NF-κB heterodimers. As a result, IκBβ is exclusively cytoplasmic in unstimulated cells (as compared to the nuclear-cytoplasmic shuttling of IκBα) [252], [251].

In order to understand the contribution of IκBβ to regulation of NF-κB in the physiological context, an IκBβ knockout mouse was generated by us (and another group) [186],

[187]. These mice are resistant to LPS-induced septic shock as they produce less TNFα [186] and IL-1β [187] in response to LPS. They are also resistant to collagen-induced arthritis. It is clear from the animal model that IκBβ has distinct functions in the cytoplasm and nucleus. Cytosolic IκBβ acts as a traditional inhibitor and inhibits basal activation of NF-κB. It does so by sequestering p65:cRel complexes in the cytoplasm of unstimulated cells. Upon stimulation with LPS, IκBβ is degraded and the dimer translocates to the nucleus. Consistent with our decade-long hypothesis, hypophosphorylated IκBβ is synthesized and interacts with p65:cRel in the nucleus. The IκBβ-p65-cREl complex binds to the DNA at specific κB sites (κB2 promoter region) leading to increased transcription of specific genes like TNFα [186] and IL-1β [187].

While it has been speculated for a long time that IκBα and IκBβ are quite different in their functions, the physiological relevance of this was unclear. Now both biochemical and genetic evidence is available to show that while IκBα inhibits inflammation, IκBβ can both activate and inhibit inflammation [186] depending on the context.

4.7: Conclusion and Perspective

While their functions may partially overlap, murine genetic models have now conclusively established the non-redundant functions of the three typical IκBs in the physiological context. IκBα-knockout mice die 7–8 days after birth from severe dermatitis and extensive granulopoiesis [368] [446]. Expectedly, upon stimulation with lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNFα), these cells show sustained upregulation of NF-κB (and IκBε) [37]. These mice have defects in NF-κB signaling in B-cells and in formation of secondary lymphoid tissues. In contrast, IκBβ-knockout or IκBε- knockout mice are viable and have relatively minor differences in immune cell composition as compared to their wild type

littermates. The IκBε-knockout mice show increased expression of certain cytokines and specific Ig isotypes [60, 182]. Remarkably, work in our laboratory has shown that mice lacking IκBβ are resistant to LPS-induced septic shock and collagen-induced arthritis [186, 187]. This result would have been unexpected based on the previous paradigm of inhibitory, sequestering function of IκBs.

It is possible that specific functions of the typical IκBs could be contingent on their selectivity of interaction with specific NF-κB homo- or heterodimers. For example, IκBα binds to heterodimers containing p50, p65, and c-Rel [447] [448] [422]. Multiple studies reveal that it also exhibits the greatest efficiency in removing the active heterodimers from the nucleus, thus blocking transcription [373] [252] [191]. In contrast, IκBβ binds preferentially to cRel and p65 containing heterodimers. IκBβ is also a relatively weaker inhibitor of NF-κB subunits binding to DNA *in vitro* [34] [252] [191]. Additionally, it is important to understand that the stimulus-dependent dynamics of degradation and subsequent regeneration of IκBα and IκBβ is substantially different. As shown in **Figure 4.3,** IκBα is degraded and resynthesized within an hour of stimulation. In spite of this rapid resynthesis of IκBα, NF-κB continues to stay activated possibly as a result of the delayed activity of IκBβ. This difference in the temporal control of IκBα and IκBβ might be because IκBβ is constitutively phosphorylated. The pathway controlled by IκBα is well understood. In order to dissect the pathway controlled by IκBβ , it is important to know the site of constitutive phosphorylation, the kinase and the biological significance of this phosphorylation (**Figure 4.3)**. This is going to be the focus of my next chapter

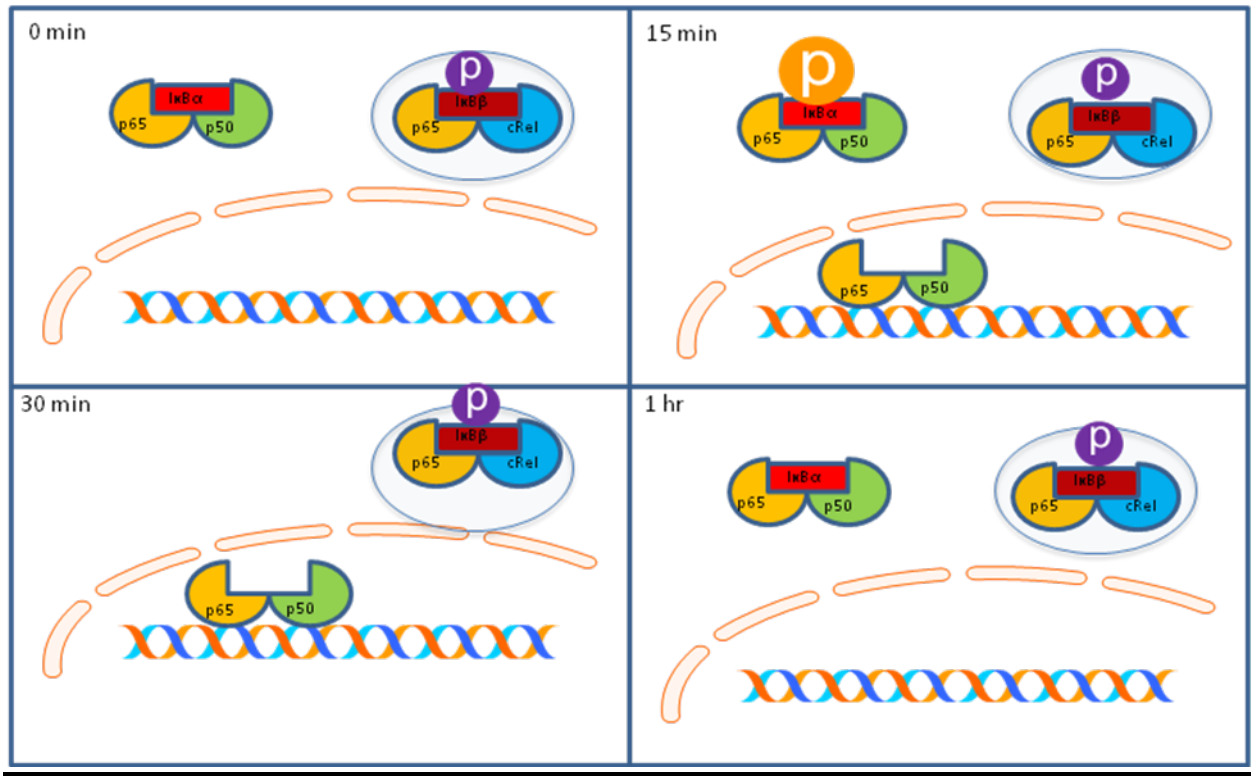


Figure 4.3: Distinct temporal control by IκBα and IκBβ (modified from [186])

Chapter 5:

GSK-3β constitutively phosphorylates IκBβ at Ser-346 to downregulate basal NF-κB activation

5.1: Abstract

It has been shown that unlike IκBα, IκBβ can both inhibit and activate the inflammatory response [186]. We hypothesize that this may be because IκBβ exists in two forms, a constitutively phosphorylated form and an unphosphorylated form. However the site of phosphorylation and the kinase is unknown, hindering a detailed characterization of the pathway mediated by IκBβ. Here, by mass-spectrometric analysis of immunoprecipitated IκBβ, we show that IκBβ is phosphorylated at Ser-346 in quiescent cells but not in cells stimulated with LPS. The kinase phosphorylating IκBβ at Ser-346 is Glycogen Synthase Kinase 3 (GSK3) which is active in resting cells, but inactivated upon stimulation with LPS. IκBβ phosphorylated at Ser-346 masks the NLS of p65, thereby sequestering the p65:cRel complex to the cytoplasm while unphosphorylated IκBβ cannot mask the NLS of p65. Consequently, phosphorylated IκBβ is anti-inflammatory while unphosphorylated IκBβ is pro-inflammatory. Additionally, Ser-346 also harbors a SNP, and preliminary experiments have suggested that it might be deleterious in patients with invasive pneumococcal disease; though the role of the SNP in the disease is not clear. It has also been predicted to be “highly damaging” by multiple softwares like Polyphen and Sift. We show that mice succumb to sub-lethal doses of LPS if phosphorylation of IκBβ is abrogated. The death occurs from increased production of TNFα caused by unphosphorylated IκBβ. Finally, a new biological pathway for the regulation of IκBβ has been proposed.

5.2: Introduction

NF-κB is an inducible transcription factor and is known to play a critical role in regulating multiple biological processes including development, survival and mediates the immune response in response to multiple pathogenic insults [25]. Once activated, NF-κB upregulates genes that encode for different kinds of antiapoptotic proteins, cytokines and other molecules involved in immunoregulation. The NF-κB family consists of five members: p50, p52, p65, cRel and RelB. They can form homodimers or heterodimers that are inactive in the basal state, typically sequestered in the cytoplasm in a complex with the inhibitor of κB (IκB) proteins. NF-κB is activated by a wide range of stimuli. It is remarkable that all these stimuli converge to activate a single kinase complex, the IκB kinase complex (IKK), which phosphorylates the IκBs, targeting them for proteasomal degradation [33]. The NF-κB dimers are now to free to enter the nucleus, bind to specific κB promoter sites on the DNA, and modulate transcription of target genes [187]. NF-κB functions in an intricate and context-dependent manner to selectively upregulate specific genes in response to specific stimuli.

The primary inhibitors of NF-κB in the resting state are the IκB proteins. Consequently, ever since their initial discovery [24], much attention has been paid to understanding their function and mechanism of action. This has been an arduous task given that there are many members in the IκB family, including IκBα, IκBβ, IκBε, IκBγ, p100, p105, Bcl-3 and IκBζ [19], all of which have multiple ankyrin repeats in common. Amongst the IκBs, IκBα and IκBβ are the major inhibitors that sequester NF-κB to the cytoplasm in quiescent cells [34], [447]. Both IκBα and IκBβ also have a carboxy-terminal PEST domain that is rich in proline, glutamic acid, serine

and threonine. The biggest difference between IκBα and IκBβ in resting conditions is that IκBβ (but not IκBα) is constitutively phosphorylated in the basal state. Upon stimulation, both the inhibitors are inducibly phosphorylated at two conserved serine residues in their N-terminal signal-response domain, leading to their degradation and subsequent resynthesis, although with markedly different kinetics. Much of the work in the field has focused on understanding the mechanism of IκBα which is the prototype inhibitor undergoing rapid degradation and subsequent resynthesis (within an hour of stimulation) [48]. However, NF-κB remains induced despite the rapid resynthesis and accumulation of IκBα after stimulation [35].

Thus in spite of their structural similarities, IκBα and IκBβ have different functions [34], [191]. While IκBα–NF-κB complexes undergo nuclear- cytoplasmic shuttling under resting conditions, IκBβ–NF-κB complexes are entirely cytoplasmic [191], [251], [252]. Almost all known stimuli transiently activate NF-κB by rapidly degrading IκBα which is then almost immediately resynthesized in a NF-κB–dependent negative-feedback loop. The resynthesized IκBα travels to the nucleus, binds to the DNA-bound NF-κB dimer (mainly p65:p50 heterodimer), exporting it to the cytoplasm [50], [368], [60]. In contrast, IκBβ is not a NF-κB dependent gene. Moreover, it is degraded in a delayed fashion (2 hrs) in specific cell-types by a subset of stimuli like LPS or IL-1, which are known to cause persistent activation of NF-κB [34], [449]. The activation is persistent as the NF-κB dimer regulated by IκBβ (p65/cRel) is intransigent to the newly synthesized IκBα [34]. Following degradation of the constitutively phosphorylated IκBβ, IκBβ is resynthesized in stimulated cells in an unphosphorylated form [35], [253]. The newly synthesized unphosphorylated IκBβ forms a stable complex with p65:cRel in the nucleus [34], [191], [189], [423], [35]. Along with biochemical evidence, crystallographic studies have suggested that this complex in the nucleus might actually bind

stably to DNA [192]. Since this complex is resistant to IκBα , it was believed that the complex might even promote the transcription of certain genes [191], [35], [188]. This idea was indeed quite revolutionary at the time, as it suggested that an inhibitor of NF-κB could actually chaperone NF-κB dimers and turn on (instead of inhibit) transcription of some genes.

About a decade later, the IκBβ knockout mouse was generated by two groups, and it was found that unphosphorylated IκBβ complexes with the p65:cRel heterodimer; and the complex indeed binds to the κB2 promoter in the nucleus to prolong the expression of certain genes like TNFα [186] and IL-1β [187]. As a result IκBβ knockouts are resistant to LPS-shock and collagen-induced arthritis. Thus, unlike IκBα , IκBβ can both inhibit and activate the inflammatory gene response *in vivo*.

We hypothesize that this functional difference between IκBα and IκBβ is because IκBβ (but not IκBα) is constitutively phosphorylated in the quiescent state. Thus, IκBβ can exist in two forms: a phosphorylated form (which exists in unstimulated cells and has an anti-inflammatory role) and an unphosphorylated form (which is pro-inflammatory and appears after stimulation). **My aim in this chapter in to discover the site of phosphorylation, the kinase and the effect of phosphorylation.**

By mass-spectrometric analysis of endogenous IκBβ, we discovered that the constitutive site of phosphorylation is Ser-346 in the PEST domain. While determining the kinase, it became obvious that the kinase should be active in the basal state under unstimulated conditions but inactivated upon stimulation with LPS. Such a kinase is the glycogen synthase kinase 3 (GSK-3) [450], [451] (**Figure 5.1**), which was discovered as the kinase that phosphorylates and inactivates the enzyme glycogen synthase, thus having a critical role in the biosynthesis of

glycogen. It was originally isolated from skeletal muscle [452], [453], [454]. Since then, GSK3 has been shown to play a critical role in regulation of many biological processes including development, cell cycle control, differentiation, cell motility and microtubule function, cell adhesion, proliferation, survival and inflammation [455], [456], [457]. Commensurate with its diverse functions, dysregulation of GSK3 has been implicated in many diseases including diabetes, cancer and Alzheimer disease.

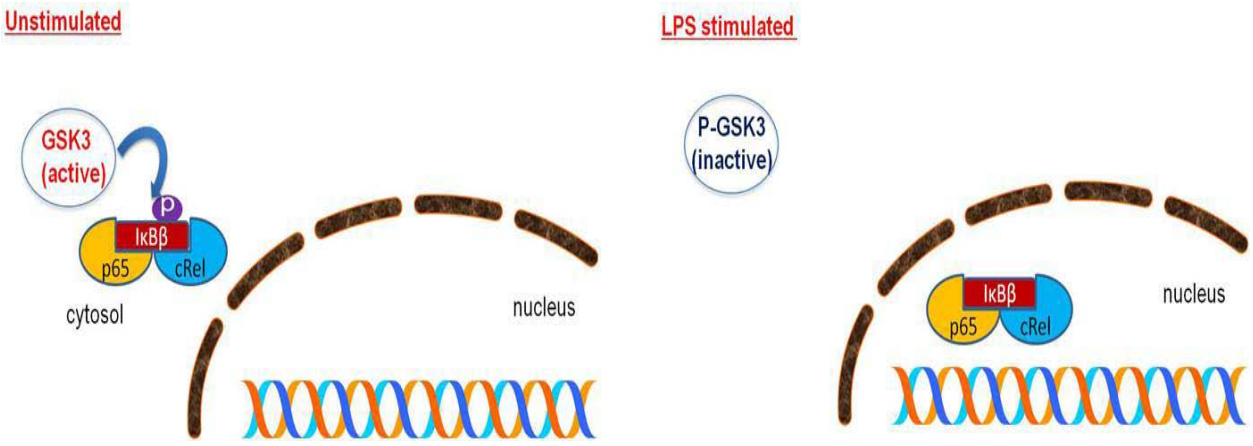


Figure 5.1: Hypothesis of GSK-3β -IκBβ axis

GSK-3 is a serine/threonine kinase and exists as two homologous proteins, GSK-3α and GSK-3β **(Figure 5.2)**. These two isoforms have an overall homology of 85%, and their kinase domains are 98% homologous [458]. It participates in a myriad of signaling pathways that are mediated by different transcription factors like NF-ATc, cyclin D1, cJun and β-catenin [459], [460], [461], [462]. It is best known for it's role in the PI3-kinase/Akt pathway and Wnt signaling. In the PI3-kinase pathway, Akt phosphorylates GSK3 thereby inhibiting GSK3 in

stimulated cells [463]. In Wnt signaling, constitutively active GSK-3β forms a complex with adenomatous polyposis coli (APC) protein, axin and β-catenin. In unstimulated cells, GSK-3β is active and phosphorylates β-catenin, leading to the degradation of β-catenin [464], [465]. As a result, β-catenin mediated transcript is suppressed in resting cells. Wnt signaling leads to inactivation of GSK-3β and disruption of the complex, stabilizing β-catenin and inducing transcription of β-catenin target genes [466].

Although GSK-3α and GSK-3β are homologous proteins, they are encoded by two distinct genes [458]and are non-redundant. GSK-3β knockout mice are embryonically lethal, and GSK-3α cannot compensate for the deficiency of GSK-3β. The GSK-3β knockout mice die around day 16 from TNFα-dependent liver degeneration [467]. The first hint that GSK-3β may cross-regulate the NF-κB pathway came from the fact that these mice closely resemble mice deficient in p65 or IKKβ [73], [72]. The degradation of IκBα is not affected in these mice, suggesting that GSK-3β affects an IκBα independent pathway. In contrast, mice lacking GSK-3α are viable [468], [469]. They are however more sensitive to insulin and have abnormal brain structures. The functional differences between the two isoforms have also been demonstrated in *Drosophila* where overexpression of GSK-3β, but not GSK-3α, could rescue a mutant lacking a GSK3 homolog [470], [471], [450].

The crystal structure of GSK-3β has been determined by three independent groups and suggests that GSK-3β has a preference for phosphorylating primed substrates (i.e, substrates that have been pre-phosphorylated by other kinases) [472], [473], [474]. While not strictly required, priming phosphorylation typically increases the efficiency of subsequent phosphorylation by GSK-3β by ~100 fold as compared to non-primed substrates [475]. This is because the primed

phosphate binds to a positively charged pocket in GSK-3β (consisting of residues R96, R180 and K205). This binding orients the kinase domain of GSK-3β for optimal phosphorylation. Mutating Arg96 disrupts the pocket, and inhibits binding and consequent phosphorylation of primed substrates [476]. Substrates that do not need priming have negatively charged residues that mimic the priming phospho-residue. The site of priming is typically three residues to the C-terminus of serine/threonine targeted by GSK-3β. Hence, the consensus sequence of substrates phosphorylated by GSK-3β is Ser/Thr—X—X—X-Ser/Thr-P, where the first S/T is the site of phosphorylation by GSK-3β; and the last residue is the S/T primed by some other kinase [477]. A prototypical priming kinase is casein kinase II (CK2) for the substrate glycogen synthase. This initial phosphorylation of glycogen synthase by CK2 is required for subsequent phosphorylation by GSK-3β [478], [479]. Other protein kinases that resemble GSK-3β also require primed phosphorylation of their substrates. They include ERK2, CDK2 and p38γ [480], [481], [482]. However, GSK-3β has many important substrates that do not need priming including axin [483], [476] tau [484], APC [485], [486] and presenlin-1 [487].

GSK-3β has been shown to be inhibited by phosphorylation at Ser-9, while GSK-3α is inhibited by phosphorylation at Ser-21. Different kinases can carry out this inhibitory phosphorylation including protein kinase A (PKA), Akt/protein kinase B (PKB) and protein kinase C (PKC) [463], [488], [489]. The crystal structure of GSK3 shows that GSK3 phosphorylated at Ser-9/21 resembles a primed pseudosubstrate, and the negatively charged phospho-group (at Ser-9/21) can now bind intramolecularly to the positively charged pocket. As a result, the substrates of GSK3 can no longer bind to the positively charged pocket. This inhibits phosphorylation of substrates. However, the physiological relevance of this inhibitory

phosphorylation is not entirely clear as knock-in mice in which these serines are mutated to alanines are viable and non-diabetic [490].

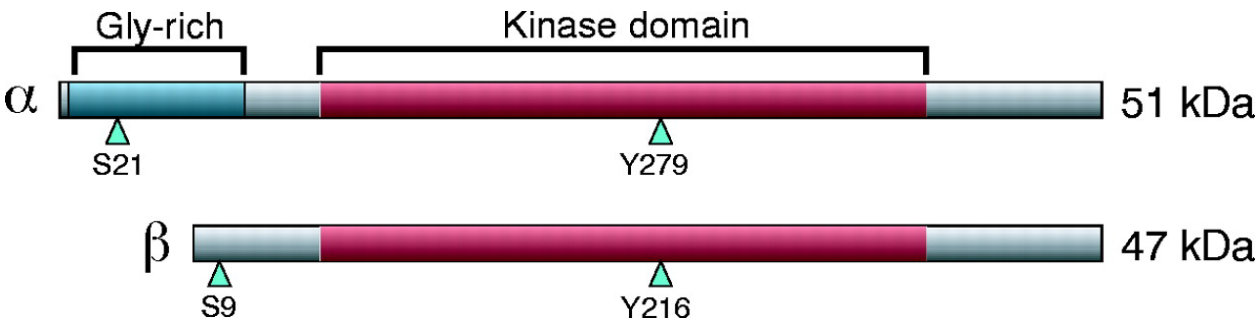


Figure 5.2: Structure of GSK-3α and GSK-3β [456]

Biochemical studies using luciferase reporter assays and EMSA on GSK-3β knockout MEFs have suggested that GSK-3β may be involved in activating NF-κB. Other studies have also shown that inhibitors of GSK-3β decrease the production of proinflammatory cytokines like TNFα during acute systemic inflammation [491], in plural exudates [492] and in the colon [493]. However, this is rather surprising as several stimuli that activate NF-κB (like TNFα, LPS, IL-1) actually lead to the inactivation of GSK-3β (by Akt mediated phosphorylation) [456]. In apparent contrast to reports using GSK3 inhibitors, multiple reports have shown that Akt limits the production of proinflammatory cytokines [494], [495], [496], [497].

Indeed in contrast to results in GSK-3β knockout MEFs, GSK-3β has been shown to inhibit IKK thereby inactivating NF-κB in neurons [498], [499]. In addition to neurons, the inhibitory regulation of NF-κB has also been demonstrated in other tissues. Overexpression of

GSK-3β has been shown to inhibit expression of TNFα in lungs, hearts and endothelial cells of LPS-injected mice [500]. Independent reports have shown that GSK-3β could attenuate TNFα expression in response to LPS in cardiomyocytes [501], and IL-6 production in response to IL-17 [502]. There have been suggestions that the inhibitory activity of GSK-3β is mediated by phosphorylation of p65 [503], [504] or p105 [505]. The authors use a peptide array approach to show that GSK-3β phosphorylates p65 at Ser-468 and this leads to downregulation of NF-κB. Besides p65, GSK-3β has also been shown to phosphorylate and stabilize p105 in quiescent cells [505], leading to suppression of NF-κB. This phosphorylation also primes the p105 for degradation by IKK in response to TNFα signaling. TNFα signaling not only inactivates GSK-3β , but also activates IKK leading to proteasomal processing of p105.

It is possible that GSK-3β specifically affects the expression of only a subset of NF-κB-target genes. A report has demonstrated the requirement of GSK-3β for the localization of p65 to the promoter region of some of the genes regulated by NF-κB using chromatin immunoprecipitation assays [506]. Another report has identified 74 early-response genes whose transcription is dependent on activation of PI3-kinase in response to stimulation with to growth factor [507]. Out of these, 12 genes could be induced by inhibition of GSK-3 even without stimulation with growth factor [508]. Computational and biochemical approaches have shown that GSK-3β inhibits NF-κB in the resting state. However GSK-3β may also be required for NF-κB activation in response to stimulation by different ligands [509].

Although GSK-3β is generally considered to be pro-inflammatory, one of the confounding findings have been that LPS stimulation activates NF-κB while simultaneously inactivating GSK-3β. We report the discovery of a novel pathway mediated by IκBβ, which

shows how inactivation of GSK-3β in response to LPS stimulation can selectively upregulate certain NF-κB dependent genes like TNFα.

5.3: Results

5.3.1: IκBβ is constitutively phosphorylated at Ser-346 in quiescent cells

1) IκBβ undergoes degradation and resynthesis in response to LPS stimulation

To demonstrate stimulus-dependent degradation and resynthesis of IκBβ, 70Z/3 cells were stimulated with 1μg/ml of LPS. In agreement with previous results [186], [35], we found that IκBβ is constitutively phosphorylated in the unstimulated state (slower migrating upper band). Upon stimulation, the phosphorylated IκBβ is degraded followed by the accumulation of unphosphorylated IκBβ (faster migrating lower band) as shown in **Figure 5.3A**. The time points were chosen carefully in order to identify a time point where IκBβ is entirely in the unphosphorylated state. We determined that IκBβ is almost entirely unphosphorylated at 4.5 hours. The stimulation with LPS was also carried out in the presence of cycloheximide. Our results agree with previous results [35] and show that the appearance of the unphosphorylated IκBβ depends on the synthesis of new unphosphorylated IκBβ protein (as opposed to being formed from the degradation of phosphorylated IκBβ).

As opposed to IκBβ which exhibits a delayed kinetics, IκBα is degraded and synthesized rapidly within an hour (**Figure 5.3A)**.

2) IκBβ is phosphorylated at conserved residue Ser-346 in the quiescent state but not in stimulated cells

Since IκBβ is present entirely in the phosphorylated and unphosphorylated state at 0 and 4.5 hours, we decided to immunoprecipitate and determine the site of phosphorylation by mass-spectrometric analysis. 70Z/3 cells were chosen as these are suspension cells, easy to grow and has a higher yield of endogenous proteins. Hence, we immunoprecipitated endogenous IκBβ at these time points from 70Z/3 cells **(Figure 5.3B)** and analyzed it by western blot. After this, we scaled up the immunoprecipitations reaction and ran it on a 8% SDS-PAGE gel **(Figure 5.3B) .** The bands containing the IκBβ protein was excised and analyzed by mass-spectrometry. Mass-spectrometric analysis revealed that IκBβ is phosphorylated at Ser-346 in the PEST domain of IκBβ only in unstimulated cells, but not under stimulated conditions **(Figure 5.3B).** Ser-346 is conserved in mice, rats, chinese hamster, chimpanzee and humans**.** Additionally, Ser-346 is the site of a SNP ("rs11551804") in humans (mutated to leucine), and one report has shown that it is in patients with invasive pneumococcal disease , though it's role in the disease is unclear [510]. There is another SNP in the PEST domain where arginine at the 339th position is mutated to a tryptophan ("rs17886215"). Both these SNPs have been predicted by multiple softwares ((Polyphen, Sift, etc) to be "highly damaging" SNPs. This is predictable as the mutated leucine cannot be phosphorylated. This confirmed our initial prediction that the site of constitutive phosphorylation would have some important downstream functions. In this context, not many disease-associating mutations of typical IκBs have been found till date, perhaps because redundancy of IκB proteins [16].

3) Characterization of phospho-specific antibody against Ser-346 of IκBβ

We next generated an antibody against phospho-S346-IκBβ by injecting a peptide containing the Phospho-Ser-346-IκBβ peptide in rabbit. The phospho-specific antibody detected

a band corresponding to endogenous phospho-IκBβ in wild type macrophages and MEFs (under unstimulated conditions) but not in the IκBβ knockout cells as determined by western blot analysis **(Figure 5.3C).** Lambda phosphatase treatment of IκBβ abrogated the recognition by phospho-specific antibody, thereby demonstrating the integrity of the phospho-Ser-346 antibody **(Figure 5.3C).** The specific band was also detected in IκBβ$^{-/-}$ MEF transfected with WT IκBβ, but not with a S346A-IκBβ mutant **(Figure 5.3C)**.

We confirmed the LPS-dependent degradation and resynthesis of phospho-IκBβ using the phospho-S346 in intact cells. **(Figure 5.3D)**.

4) Phosphorylation of IκBβ at Ser-346 masks the NLS of p65

While NF-κB-IκBα complexes shuttle between the cytoplasm and nucleus in resting cells, NF-κB-IκBβ complexes are exclusively cytoplasmic. A previous report has suggested that the NLS of p65 is exposed in a complex with recombinant IκBβ, but not with mammalian IκBβ [35]. Hence, we hypothesized that phosphorylation at Ser-346 may be responsible for masking the NLS of p65.

To test if the unphosphorylated IκBβ exposes the NLS of p65, we incubated recombinant or mammalian IκBβ (wt, S346A or S346D analogs) with p65. Immunoprecipitation of p65 was carried out using two different antibodies, one that recognizes a 20 amino-acid C-terminal peptide of p65 (generic p65 antibody) and another that recognized the NLS of p65. Previous reports have showed that the NLS-specific antibody fails to recognize the p65 NLS in a p65-IκBα complex [366], [35] as the complex masked the NLS of p65.

Figure 5.3E shows that the p65 NLS antibody was able to efficiently immunoprecipitate p65 only when incubated with recombinant wild-type or S346A-IκBβ, but not for S346D-IκBβ. Hence, this experiment with recombinant IκBβ demonstrates that the phospho-mimetic S346D analog indeed masks the NLS of p65. However, we were concerned that this result may be because of differences between the two antibodies in binding to IκBβ or enhanced binding of S346D-IκBβ to p65. However, both the p65 antibodies pulled down equal amounts of the wt and S346A IκB analogs. Additionally, all the three IκB analogs are co-immunoprecipitated equally with the generic p65 antibody. This shows that the inability of the NLS-antibody to pull-down p65 when incubated with S346D-IκBβ is indeed because the phosphorylation at Ser-346 masks the NLS of p65 and not because of different binding efficiencies between the two antibodies or tighter binding of the S346D-IκBβ with p65.

To confirm that the differences between phospho-S346-IκBβ and unphosphorylated IκBβ is not somehow due to anomalous protein expression in bacteria, we repeated the experiment with the three IκBβ analogs purified from mammalian cells (**Figure 5.3E**). In this case, the p65 NLS antibody was able to efficiently immunoprecipitate p65 only when incubated with the unphosphorylated S346A-IκBβ analog, but not the wild-type or S346D-IκBβ. This is because the wild-type analog is basally phosphorylated in mammalian cells while the S346D-IκBβ is a phospho-mimetic. We confirmed that this was because phosphorylation masks the NLS of p65, and not because of differences between the two antibodies or unequal binding of the IκB-analogs to p65.

5.3.2: GSK3 phosphorylates IκBβ at Ser-346 *in vitro* and *in vivo*

1) GSK3 phosphorylates IκBβ *in vitro*

Recombinant GST tagged wt, S346A and S346D IκBβ analogs were expressed in BL21 cells and purified using standard protocols. Both GSK-3α and GSK-3β **(Figure 5.4A)** immunoprecipitated from macrophages and MEFs phosphorylated recombinant IκBβ *in vitro* in the presence of γ^{32}-ATP, and this phosphorylation was blocked by two specific GSK3 inhibitors in a dose-dependent manner**.** The two generic GSK3 inhibitors used are 6-bromoindirubin-30-acetoxime and a membrane-permeable GSK-3 peptide inhibitor (Calbiochem). Both have an IC50 value in the nanomolar range and are highly specific inhibitors of GSK3 (has a high selectivity even for proteins that have a similar structure like CDK1). The inhibitors are used at a concentration of 20nM and 100nM [511]. The phosphorylation was inhibited in the presence of phosphatase. The negative control using IGG1 isotype antibody could not phosphorylate the recombinant IκBβ. Recombinant GSK-3β could also phosphorylate recombinant IκBβ and this phosphorylation is decreased in the presence of the GSK3 inhibitors **(Figure 5.4B)**.

2) IκBβ is an unprimed substrate of GSK3

WT-GSK-3β , the kinase-mutant analog of GSK-3β (KM-GSK-3β) and R96A—GSK-3β was reconstituted into GSK-3β knockout MEFs and the immunoprecipitated analogs was used to phosphorylate recombinant IκBβ in the presence of γ^{32}-ATP **(Figure 5.4C)**. While the KM-GSK-3β could not phosphorylate IκBβ, both the WT and R996A analog phosphorylates IκBβ. The R96A-GSK3β phosphorylates only substrates that do not need priming, but not

substrates that need prior priming by other kinases [476], [511]. Hence we conclude that IκBβ does not need priming by other kinases to be phosphorylated by GSK-3β.

3) GSK3 phosphorylates IκBβ at Ser-346 *in vitro*

γ^{32}-ATP was not incorporated into recombinant S346A-IκBβ upon incubation with either immunoprecipitated GSK3 or recombinant GSK-3β , meaning that the S346A-IκBβ cannot be phosphorylated by GSK3. **Figure 5.4D** shows the kinase assay in the presence of immunoprecipitated GSK-3α and GSK-3β (from two different mammalian cell lines, BMDMs and MEFs). **Figure 5.4E** shows the kinase assay with recombinant GSK-3β which phosphorylated the wild-type but not the recombinant S346A-IκBβ.

Phosphorylation at Ser-346 was also checked using the phospho-Ser-346 antibody. The antibody detected phosphorylation of recombinant GST-IκBβ (**Figure 5.4F**) after incubation of GST-IκBβ with GSK-3β immunoprecipitated from MEFs and macrophages in presence of ATP. Phosphatase (lambda phosphatase) treatment of GST-IκBβ previously incubated with GSK-3β abrogated its recognition by the phospho-Ser-346 antibody. This shows that GSK3 phosphorylates IκBβ specifically at Ser-346.

4) GSK3 phosphorylates IκBβ at Ser-346 *in vivo*

To directly determine whether GSK3 phosphorylates IκBβ at Ser-346 *in vivo*, BMDMs were treated with two distinct cell-permeable GSK3 inhibitors. The efficient inhibition of GSK3 was confirmed by the increased levels of β-catenin in GSK3-inhibited BMDMs. The levels of IκBβ phosphorylation at Ser-346 decreased in a dose dependent manner **(Figure 5.4G)**. The inhibitors did not affect the levels of IκBα , in agreement with previous studies [73], [72], [506],

which suggested that the NF-κB modulating activity of GSK3 was independent of IκBα . We next used a shRNA that has been reported to deplete both the isoforms of GSK3 [511]. We find that the depletion of GSK-3β stabilizes β-catenin, in agreement with multiple previous studies [459], [460]. Depletion of both the isoforms using shRNA markedly decreases the amount of phosphorylated IκBβ (**(Figure 5.4H)**. Additionally, the phosphorylation of IκBβ at Ser-346 increases when IκBβ$^{-/-}$ MEFs were co-transfected with IκBβ along with GSK-3β **(Figure 5.4I)**.

To determine the levels of phosphorylation and degradation kinetics in WT, GSK-3β$^{-/-}$ and GSK-3α$^{-/-}$ MEFs, we stimulated these cells with LPS **(Figure 5.4J)**. We found the presence of unphosphorylated IκBβ protein in unstimulated GSK-3β$^{-/-}$ MEFs. While the isoforms may partially compensate for each other, GSK-3β seems to be exclusively responsible for phosphorylating the newly synthesized, unphosphorylated IκBβ (as determined from the presence of unphosphorylated IκBβ six hours after stimulation).

We also checked the degradation kinetics of IκBβ in MEFs where both the isoforms have been depleted using a commercially available inhibitor of GSK3 (6-bromoindirubin-30-acetoxime at a concentration of 50nM) and shRNA (**Figure 5.4K)**. The amount of phosphorylates IκBβ is substantially decreased in both unstimulated and stimulated cells, thereby confirming GSK3 as the kinase for IκBβ.

5.3.3: The β-inhibitory peptide (BIP) selectively abrogates phosphorylation of IκBβ by GSK-3β by decreasing the association of GSK-3β with IκBβ

1) Differential association of two isoforms of GSK3 with IκBβ in stimulated and unstimulated cells

Reciprocal immunoprecipitations of IκBβ with GSK3 and GSK3 with IκBβ shows that GSK3α/β and IκBβ physically associate with each other both in unstimulated and stimulated cells **(Figure 5.5A and 5.5B)**.

Co-immunoprecipitation of IκBβ using antibodies against GSK-3α and GSK-3β show that while GSK-3β associates with IκBβ under both stimulated and unstimulated circumstances, the interaction of GSK-3α with IκBβ is markedly decreased in stimulated cells (**Figure 5.5A)**.

2) GSK-3β but not GSK-3α is associated with the IκBβ:p65:cRel complex in stimulated cells

Previous work has shown that IκBβ exists in a trimeric complex with p65:cRel [186]. We performed sequential immunoprecipitations by first immunoprecipitating IκBβ, and then immunoprecipitating the eluted IκBβ complexes with anti-p65 antibody. cRel was found to be present in the anti-p65 immunoprecipitate both in stimulated and unstimulated cells, confirming the existence of the IκBβ:p65:c-Rel complex. None of the GSK3 isoforms interact with the trimeric complex in unstimulated cells, thereby suggesting that GS3 does not phosphorylate IκBβ in a complex under resting conditions. Only GSK-3β (but not GSK-3α) selectively associates with this complex upon stimulation **(Figure 5.5B).** This confirms the previous results and show that while the two GSK3 isoforms may be redundant in phosphorylating IκBβ in the quiescent state, only GSK-3β associates with the unphosphorylated-IκBβ:p65:cRel complex and phosphorylates IκBβ in stimulated cells.

3) The PEST domain of IκBβ is necessary for interaction with GSK3

Next, we mapped the domain of IκBβ required for interacting with GSK3. The full length IκBβ, but not the PEST deleted IκBβ, could interact with GSK3; demonstrating that the PEST domain of IκBβ is required for association with GSK3 **(Figure 5.5C)**.

4) β-inhibitory peptide (BIP) selectively inhibits the phosphorylation of IκBβ (but not β-catenin) by GSK-3β by decreasing the association of GSK-3β with IκBβ both *in vitro* and *in vivo*

GSK-3β may molecularly regulate NF-κB in various ways as have been suggested by different groups in the past. In order to specifically study the effect of the GSK-3β-IκBβ axis, we designed a peptide spanning Ser-346 in the PEST domain that abrogated the phosphorylation of IκBβ at Ser-346 (by GSK-3β). A phosphorylated version of the peptide (mutant peptide with phosphorylation at Ser-346) was also designed and was found to have no effect on the phosphorylation.

Kinase assays showed that the peptide inhibited the phosphorylation of IκBβ at Ser-346 by GSK-3β in a dose-dependent manner. GSK-3β (**Figure 5.5D)** or GSK-3α **(Figure 5.5E)** were immunoprecipitated from macrophages and incubated with recombinant GST-IκBβ *in vitro* in the presence of γ^{32}-ATP and the peptide. The peptide (but not the mutant-peptide) inhibited phosphorylation of IκBβ by GSK-3β in a dose-dependent manner **(Figure 5.5D)**. However, phosphorylation by GSK-3α was only modestly affected in the presence of the peptide **(Figure 5.5E)**. The peptide also reduced the phosphorylation of GST-IκBβ by recombinant GSK-3β in the presence of γ^{32}-ATP although it did not affect the autophosphosphorylation of GSK-3β **(Figure 5.5F)**. This suggested that the peptide might be a specific inhibitor of IκBβ phosphorylation. We confirmed that the peptide was remarkably selective in inhibiting the

phosphorylation of GST-IκBβ as it also did not affect the phosphorylation of GST-β-catenin primed with casein-kinase **(Figure 5.5G)**.

We discovered that the peptide acted by abolishing the association of GSK-3β with IκBβ both *in vitro* and *in vivo*. The mutant peptide was found to have no effect on the association of the molecules either *in vitro* or *in vivo*. We first incubated recombinant GSK-3β with recombinant GST-IκBβ in presence of the peptide and carried out a pull-down experiment using glutathione beads. The association of GSK-3β with GST-IκBβ was completely abolished at higher concentrations of the peptide. Reciprocal pull-down using an antibody against GSK-3β also abolished the amount of GST-IκBβ associated with GSK-3β at similar peptide concentrations **(Figure 5.5H)**.

We also wanted to see if the peptide could inhibit the GSK-3β-IκBβ interaction in intact cells. Hence, we made a cell-permeable version of the peptide (and mutant-peptide) by fusing the peptide with a sequence derived from the Antennapedia homeodomain that has been shown to mediate membrane translocation [512], [513]. Reciprocal immunoprecipitation reactions using antibodies directed against both GSK-3β and IκBβ showed that the interaction was indeed abrogated in the presence of the peptide **(Figure 5.5I)**. The association between GSK-3α and IκBβ was largely unaffected in the presence of the peptide, and is probably the reason why the peptide fails to inhibit phosphorylation by GSK-3α . The peptide did not affect the interaction of p65 and cRel with IκBβ. While higher doses of the peptide did result in some unphosphorylated IκBβ, it is not enough to ablate the phosphorylated form already present. This shows that the peptide acts by preventing the phosphorylation of newly synthesized unphosphorylated IκBβ, but does not trigger degradation of the pre-formed phosphorylated-IκBβ already present in cells.

The levels of β-catenin was not affected by the addition of the peptide, confirming that the peptide is not a global inhibitor of GSK-3β and is a specific inhibitor of the kinase activity directed towards IκBβ.

5.3.4: Functional consequences of GSK3β phosphorylating IκBβ at Ser-346

1) Unphosphorylated IκBβ selectively activates specific NF-κB target genes (like TNFα) while inhibiting others in response to LPS stimulation

Luciferase assay was performed by co-transfecting wt-IκBβ, S346A-IκBβ or S346D-IκBβ along with pBIIX-luciferase and Renilla luciferase in IκBβ$^{-/-}$ MEFs. All the analogs are expressed in equal amounts as determined by western-blot analysis. While both wild type and S346D analogs suppressed NF-κB activity, the S346A analog failed to inhibit NF-κB **(Figure 5.6A)**.

To explore if S346A-IκBβ positively regulates all NF-κB dependent genes (or only a subset), we also did a luciferase assay with the luciferase gene downstream of the promoters of TNFα and IL-6 (pTNF-luciferase and pIL6-luciferase) **(Figure 5.6B)**. We first confirmed equal expression of the different IκBβ analogs. Remarkably, the results indicate that the S346A-IκBβ selectively increases the expression of the luciferase gene downstream of the TNFα promoter, but not the IL-6 promoter. In contrast, the phosphomimetic S346D-IκBβ inhibits expression of luciferase downstream of both the promoters.

These results were confirmed by ELISA analysis. We reconstituted IκBβ$^{-/-}$ BMDMs with GFP and wt-IκBβ, S346A-IκBβ or S346D-IκBβ. After confirming that the IκBβ$^{-/-}$ BMDMs express equal amounts of the IκBβ analogs, we stimulated them with LPS. Upon LPS stimulation, IκBβ$^{-/-}$ macrophages failed to make TNFα (but produced normal IL-6) in agreement with previous results [186]. This effect was rescued by expression of wt-IκBβ and increased in S346A-IκBβ containing cells; however the phosphomimetic S346D analog suppressed production of TNFα. The production of IL-6 was not influenced by the different analogs, suggesting that the pathway is specific for TNFα **(Figure 5.6C)**. Since the production of TNFα was substantially increased in macrophages expressing S346A-IκBβ and almost completely abrogated in macrophages expressing S346D-IκBβ, we hypothesized that the unphosphorylated form of IκBβ may be responsible for the selective expression production of TNFα.

2) GSK-3β inhibits the expression of TNFα via a novel pathway mediated by the inhibitory phosphorylation of IκBβ

Consistent with previous studies [467], [506], total cellular NF-κB activity was strongly downregulated (75%) in GSK-3β $^{-/-}$ MEFs as determined by luciferase assay (**Figure 5.6D**). In order to determine if all NF-κB mediated genes were affected similarly, we repeated the assay with the luciferase gene downstream of the IL-6 and TNFα promoter (**Figure 5.6E)**. While luciferase was also strongly downregulated (~70%) downstream of the IL-6 promoter in GSK-3β $^{-/-}$ MEFs, the downregulation downstream of the TNF-promoter was relatively mild (~15%). However, overexpressing the phosphomimetic S346D-IκBβ (but not the S346A-IκBβ) abrogated the activation of luciferase downstream of the TNF-promoter. GSK-3β is known to upregulate the secretion of TNFα and other pro-inflammatory cytokines (like IL-6) by an IκBβ -independent

mechanism. Our results suggest that GSK-3β might be playing a dual role in selectively downregulating TNFα (but not IL-6) via a different pathway mediated by inhibitory phosphorylation of IκBβ .

To confirm the results obtained from luciferase assays, we also measured the cytokines secreted by wild-type and GSK-3$\beta^{-/-}$ MEFs in response to stimulation with 10ng/ml of LPS (**Figure 5.6F)**. Not surprisingly, secretion of both TNFα and IL-6 was reduced in GSK-3$\beta^{-/-}$ MEFs (expressing GFP as control) as compared to wild type MEFs (expressing GFP) in agreement with previous studies [491]. However, the relative downregulation of IL-6 was six times more than TNFα. Co-expression of S346D-IκBβ (but not S346A-IκBβ) was required to suppress TNFα to comparable levels.

Intriguingly, the suppression of TNFα secretion by S346D-IκBβ was more in GSK-3$\beta^{-/-}$ MEFs as compared to wild-type MEFs. This is because the expression of S346D-IκBβ in wild type MEFs suppresses the secretion of TNFα mediated by the IκBβ pathway, but not the IκBβ -independent pathway. However, the expression of TNFα in GSK-3$\beta^{-/-}$ is caused only by the presence of unphosphorylated IκBβ stabilizing the p65:cRel heterodimers at the TNF promoter. Hence, expression of S346D-IκBβ almost completely suppresses the expression of TNFα.

3) <u>β-inhibitory peptide (BIP) selectively increases the production of TNFα but not IL-6 in BMDMs in response to LPS stimulation</u>

In order to confirm our hypothesis that the unphosphorylated IκBβ selectively increases the production of TNFα and study the specific effects of the GSK-3β -IκBβ pathway, we stimulated wt-BMDMs with LPS in the presence and absence of BIP. While administration of

the peptide alone was not enough to convert IκBβ entirely to the unphosphorylated form, IκBβ was almost exclusively in the unphosphorylated state after stimulation with LPS in the presence of the peptide **(Figure 5.6G)**; hence the BIP is a great tool for studying the physiological effects of unphosphorylated IκBβ. The stimulation with LPS is required to degrade the 'preformed' phosphorylated IκBβ already present in cells. While the levels of β-catenin increase upon addition of LPS, it does not increase any further upon addition of the peptide.

The amounts of TNFα produced by LPS-stimulated BMDMs increases with the addition of the peptide in a dose-dependent manner **(Figure 5.6H)**. In contrast, the amounts of IL-6 produced is relatively unaffected. Previous results have suggested that the κB2 site in the TNFα promoter is required for optimal transcription of TNFα [186]. Hence, we carried out an EMSA using a κB2 probe and found the enhanced recruitment of p65:cRel heterodimer to the κB2 site in the presence of the peptide in LPS stimulated BMDMs **(Figure 5.6I)**. Our results confirm previous suggestions that unphosphorylated IκBβ associates with p65:cRel and this trimeric complex binds to the κB2 site in the TNFα promoter to augment transcription of TNFα.

4) Mice succumb to elevated levels of caused by hypophosphorylated IκBβ in response to sub-lethal doses of LPS

To test the consequences of the Ser-346 phosphorylation in animals, we challenged mice with sub-lethal dose (18mg/kg of animal weight) of LPS in the presence and absence of the peptide. Our results show that mice succumb to the sub-lethal dose in the presence of the peptide **(Figure 5.6J)**. The mice do not die in the presence of LPS alone or when LPS is injected with the mutant peptide. The mice die only when LPS is injected with the peptide. Tunnel-staining of liver and spleen (as shown in **Figure 5.6K**) show increased signs of cell-death. We examined the

serum levels of acute phase cytokines like TNFα, IL-1β and IL-6. TNFα peaked early after an hour while both IL-6 and IL-1β peaked around 2 hours, in agreement with previous studies [186]. Levels of IL-6 and IL-1β did not change substantially in the peptide-treated mice, but production of TNFα was strikingly increased in these animals (**Figure 5.6L**). Immunostaining also shows accumulation of TNFα and IL-6 in the liver and spleen of these animals (**Figure 5.6M**).

To confirm that the effect of the peptide was mediated by IκBβ and not through any non-specific effects, we also challenged IκBβ$^{-/-}$ mice with peptide in the presence of LPS. The IκBβ$^{-/-}$ mice do not succumb to LPS shock in the presence of the peptide unlike the wild-type mice (**Figure 5.6N**). Wild type mice (but not the IκBβ$^{-/-}$ mice) show elevated levels of TNFα in their serum as measured by ELISA (**Figure 5.6O**) and immunostaining (**Figure 5.6P**). Tunnel staining of liver and spleen also confirm increased cell death in wt but not in IκBβ$^{-/-}$ mice (**Figure 5.6Q**). Our results show that unphosphorylated IκBβ is pro-inflammatory and specifically upregulates the expression of TNFα.

5.3.5: A biological pathway for regulation of IκBβ

To uncover a model by which phosphorylation by GSK3 regulates IκBβ, we examined if immunoprecipitated nuclear and cytoplasmic GSK3 can phosphorylate IκBβ under basal and stimulated conditions. We extracted the nuclear and cytoplasmic fractions of BMDMs under both stimulated and unstimulated conditions as shown in **Figure 5.7 (right panel)**. We confirmed the compartmentalization of HDAC1 and β-tubulin to the nucleus and cytoplasm respectively. IκBβ is phosphorylated in unstimulated cells and is completely cytoplasmic. In stimulated cells, the newly synthesized unphosphorylated IκBβ is localized to the nucleus. Total

GSK3 is distributed both in the nucleus and cytoplasm in stimulated and unstimulated cells. LPS stimulation inactivates GSK-3β by phosphorylating it at Ser-9 [463], [488], [489], and this inactive GSK-3β is present only in the cytoplasm. While LPS stimulation generally inactivates GSK-3β, nuclear GSK-3β is less phosphorylated and more active than cytoplasmic GSK-3β in stimulated cells.

Equal amounts of GSK-3β were immunoprecipitated from whole cell extracts, nuclear and cytosolic fractions of both unstimulated and stimulated cells. A kinase assay was then performed with GST-IκBβ in the presence of γ^{32}-ATP **(Figure 5.7 (left panel)**. GSK-3β phosphorylates IκBβ to a much greater extent in unstimulated cells in both the nucleus and the cytoplasm. This is expected as GSK3 is active in the quiescent state and present almost equally in both compartments. However at 4.5 hrs, GST-IκBβ can be phosphorylated only by the nuclear GSK-3β. This is because the cytosolic GSK-3β is predominantly phosphorylated (S9) and inactive.

5.4: Biological model of GSK-3β regulating IκBβ

We propose a model **(Figure 5.8)** where IκBβ is phosphorylated in the quiescent state by GSK-3β (both nuclear and cytosolic) at Ser-346 (**Figure 5.8, top-left**). Phosphorylated-IκBβ masks the NLS of p65, hence the phosphorylated-IκBβ:p65:cRel complex is cytoplasmic in the resting state (**Figure 5.8, top-left**). Upon LPS stimulation, GSK-3β is inactivated and phosphorylated IκBβ is proteasomally degraded. IκBβ is resynthesized in the unphosphorylated state and cannot mask the NLS of p65. As a result, the trimeric unphosphorylated-IκBβ:p65:cRel trimeric complex is found in the nucleus, where it drives the expression of proinflammatory

genes like TNFα (**Figure 5.8, top-right**). Nuclear GSK-3β associates with this complex and phosphorylates IκBβ (**Figure 5.8, bottom-left)**. The phosphorylation masks the NLS of p65, translocating the phosphorylated-IκBβ:p65:cRel complex back to the cytoplasm (**Figure 5.8, bottom-right)**.

GSK3 does not associate with the phosphorylated-IκBβ:p65:cRel trimeric complex in the quiescent state (**Figure 5.5B and Figure 5.8, top-left**), hence it seems that GSK3 does not phosphorylate IκBβ in the complex under resting conditions. However nuclear GSK-3β associates with this complex after stimulation (**Figure 5.5B and Figure 5.8, top-right and bottom panels**), suggesting that GSK-3β phosphorylates the newly synthesized, unphosphorylated-IκBβ that is associated with p65:cRel heterodimer.

5.5: Discussion

It had long been known that IκBβ is constitutively phosphorylated [35]. The phosphorylated IκBβ is degraded upon stimulation with LPS, and the new IκBβ is resynthesized in a hypophosphorylated form. This hypophosphorylated form was hypothesized to be pro-inflammatory in an *in vivo* model [186]. We discovered that GSK3 associates with the PEST domain of IκBβ and phosphorylates IκBβ at Ser-346 both *in vitro* and *in vivo*. IκBβ does not need priming by any other kinase. This is not surprising as IκBβ was efficiently phosphorylated by both GSK-3α and GSK-3β in the absence of any other kinase (unlike β-catenin which could not be phosphorylated at all in the absence of priming by CK2). The presence of two aspartic acids at residues 355 and 356 might be providing the negative charges for the 'positively charged' kinase binding pocket of GSK3.

The redundancy of IκBs may be a reason for the dearth in disease-associating mutations that have been discovered till date with these proteins [16]. Another reason could be that the SNPs associated with the IκB proteins are so drastic that they may result in early lethality. Remarkably, a 'highly dangerous' SNP was found at Ser-346 of IκBβ and reported in patients suffering from invasive pneumococcal disease, though it is not clear if the SNP is linked to the disease [510].

We generated a phospho-specific antibody against the site of phosphorylation, and showed the degradation and reappearance of endogenous phosphorylated IκBβ in response to LPS stimulation. The phosphorylation masks the NLS of p65, thus sequestering it to the cytoplasm. This is why the phosphorylated form is anti-inflammatory. Phosphorylation does not affect the binding to p65 and all the three analogs of IκBβ (wild type, S346A and S346D) bind equally to p65. This is because the binding of IκBβ to p65 is mediated only by the ankyrin repeats of IκBβ and Rel-homology domain of p65 [34], [370].

While both GSK-3α and GSK-3β can phosphorylate IκBβ *in vitro*, GSK-3β is more physiologically relevant *in vivo*. It is not surprising that both the isoforms phosphorylate IκBβ *in vitro* given the high degree of homology (98%) in their kinase domains [458]. Given it's importance in regulating NF-κB, it is also not surprising that GSK-3β would be expected to be more important in an *in vivo* setting. This is additionally borne out by the observation that only GSK-3β is associated with the trimeric unphosphorylated-IκBβ:p65:cRel complex in stimulated cells. It is possible that GSK-3β downregulates NF-κB in resting cells by phosphorylating p65 [503], [504], p105 [505] and IκBβ (according to our findings).

We also discovered a peptide (BIP) that selectively abrogates the phosphorylation of IκBβ (but not other GSK3 substrates) by inhibiting the association between GSK-3β and IκBβ, and show that IκBβ is unphosphorylated in the presence of the peptide. BIP does not inhibit *in vitro* phosphorylation by GSK-3α. Administration of the peptide alone does not completely lead to the formation of unphosphorylated IκBβ and an accompanying stimulation with LPS is required for complete de-phosphorylation. This may be because a sufficiently high dose of the peptide (alone) has not been administered. Alternatively, it may be because the peptide only inhibits the phosphorylation of newly synthesized unphosphorylated IκBβ. Concurrent stimulation with LPS is required to degrade the already phosphorylated IκBβ (**Figure 5.8, top-left**).

Mice succumb to sublethal doses of LPS in the presence of the peptide because of increased secretion of TNFα (but not IL-6 or IL1 β). GSK-3β is known to be important for the upregulation of TNFα (and other cytokines) by an IκBβ independent pathway; the peptide does not affect this pathway. The peptide additionally inhibits the phosphorylation of IκBβ, thereby activating TNFα synergistically by the described IκBβ-mediated pathway. This is probably the reason why the peptide is so effective and death with accompanying upregulation of TNFα. This also demonstrates the importance of unphosphorylated IκBβ in regulating expression of specific proinflammatory cytokines like TNFα.

GSK-3β generally abets inflammation, and GSK-3$\beta^{-/-}$ mice are embryonically lethal resulting from a failure to activate the pro-survival NF-κB pathway [467]. GSK-3β inhibitors are increasingly used in therapeutics with the expectation that they would inhibit the production of pro-inflammatory cytokines. Our results show that while they are effective in abrogating the

production of some inflammatory cytokines like IL-6, they are not as effective in abolishing production of TNFα. This is because of our finding that GSK-3β also plays a physiological role in inhibiting the production of TNFα in response to LPS, something that might be important in inhibiting persistent inflammation (**Figure 5.8, bottom panels**). Infact, the side-effects of GSK-3β inhibitors may be manifested by their failure to abrogate expression of TNFα. Developing drugs that mimic the effect of S346D-IκBβ in conjunction with these inhibitors might provide better therapeutic value as compared to using these inhibitors alone as it will substantially decrease the production of TNFα.

5.6: Materials and Methods

5.6.1: Cell culture, reagents and transfection

Wild-type, GSK-3α and GSK-3β null mouse embryonic fibroblasts (MEFs) were kind gifts of Prof. J. Woodgett). MEFs, immortalized BMDMs and HEK293 cells were cultured in Dulbecco's modified Eagle's medium-H supplemented with 10% fetal bovine serum. Transfection was done using Lipofectamine LTX according to the manufacturer's protocol. The commercial GSK inhibitors used were 6-bromoindirubin-30-acetoxime (Calbiochem) and membrane-permeable GSK-3 peptide inhibitor (Calbiochem). The GSK3α/β shRNA was obtained Prof. William Snider.

5.6.2: Western analysis and immunoprecipitations

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors for 20 min on ice and centrifuged at 13,000 × *g* for 10 min. Supernatants were quantitated by the BCA and 30 μg of protein was denatured in sodium dodecyl sulfate loading buffer and fractionated 8% SDS-PAGE gels. Following transfer onto PVDF membranes, the blots were blocked in 5% milk. They were then incubated with appropriate antibodies in 2.5% milk overnight at 4°C. For immunoprecipitations, cells were lysed in 25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol along with the protease/phosphatase inhibitors. For the analysis with phospho-Ser-346 antibody, the membranes was blocked in Protein-Free (TBS) Blocking Buffer (ThermoScientific) for 2 hours. After this, the antibody was diluted 1:25 (or (1:50) in the Protein-Free (TBS) Blocking Buffer and the membrane was incubated overnight.

5.6.3: Kinase assays

Lysates were immunoprecipitated overnight with GSK-3α or GSK-3β antibody. After this Protein-G-Sepharose was added for 2 hours. Immunoprecipitates were washed twice with lysis buffer and thrice with kinase reaction buffer (20 mm Hepes, pH 7.2, 10 mm $MgCl_2$, 10 mm $MnCl_2$, 1 mm dithiothreitol, 0.2 mm EGTA, and 5 μm ATP). After this, the GSK-3 kinase assay was performed at 37°C for 1 hour in 20 μl of GSK -3 kinase buffer supplemented with 4 μg of recombinant GST-IκBβ and 5 μCi of [γ-^{32}P]ATP for each reaction. When the reaction was carried out in the presence of peptides, then the peptide was directly diluted out to the reaction media. The reaction was terminated by adding 6X sample buffer and boiling for 5 min. The entire reaction was resolved on a 8% SDS-PAGE gel, and exposed to a PhosphorImager plate. Equal loading of GST-IκBβ and immunoprecipitated GSK-3 was determined by Comassie

staining and western-blot analysis respectively. As controls, GSK-3 kinase reactions were also carried out without adding the GSK-3 antibody (with the IGG1 isotype).

5.6.4: Electrophoretic mobility shift assays.

Gel shift assay was done using the nuclear fractions with the Li-cor EMSA kit as per the user manual. The κB2 and Oct1 probe was custom made.

5.6.5: LPS-induced shock and serum-cytokine measurement

LPS-induced shock was tested by intraperitoneal injection of a sublethal dose of 18 µg/g body weight LPS and monitoring for survival every six hours. In a separate identical experiment, the mice were bled at 1 hr and 2 hr after LPS treatment and the concentration of TNF-α, IL-6 and IL-1β in the serum was measured by ELISA. Peptide injections were done by intraperitonial injection of 200µg of the peptide along with LPS. ELISA analyses were performed as previously described [186], [319] with kits from BD Biosciences.

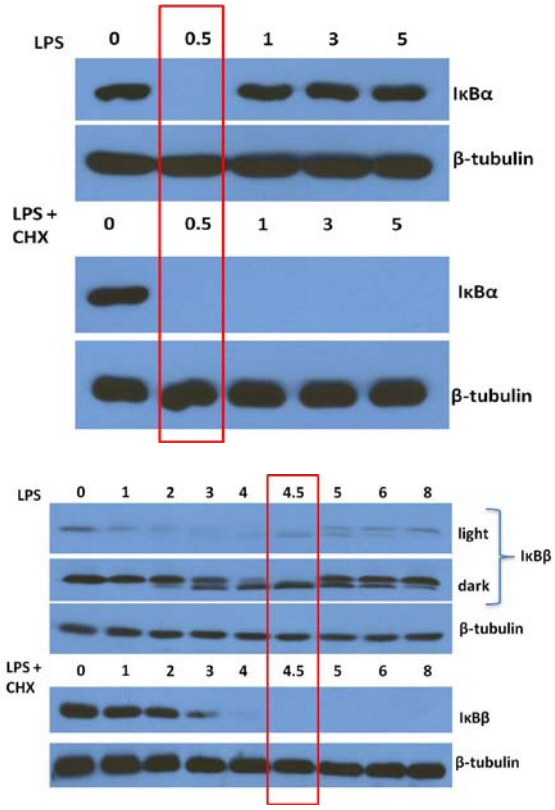


Figure 5.3A: Degradation kinetics of IκBα and IκBβ in response to LPS stimulation (boxes indicate the early and latter time course of action of IκBα and IκBβ respectively): 70Z/3 cells were stimulated with 1µg/ml LPS for the indicated time points with and without cycloheximide and blotted with respective antibodies.

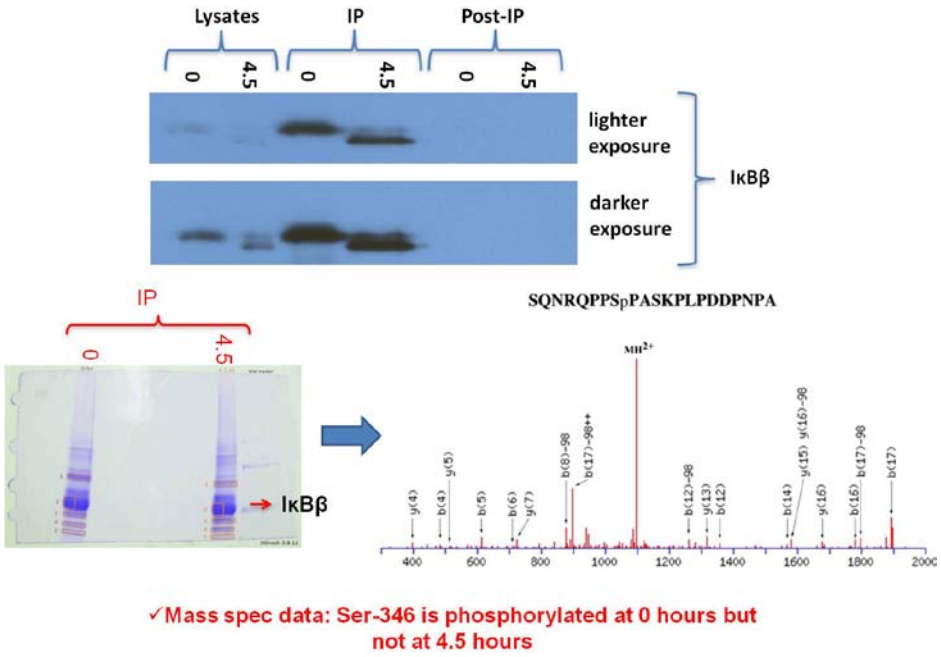


Figure 5.3B: Mass-spectrometric analysis shows that IκBβ is phosphorylated at Ser-346 in resting cells (70z/3 cells were treated with 1µg/ml of LPS for the indicated time points and immunoprecipitated using the generic IκBβ antibody). The MH^{2+} peak denotes that the serine at position 346 is phosphorylated in unstimulated cells.

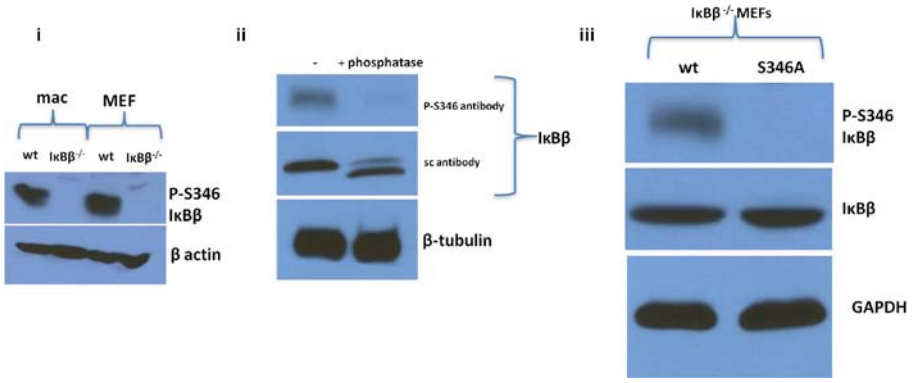


Figure 5.3C: Characterization of phospho-antibody: i) western blot showing the presence of endogenous phospho-Ser-346-IκBβ; wild—type and IκBβ$^{-/-}$ BMDMs and MEFs were lysed and 200 µgs of lysate was run out on a 8% gel (ii) Recognition by phospho-antibody abrogated by phosphatase treatment (200 µgs of wild-type BMDM lysate was treated with and without lambda-phosphatase and blotted with the respective antibodies) (iii) Phospho-antibody recognizes phospho-Ser-346 in IκBβ$^{-/-}$ MEFs transfected with wt-IκBβ but not S346A-IκBβ (IκBβ$^{-/-}$ MEFs were reconstituted with wt-IκBβ and S346A-IκBβ; cells were lysed and blotted with the respective antibodies)

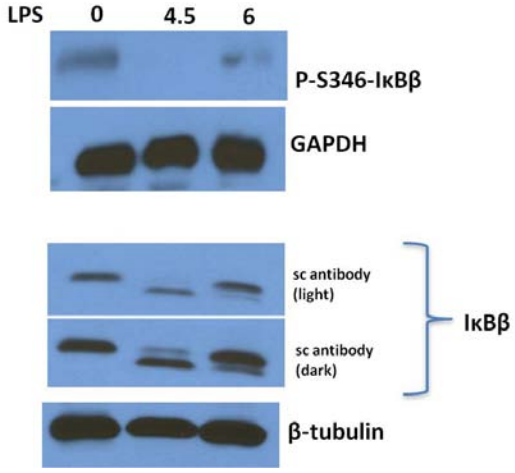


Figure 5.3D: Degradation and reappearance of endogenous phospho-Ser-346-IκBβ in response to 1µg/ml LPS stimulation (BMDMs were stimulated with 100ng/ml of LPS and 200 µgs of lysate was run out on 8% gel and blotted with phospo-specific antibody as shown in the top panel; 30 µgs of same lysate was run out on 8% gel and blotted with pan- IκBβ antibody (sc) as shown in the bottom panel)

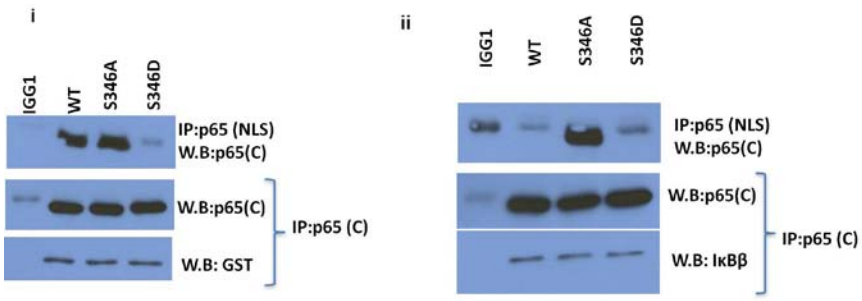


Figure 5.3E: Phosphorylation at Ser-346 masks the NLS of p65 demonstrated by the (i) expression of recombinant IκB analogs (HA-p65 was overexpressed and purified from HEK293 by eluting with peptide; purified HA-p65 was incubated for 30 minutes on ice with equal amounts of each of the three GST-tagged IκBβ analogs, diluted 10X times with co-IP buffer, immunoprecipitated with p65 (NLS) or with p65(C-terminal antibody) and blotted with respective antibodies (ii) expression of IκBβ analogs in IκBβ$^{-/-}$ MEFs (HA-tagged IκB analogs were also purified like HA-p65 from HEK293 cells, and the previous analysis was repeated using the IκBβ analogs purified from mammalian cells instead of recombinant IκBβ)

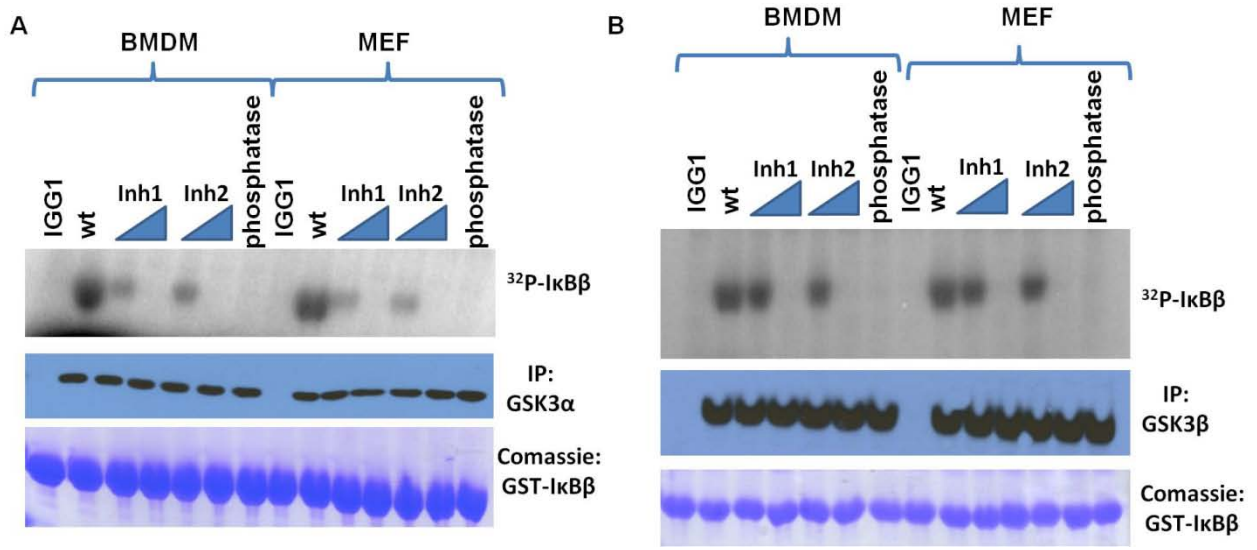


Figure 5.4A: Immunoprecipitated GSK-3α and GSK-3β phosphorylates IκBβ *in vitro* in kinase assay with γ32-ATP (Inhibitor 1 is 6-bromoindirubin-30-acetoxime and Inhibitor 2 is membrane-permeable GSK-3 peptide inhibitor (Calbiochem); both are used at a concentration of 20nM and 100nM): to confirm equal amounts of substrate and kinase in the kinase-assay, the substrate GST- IκBβ was analyzed by coomassie staining; and the amount of immunoprecipitated-kinase was run out on a separate gel and analyzed by western-blotting)

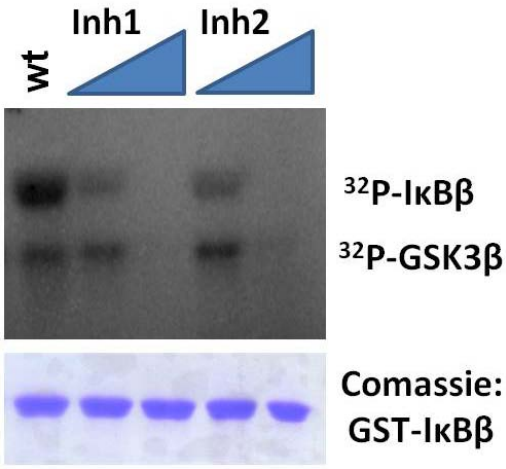


Figure 5.4B: Recombinant GSK-3β phosphorylates IκBβ *in vitro* in kinase assay with γ32-ATP (Inhibitor 1 is 6-bromoindirubin-30-acetoxime and Inhibitor 2 is membrane-permeable GSK-3 peptide inhibitor (Calbiochem); both are used at a concentration of 20nM and 100nM) in a kinase assay: the input substrate GST- IκBβ was run out on a separate gel and analyzed by coomassie staining.

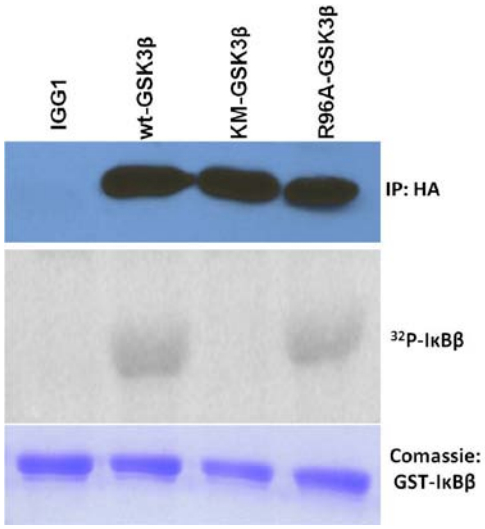


Figure 5.4C: IκBβ is an unprimed substrate of GSK-3β as wt-GSK-3β and R96A-GSK-3β but not kinase-mutant (KM) of GSK-3β phosphorylates IκBβ *in vitro* in kinase assay with γ32-ATP: (HA-tagged GSK-3β constructs were overexpressed in GSK-3β$^{-/-}$ MEFs, and immunoprecipitated with GSK-3β antibody; to confirm equal amounts of substrate and kinase in the kinase-assay, the substrate GST- IκBβ was analyzed by coomassie staining; and the amount of immunoprecipitated-kinase was run out on a separate gel and analyzed by western-blotting)

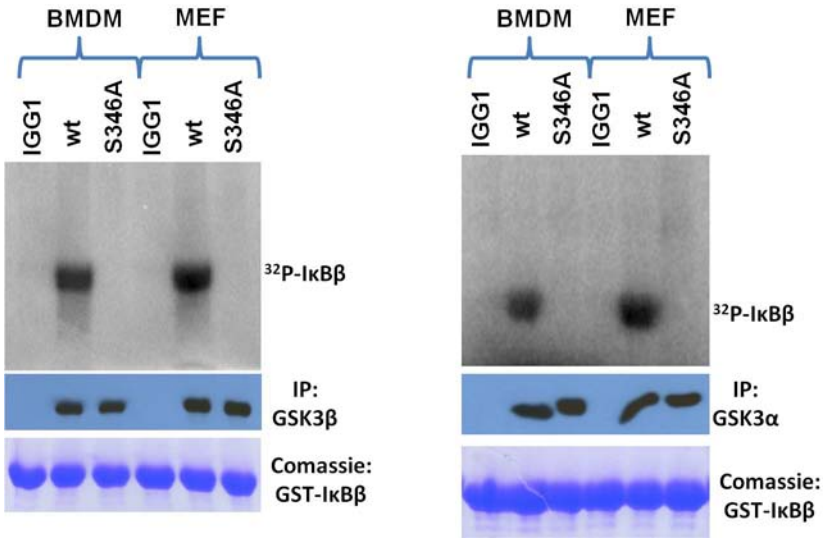


Figure 5.4D: Immunoprecipitated GSK-3β (left panel) and GSK-3α (right panel) phosphorylates Ser-346 of IκBβ (wt- IκBβ but not S346A- IκBβ is phosphorylated *in vitro* in a kinase assay with γ32-ATP*:* to confirm equal amounts of substrate and kinase in the kinase-assay, the substrate GST- IκBβ was analyzed by coomassie staining; and the amount of immunoprecipitated-kinase was run out on a separate gel and analyzed by western-blotting)

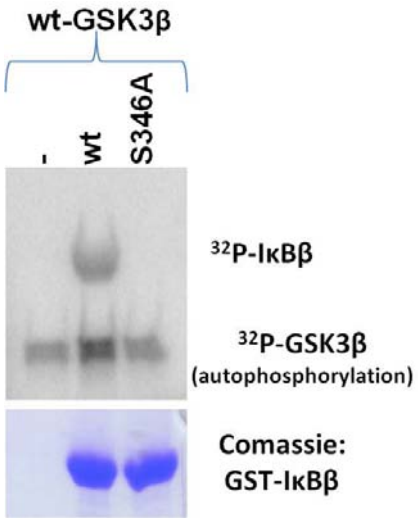


Figure 5.4E: Recombinant GSK-3β phosphorylates Ser-346 of IκBβ *in vitro* in a kinase assay with γ32-ATP: (wt- IκBβ but not S346A- IκBβ is phosphorylated; the input substrate GST-IκBβ analogs was run out on a separate gel and analyzed by coomassie staining).

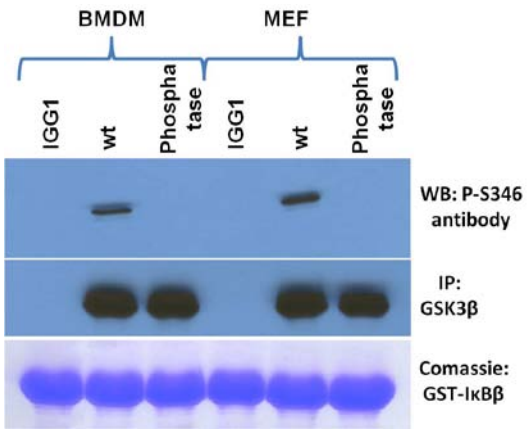


Figure 5.4F: Phospho-Ser 346-antibody recognizes IκBβ phosphorylated at Ser-346 by GSK-3β in presence of cold ATP; band corresponding to phospho-IκBβ disappears upon phosphatase treatment of GST-IκBβ previously incubated with GSK-3β (and ATP) (the input substrate GST-IκBβ was run out on a separate gel and analyzed by coomassie staining).

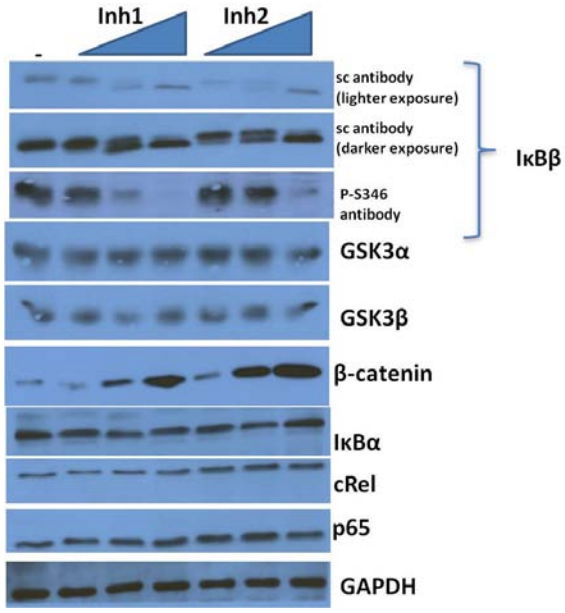


Figure 5.4G: GSK-3β phosphorylates IκBβ at Ser-346 *in vivo* in BMDM (Inhibitor 1 is 6-bromoindirubin-30-acetoxime and Inhibitor 2 is membrane-permeable GSK-3 peptide inhibitor (Calbiochem); both are used at a concentration of 20nM and 100nM); lysates were run on a 8% gel and analyzed by western-blot with respective antibodies; for the western-blot with phospho-S346 antibody, 200 µgs of lysate was used, while for the blot with sc-antibody, 20 µgs of lysate was used.

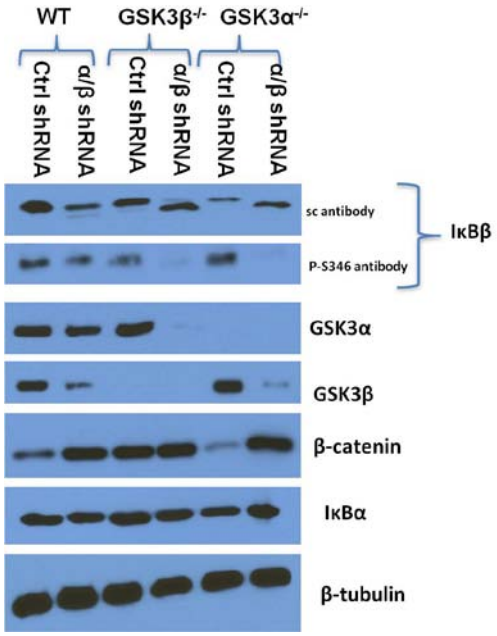


Figure 5.4H: Phosphorylation of IκBβ at Ser-346 decreases upon treatment with GSK-3α/β shRNA (lysates were run on a 8% gel and analyzed by western-blot with respective antibodies; for the western-blot with phospho-S346 antibody, 200 µgs of lysate was used, while for the blot with sc-antibody, 20 µgs of lysate was used)

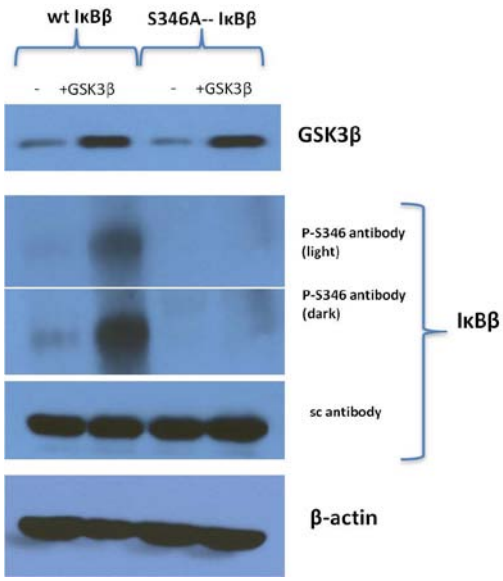


Figure 5.4I: Phosphorylation of IκBβ at Ser-346 increases upon co-transfection of GSK-3β (IκBβ$^{-/-}$ MEFs were transfected with wt-IκBβ and S346A-IκBβ; co-transfection of GSK-3β increased phosphorylation of wt-IκBβ but not in S346A-IκBβ)

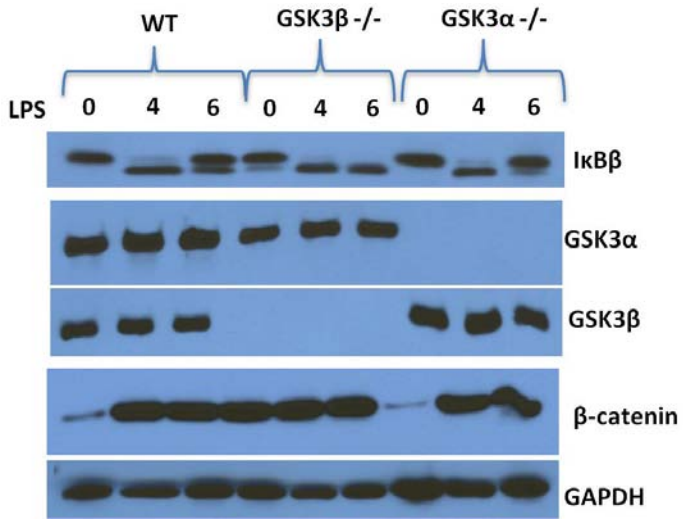


Figure 5.4J: LPS-degradation kinetics of IκBβ in WT, GSK-3β $^{-/-}$ and GSK-3α $^{-/-}$ MEFs (stimulated with 1ug/ml of LPS); lysates were run on a 8% gel and analyzed by western-blot with respective antibodies.

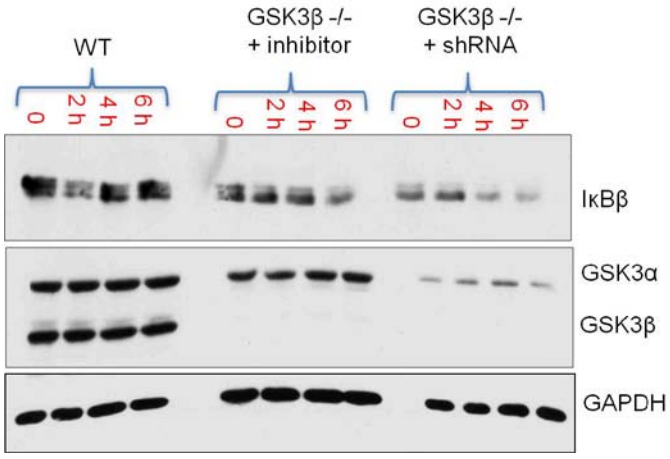


Figure 5.4K: LPS-degradation kinetics of IκBβ in MEFs where both isoforms of GSK3 have been depleted (inhibitor used is 6-bromoindirubin-30-acetoxime at a concentration of 50nM); lysates were run on a 8% gel and analyzed by western-blot with respective antibodies.

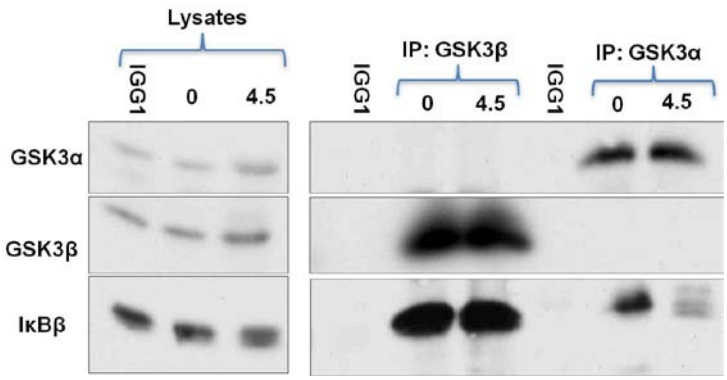


Figure 5.5A: IκBβ co-immunoprecipitates with GSK-3α and GSK-3β (BMDMs were left unstimulated or stimulated with 100ng/ml of LPS, lysed in IP-lysis buffer and incubated with IGG1 isotype (unstimulated lysate), GSK-3β antibody or GSK-3α antibody overnight; followed by the addition of Protein-G sepharose, resolved on a 8% gel and analyzed by western blotting)

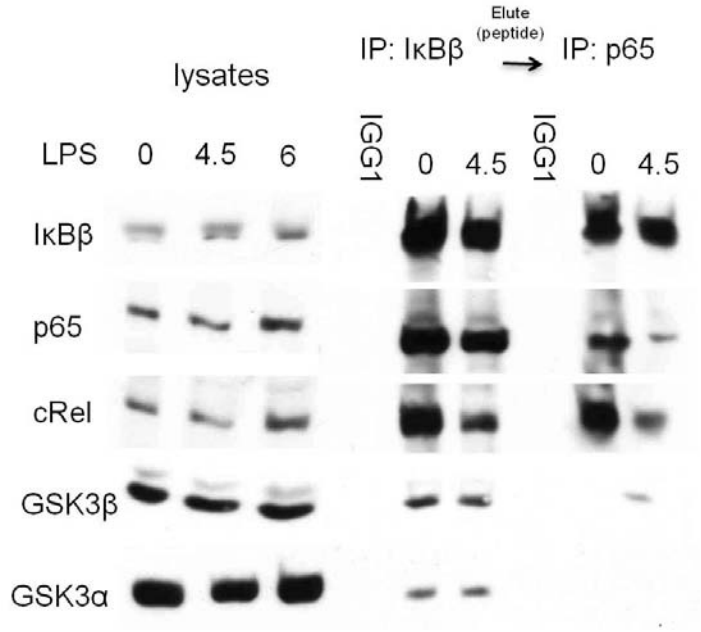


Figure 5.5B: GSK-3β is associated in a complex with hypophosphorylated-IκBβ:p65:cRel in stimulated cells (BMDMs were left unstimulated or stimulated with 100ng/ml of LPS, lysed in IP-lysis buffer and incubated with IGG1 isotype (unstimulated lysate) or IκBβ antibody overnight; followed by the addition of Protein-G sepharose, eluted with the IκBβ-peptide in a total volume of 50µl, diluted 20X times with co-IP buffer, incubated with IGG1 isotype (unstimulated lysate) or p65 antibody overnight, followed by the addition of Protein-G sepharose, resolved on a SDS-PAGE gel and analyzed by western blotting. The second immunoprecipitation with p65 determines the presence of the IκBβ:p65:cRel complex)

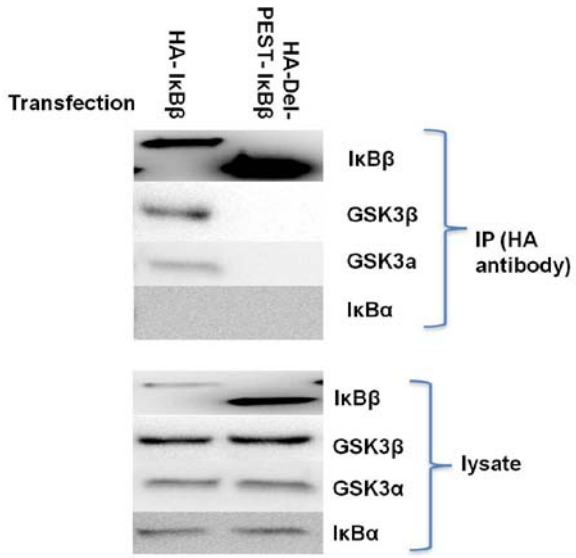


Figure 5.5C: The PEST domain of IκBβ is necessary for interaction with GSK3 (HA-tagged IκBβ constructs were overexpressed in IκBβ$^{-/-}$ MEFs, and immunoprecipitated with HA antibody overnight; followed by the addition of Protein-G sepharose, resolved on a SDS-PAGE gel and analyzed by western blotting)

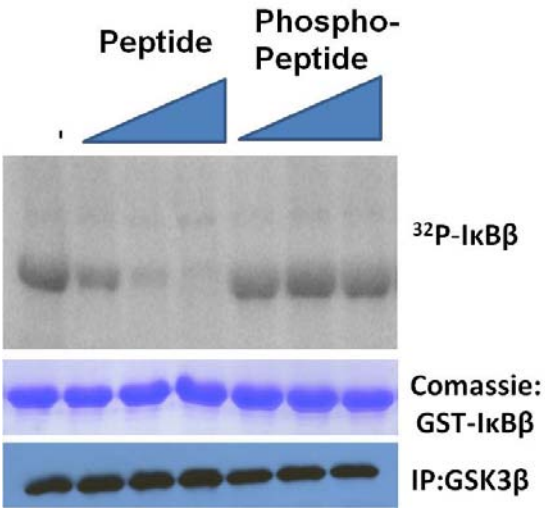


Figure 5.5D: BIP inhibits phosphorylation by immunoprecipitated GSK-3β *in vitro* in a kinase assay with γ32-ATP (peptides are used in increasing concentrations of 50µM, 200 µM and 500 µM): to confirm equal amounts of substrate and kinase in the kinase-assay, the substrate GST-IκBβ was analyzed by coomassie staining; and the amount of immunoprecipitated-kinase was run out on a separate gel and analyzed by western-blotting)

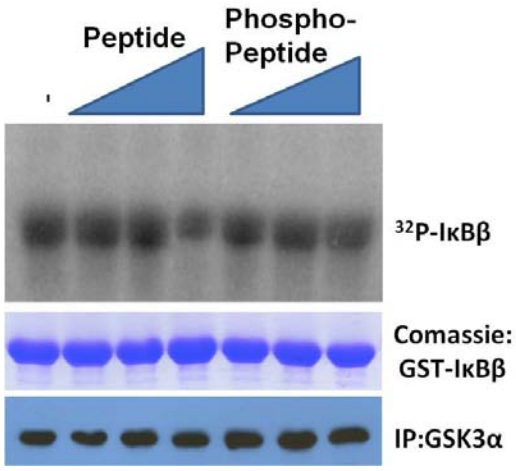


Figure 5.5E: BIP does not inhibit phosphorylation by immunoprecipitated GSK-3α *in vitro* in a kinase assay with γ32-ATP (peptides are used in increasing concentrations of 50μM, 200 μM and 500 μM): to confirm equal amounts of substrate and kinase in the kinase-assay, the substrate GST-IκBβ was analyzed by coomassie staining; and the amount of immunoprecipitated-kinase was run out on a separate gel and analyzed by western-blotting)

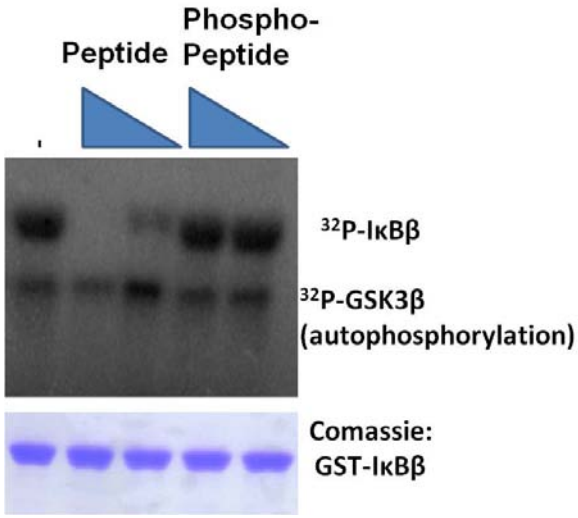


Figure 5.5F: BIP inhibits phosphorylation by recombinant GSK-3β *in vitro* in a kinase assay with γ32-ATP (peptides are used in increasing concentrations of 50μM and 500 μM): to confirm equal loading, substrate GST- IκBβ was run out on a separate gel and analyzed by coomassie staining)

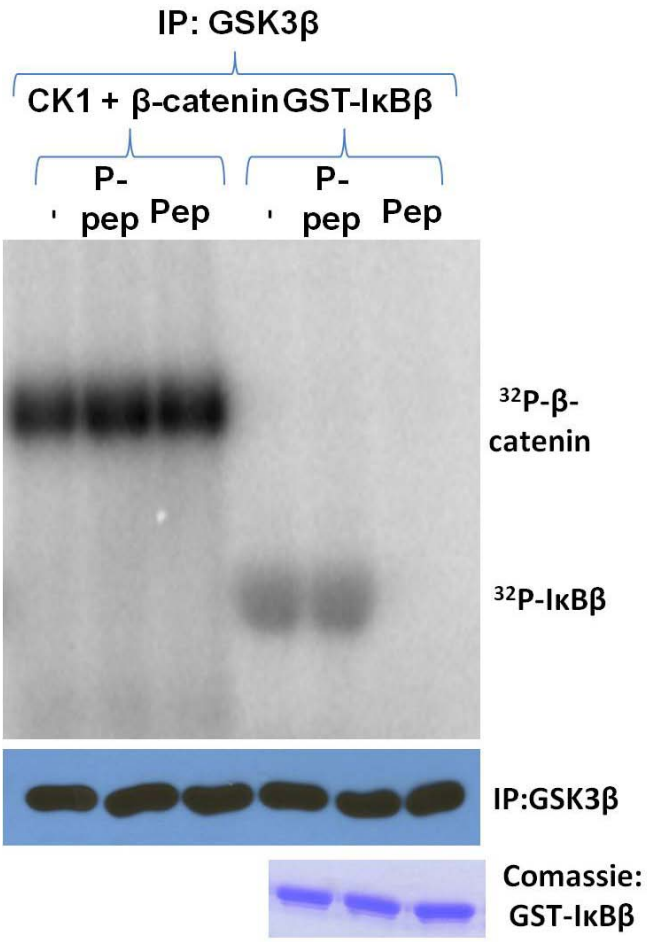


Figure 5.5G: BIP inhibits the phosphorylation of GST-IκBβ but not of GST-β-catenin in a kinase assay with γ32-ATP (concentration of peptides are 500 μM) : to confirm equal amounts of substrate and kinase in the kinase-assay, the substrate GST-IκBβ was analyzed by coomassie staining; and the amount of immunoprecipitated-kinase was run out on a separate gel and analyzed by western-blotting)

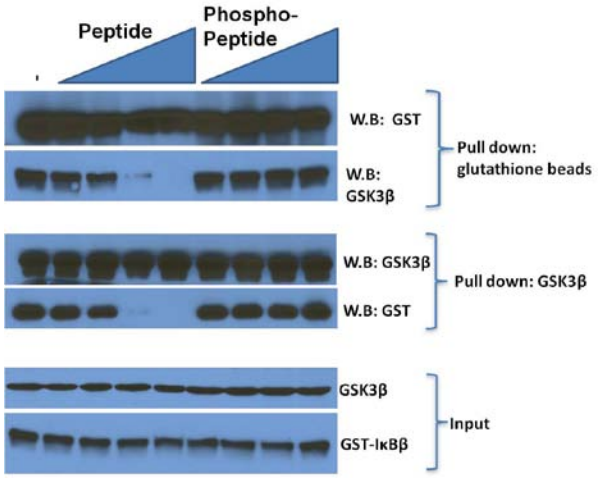


Figure 5.5H: BIP abrogates the association of GSK-3β with IκBβ *in vitro* (peptides are used in increasing concentrations of 10 µM, 50µM, 200 µM and 500 µM): Equal amounts of recombinant GSK-3β (NEB) and GST-IκBβ were incubated in the presence of peptide or mutant-peptide for 1 hour on ice in a total volume of 50µl; diluted 20X with co-IP buffer, and pulled-down overnight with glutathione beads or immunoprecipitated with GSK-3β antibody, and analyzed by western blot.

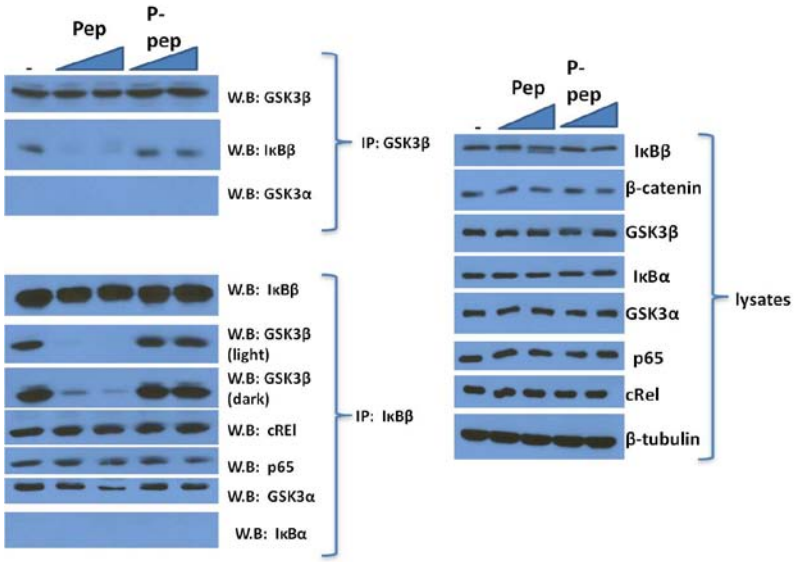


Figure 5.5I: BIP abrogates the association of GSK-3β with IκBβ *in vivo* in wt-BMDM treated with peptides as shown (peptides are used in increasing concentrations of 100µM and 200 µM): Cells were lysed in IP-lysis buffer, and immunoprecipitated with GSK-3β (upper-panel) or IκBβ antibody (lower-panel); resolved on a 8% gel and blotted with the respective antibodies (the input lysates before IP are shown in the side-panel)

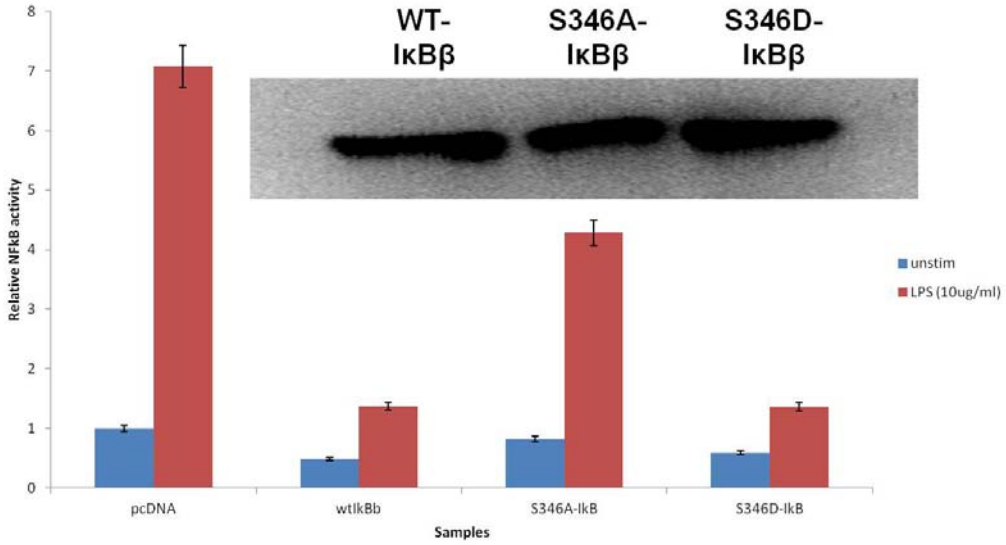


Figure 5.6A: S346A-IκBβ fails to inhibit NF-κB in luciferase assay (pcDNA or the three IκBβ analogs were transfected into IκBβ$^{-/-}$ MEFs along with κB-luciferase and Renilla construct, left unstimulated or stimulated with 10ng/ml of LPS, and relative activation of luciferase was analyzed; equal expression of the three IκBβ analogs was analyzed by western blot)

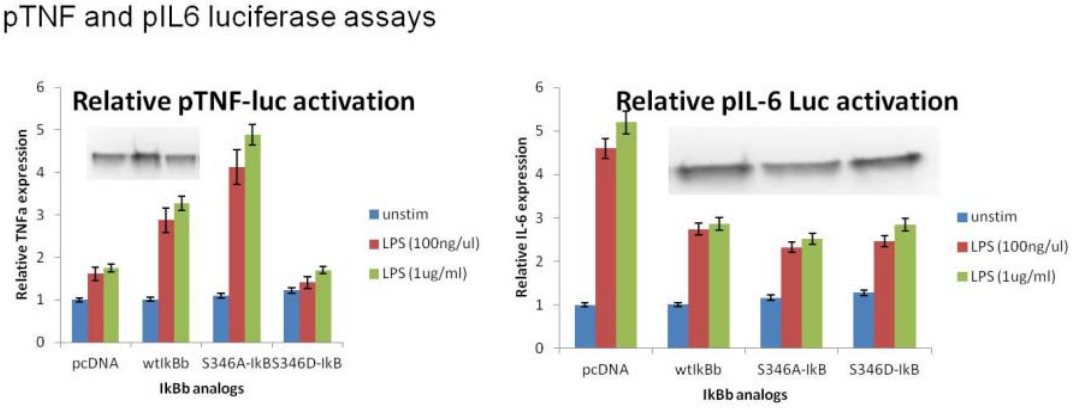


Figure 5.6B: Luciferase assay with pTNF-luciferase and pIL6-luciferase (pcDNA or the three IκBβ analogs were transfected into IκBβ$^{-/-}$ MEFs along with pTNF-luciferase construct (left) or pIL6-luciferase construct (right) and Renilla construct, left unstimulated or stimulated with 100ng/ml or 1µg/ml of LPS, and relative activation of luciferase was analyzed; equal expression of the three IκBβ analogs was analyzed by western blot in both the cases)

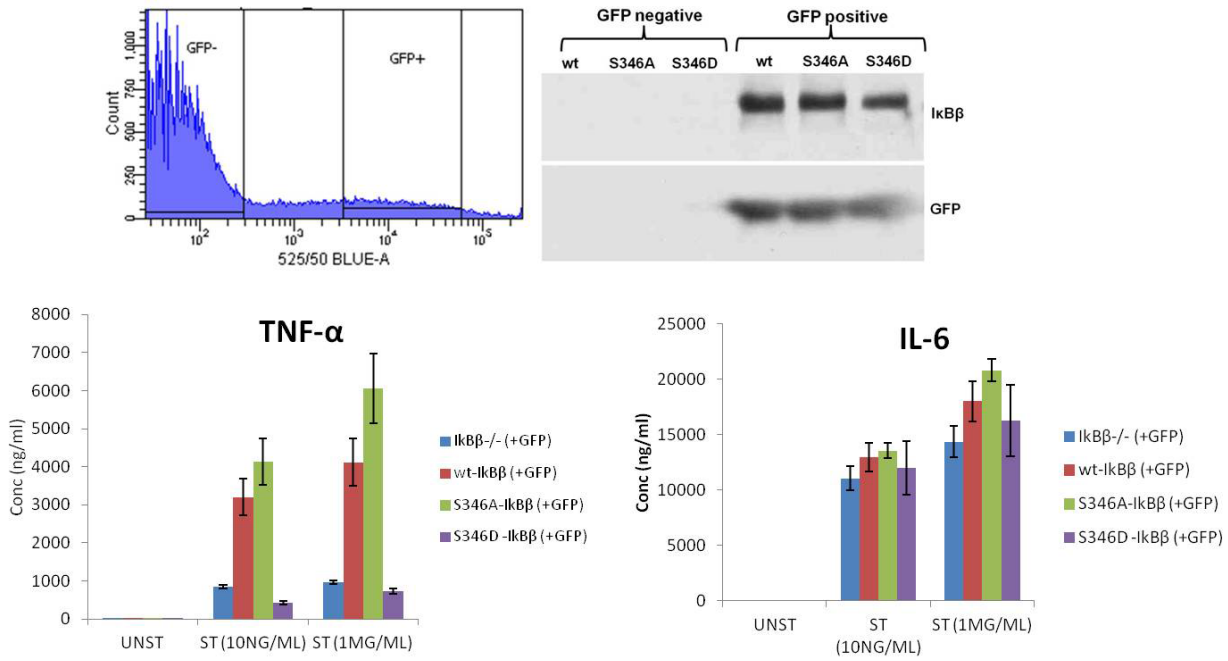


Figure 5.6C: Production of TNFα in IκBβ$^{-/-}$ BMDMs reconstituted with wt, S346A and S346D IκBβ analogs (IκBβ$^{-/-}$ BMDMs were reconstituted with GFP and the respective IκBβ analogs, cells were GFP-sorted; presence of the IκBβ analogs in GFP+ cells were confirmed by western-blot; reconstituted cells were then left unstimulated or stimulated with LPS for 20 hours, and cytokine expression was measured by ELISA)

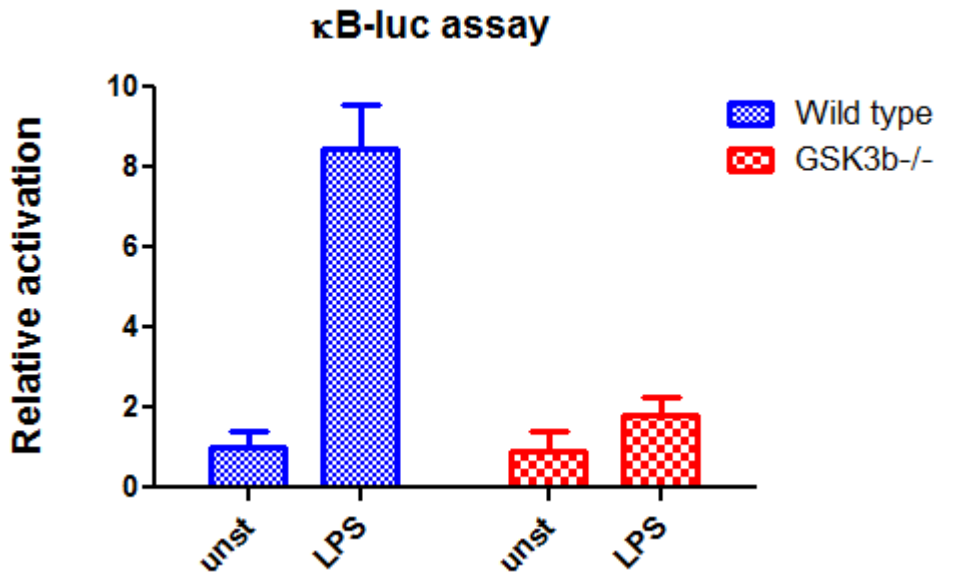


Figure 5.6D: Luciferase assay showing downregulation of NF-κB in GSK-3β $^{-/-}$ MEFs (wild type and GSK-3β$^{-/-}$ MEFs were co-transfected with κB-luciferase and Renilla construct, left unstimulated or stimulated with 10ng/ml of LPS, and relative activation of luciferase was analyzed)

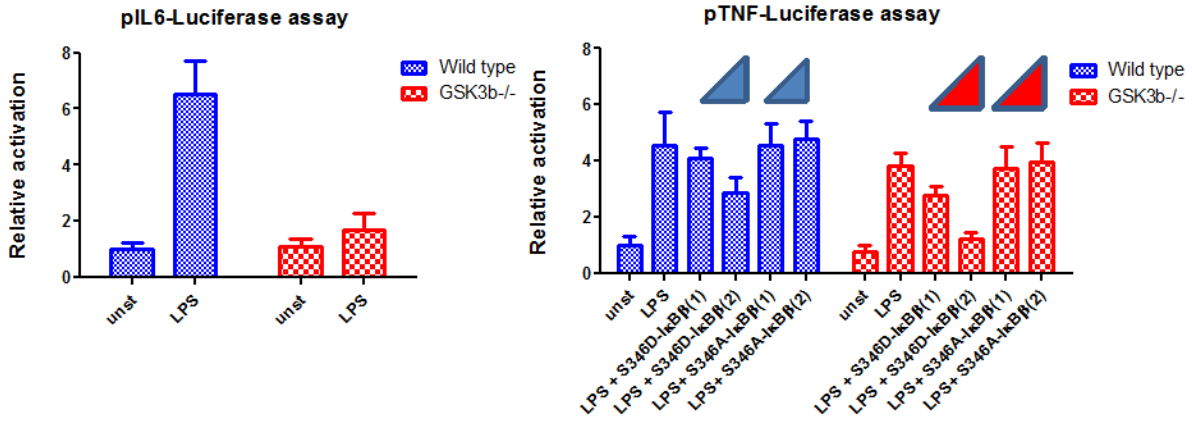


Figure 5.6E: Differential downregulation of luciferase gene downstream of IL-6 and TNF promoter in GSK-3β $^{-/-}$ MEFs (experiment was carried out as above but with pIL-6-luciferase and pTNF-luciferase instead of the generic κB-luciferase; HA-S346D-IκBβ or HA-S346A-IκBβ were co-transfected with the pTNF-luciferase construct, activation of luciferase was analyzed)

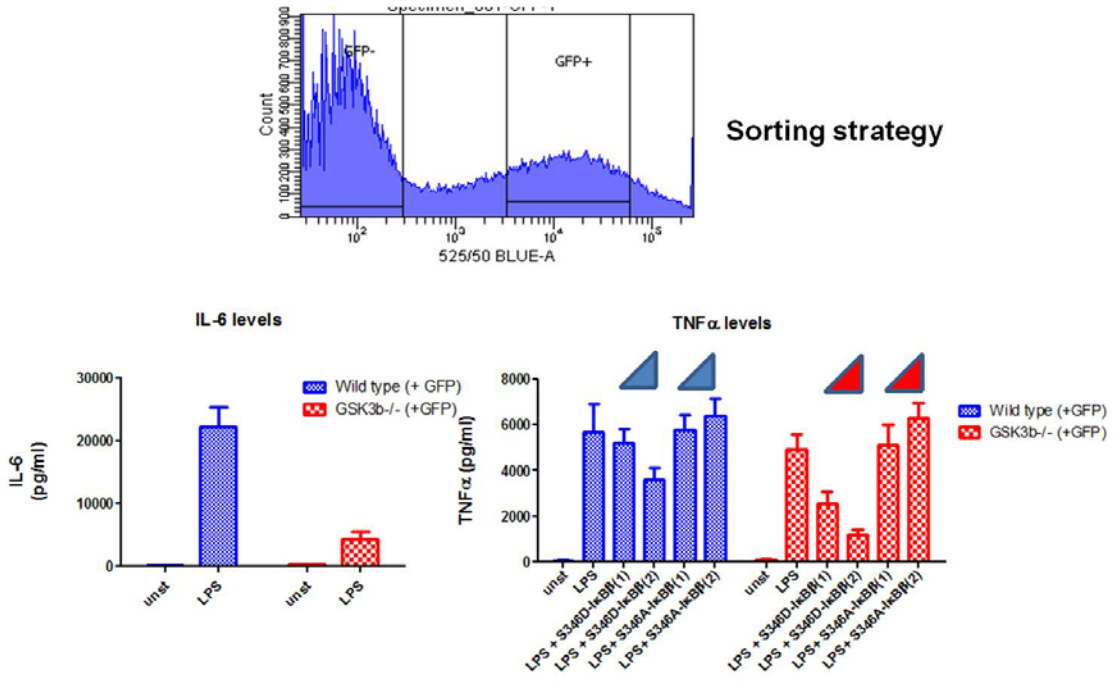


Figure 5.6F: ELISA analysis of TNFα and IL-6 of GFP-sorted wt and GSK-3β $^{-/-}$ MEFs in response to 10ng/ml of LPS (MEFs of indicated genotype were reconstituted with GFP and the respective IκBβ analogs, cells were GFP-sorted and then left unstimulated or stimulated with LPS for 20 hours, and cytokine expression was measured by ELISA)

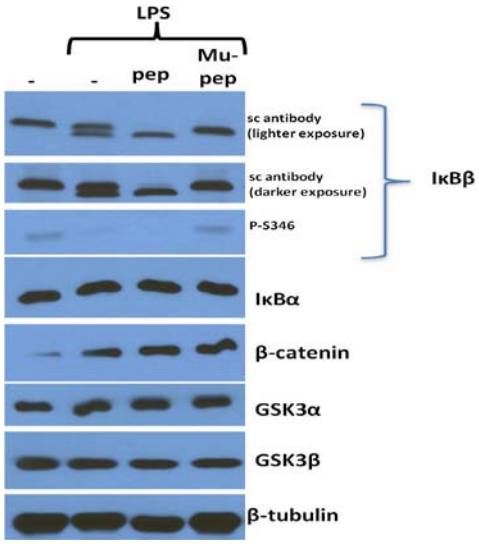


Figure 5.6G: IκBβ is entirely unphosphorylated upon stimulation with LPS in presence of peptide in BMDM (peptides used at a concentration of 200µM); for the western-blot with phospho-S346 antibody, 200 µgs of lysate was used, while for the blot with sc-antibody, 20 µgs of lysate was used

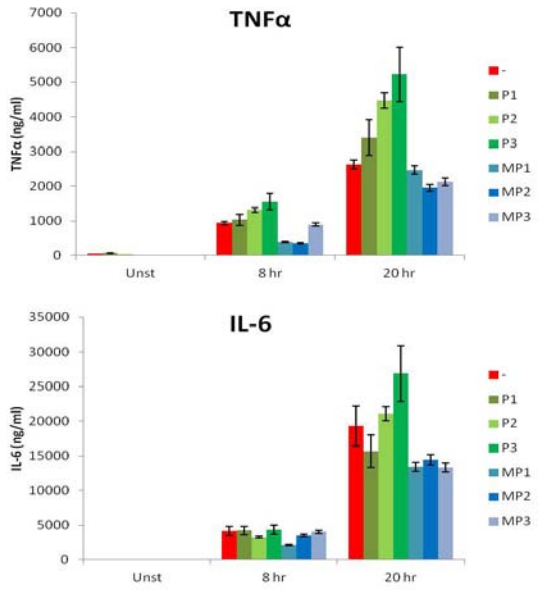


Figure 5.6H: TNFα and IL-6 production on treatment of BMDM with 10ng/ml of LPS in the presence and absence of peptide (concentrations of P1=50 µM; P2=100 µM and P3=200µM)

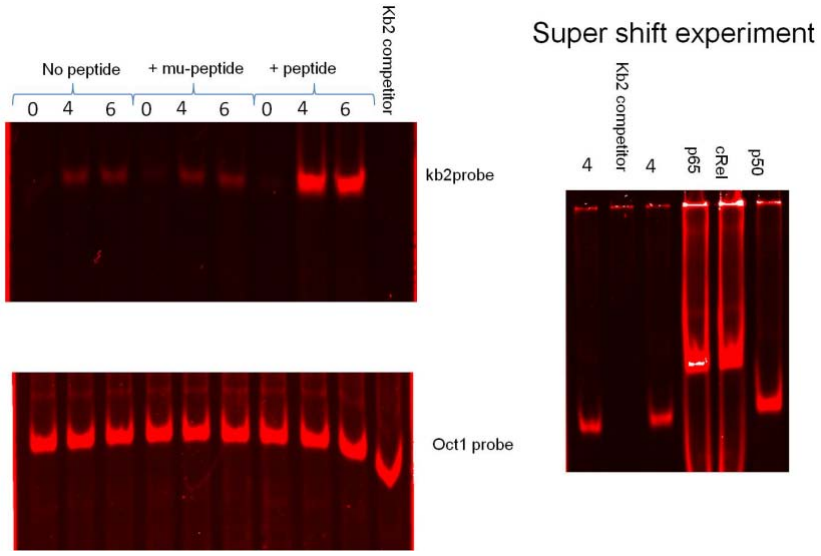


Figure 5.6I: EMSA analysis showing upregulation of p65:cRel complexes at the κB2 site in BMDMs upon stimulation with LPS in the presence of 200μM of peptide (but not mutant-peptide): gel-shift assay was carried out using nuclear fractions and κB2 probe; equal loading was confirmed by carrying out the reaction with Oct1 probe and running it on a 5% TBE gel)

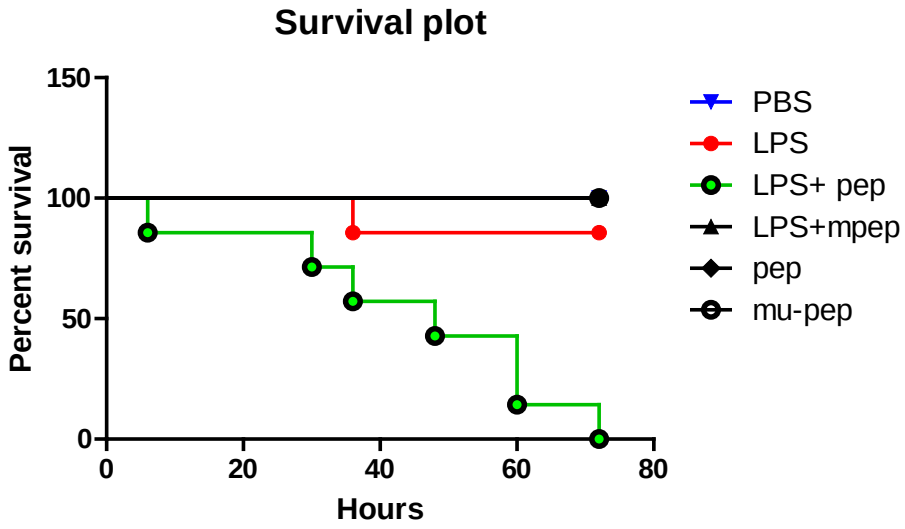


Figure 5.6J: LPS shock in wild type mice in the presence and absence of peptide (n=7); intraperitoneal injection of a sublethal dose of 18 μg/g body weight LPS was administered and monitoring for survival every six hours (mice succumb to sublethal dose of LPS only in the presence of peptide)

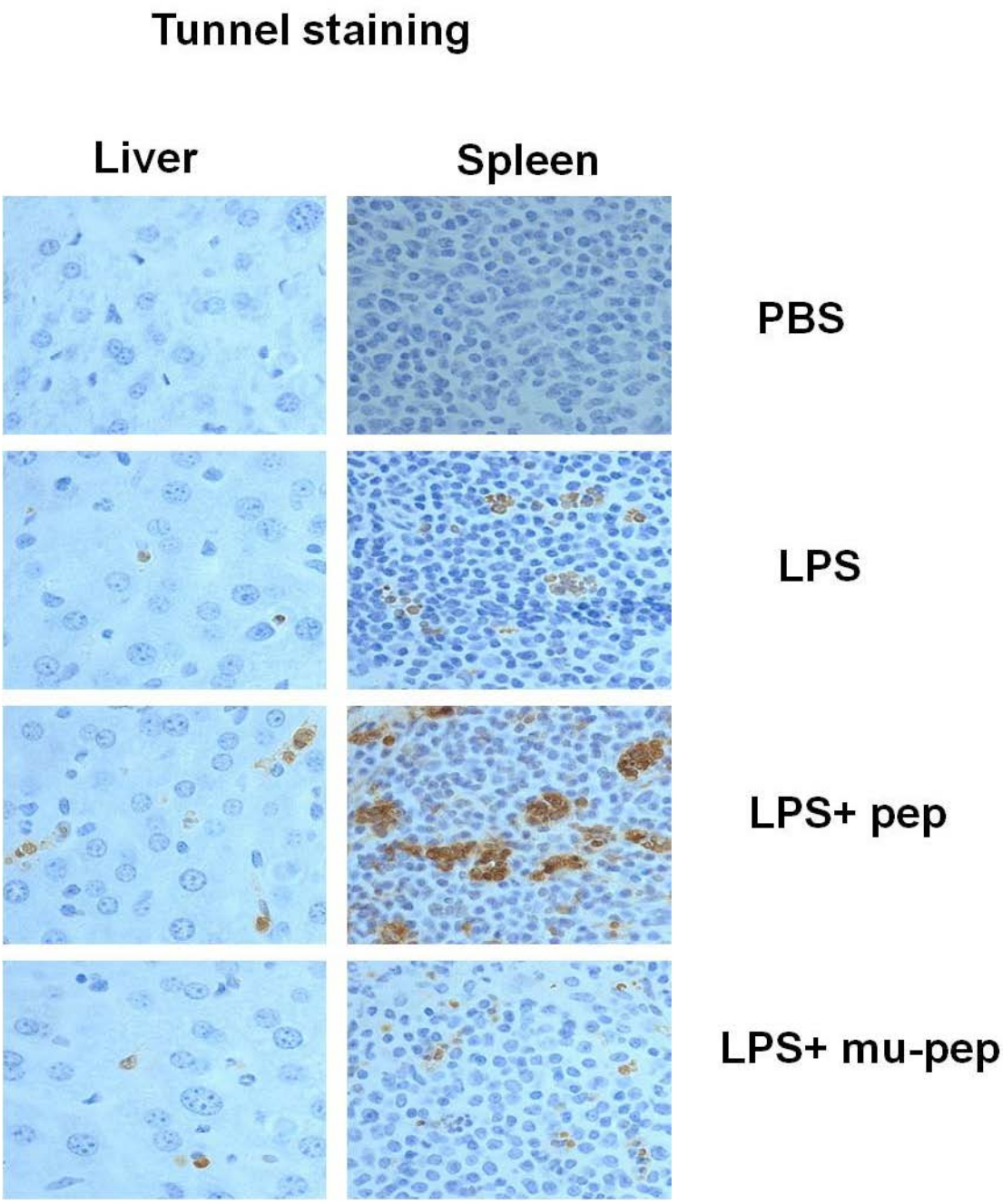


Figure 5.6K: Tunnel staining of liver and spleen in mice treated with PBS, LPS, LPS+peptide and LPS+mu-peptide (significantly increased signs of cell-death in organs of mice treated with LPS in the presence of peptide)

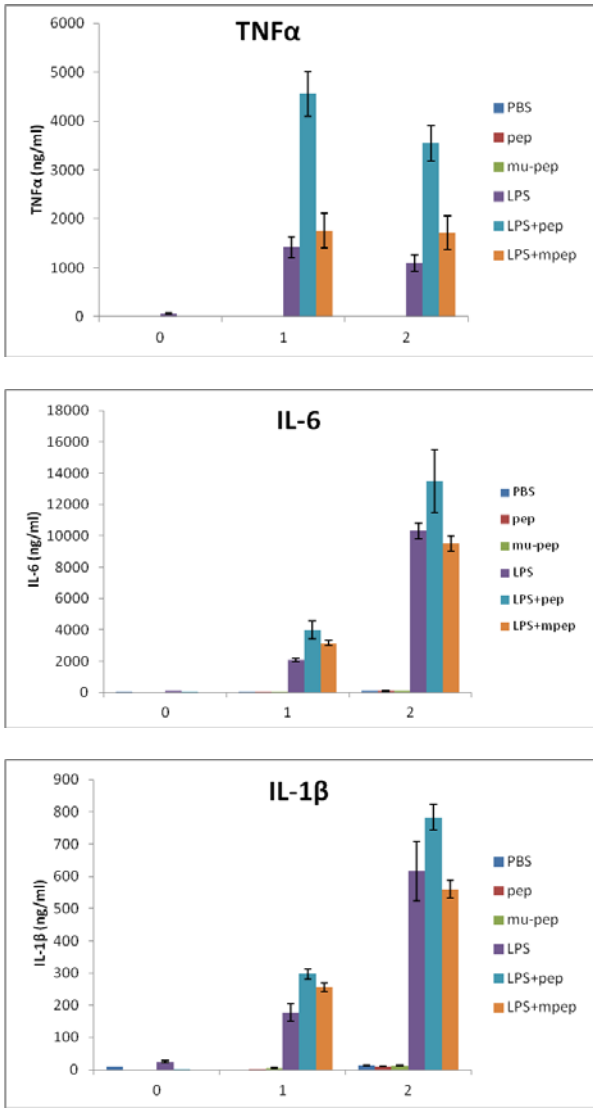


Figure 5.6L: Serum levels of TNFα, IL-6 and IL-1β in mice treated PBS, peptide (only), mutant-peptide (only), LPS, LPS+peptide and LPS+mu-peptide (significantly increased production of TNFα but not IL-6 or IL-1β in mice treated with LPS in the presence of peptide)

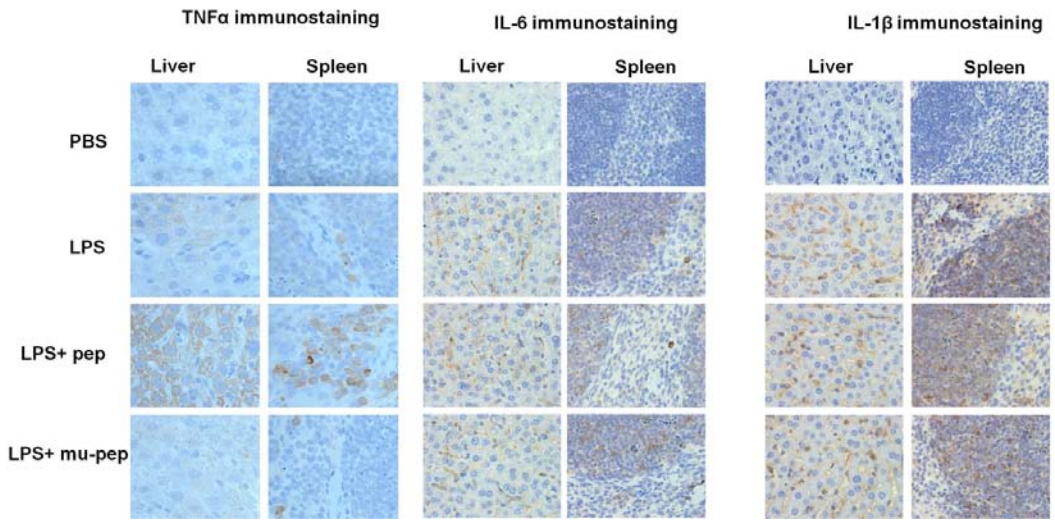


Figure 5.6M: Immunostaining of TNFα, IL-6 and IL-1β (significant upregulation of TNFα but not IL-6 or IL-1β in organs of mice treated with LPS in the presence of peptide)

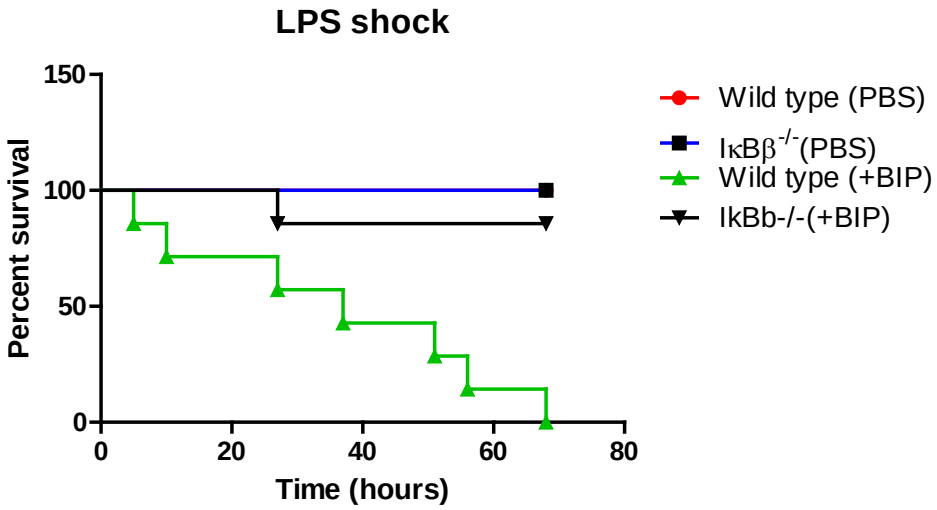
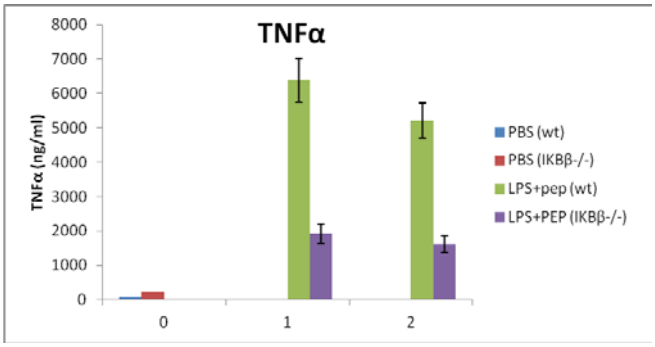
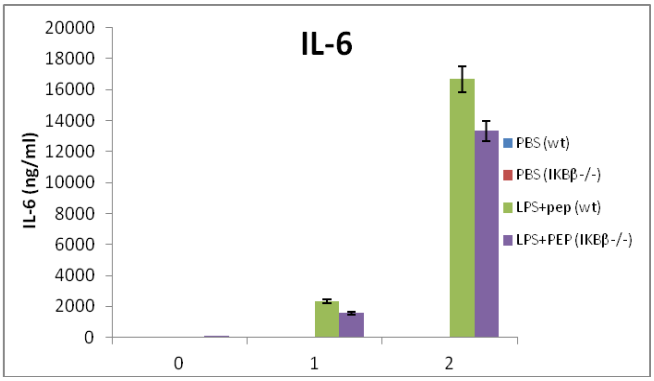


Figure 5.6N: LPS shock of wt and IκBβ$^{-/-}$ mice in the presence and absence of peptide (n=7); intraperitoneal injection of a sublethal dose of 18 µg/g body weight LPS was administered and monitoring for survival every six hours (wild type mice but not IκBβ$^{-/-}$ mice succumb to a sublethal dose of LPS in the presence of peptide)





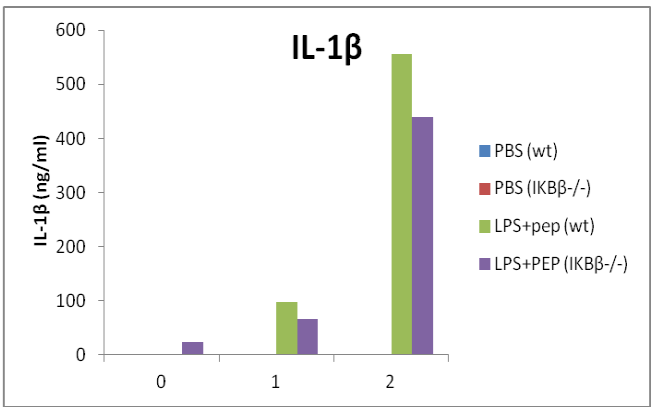


Figure 5.6O: Serum levels of TNFα, IL-6 and IL-1β in wt and IκBβ$^{-/-}$ mice (significantly increased production of TNFα but not IL-6 or IL-1β only in wild-type but not IκBβ$^{-/-}$ mice after administration of LPS in the presence of peptide)

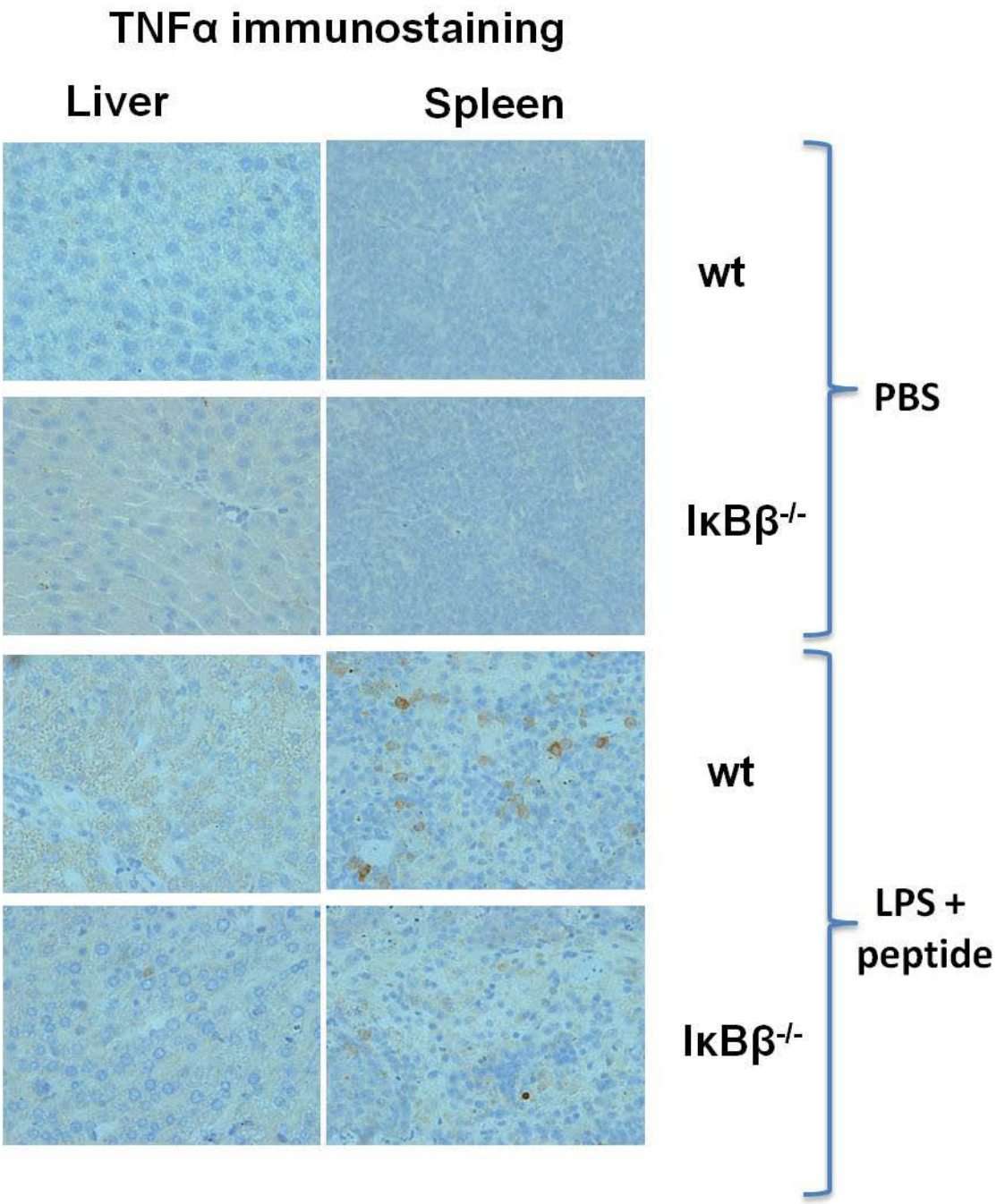


Figure 5.6P: Immunostaining of TNFα in wt and IκBβ$^{-/-}$ mice (significantly elevated expression of TNFα in organs of wild-type but not IκBβ$^{-/-}$ mice after administration of LPS in the presence of peptide)

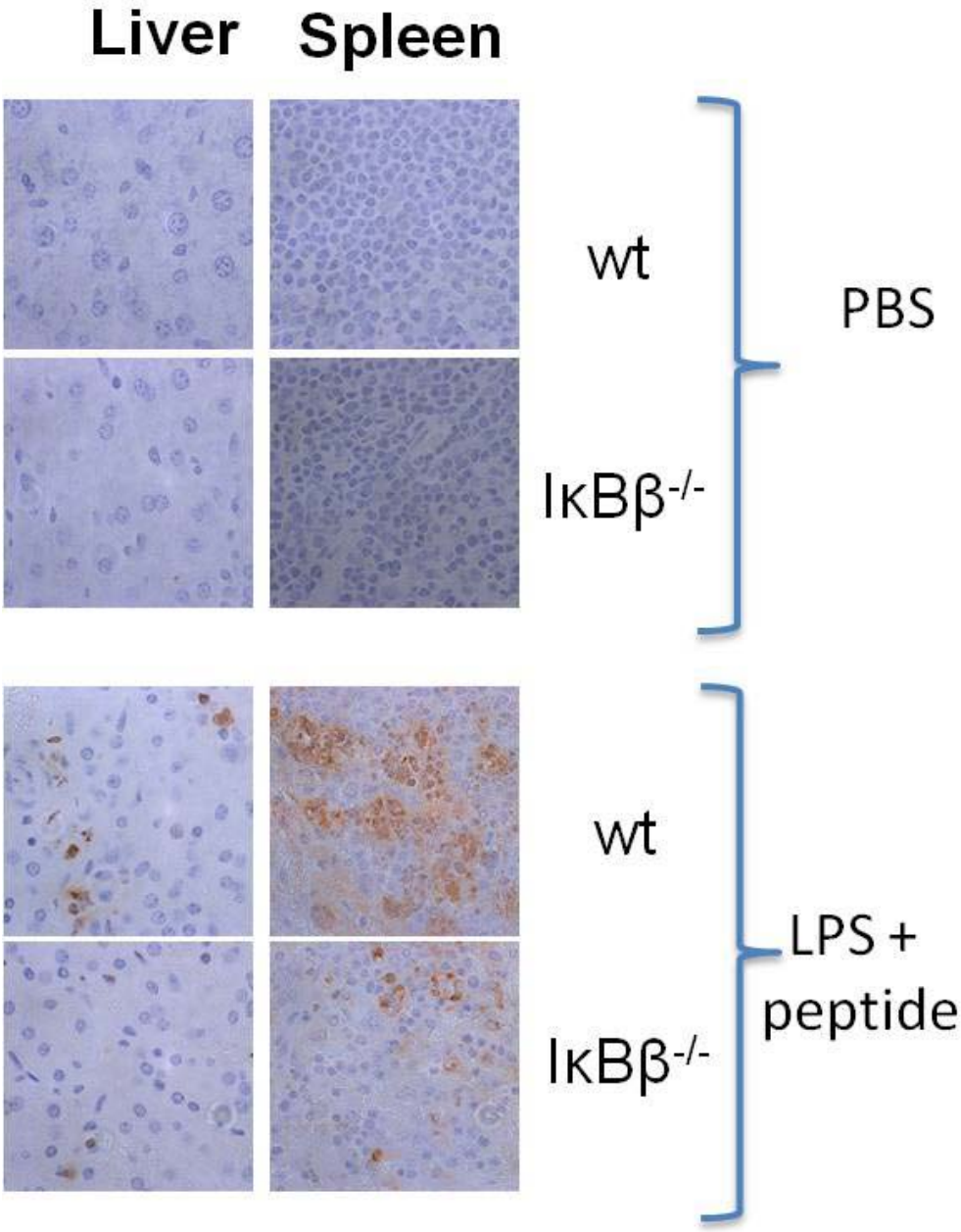


Figure 5.6Q: Tunnel staining of liver and spleen in wt and IκBβ$^{-/-}$ mice (significantly increased cell death in organs of wild-type but not IκBβ$^{-/-}$ mice after administration of LPS in the presence of peptide)

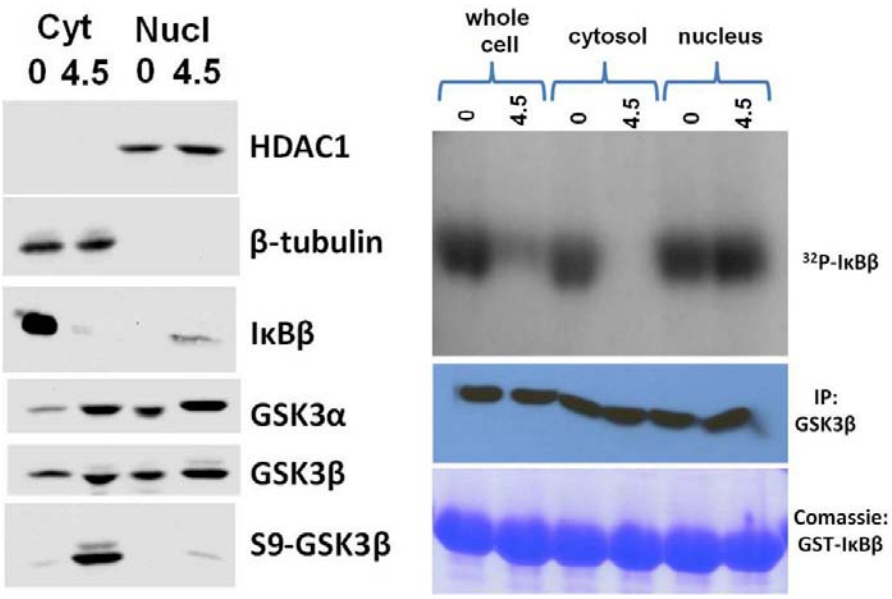


Figure 5.7: Kinase assay with immunoprecipitated nuclear and cytoplasmic GSK-3β (BMDMs were fractionated and the cytosolic and nuclear fractions are shown in the left-panel; GSK-3β was immunoprecipitated from whole-cell lysates, cytosolic or nuclear fraction; kinase-assay was performed by incubating immunoprecipitated GSK-3β with recombinant GST- IκBβ in presence of γ32-ATP (right-panel); to confirm equal amounts of substrate and kinase in the kinase-assay, the substrate GST- IκBβ was analyzed by coomassie staining; and the amount of immunoprecipitated-kinase was run out on a separate gel and analyzed by western-blotting)

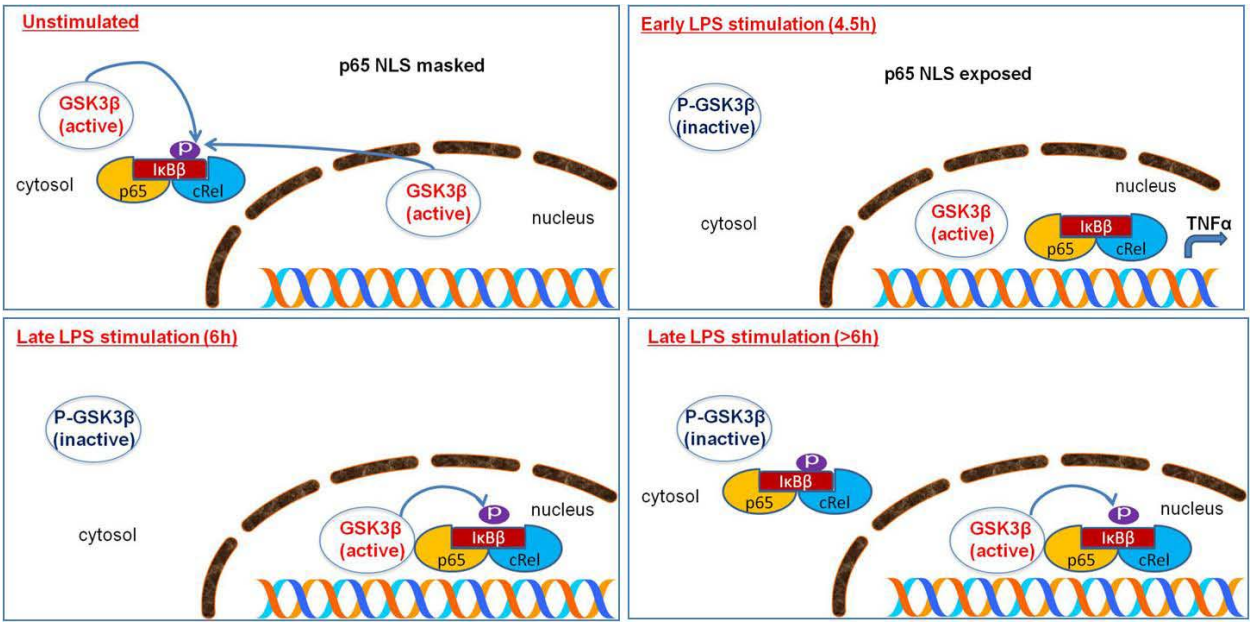


Figure 5.8: Biological model of GSK-3β regulating IκBβ

(In unstimulated cells, IκBβ is phosphorylated active GSK-3β (both nuclear and cytosolic) at Ser-346 (top-left). Phosphorylated-IκBβ masks the NLS of p65, hence the phosphorylated-IκBβ:p65:cRel complex is cytoplasmic in the resting state (top-left). During early LPS stimulation, GSK-3β is inactivated and phospho-IκBβ is proteasomally degraded following inducible phosphorylation by IKK at Ser 19 and Ser 23. New IκBβ is resynthesized in the unphosphorylated state and cannot mask the NLS of p65. As a result, the trimeric unphosphorylated-IκBβ:p65:cRel trimeric complex is found in the nucleus, where it drives the expression of proinflammatory genes like TNFα (top-right). Nuclear GSK-3β associates with this complex and phosphorylates IκBβ (bottom-left). The phosphorylation masks the NLS of p65, translocating the phosphorylated-IκBβ:p65:cRel complex back to the cytoplasm (bottom-right).

GSK3 does not associate with the phosphorylated-IκBβ:p65:cRel trimeric complex in the quiescent state (top-left; data in Figure 5.5B), hence it seems that GSK3 does not phosphorylate IκBβ in the complex under resting conditions. However nuclear GSK-3β associates with this complex after stimulation (bottom-left; data in Figure 5.5B), suggesting that GSK-3β phosphorylates the newly synthesized, unphosphorylated-IκBβ that is associated with p65:cRel heterodimer.)

Chapter 6:

Conclusion: perspectives on regulatory ubiquitination, A20 and IκBβ

This thesis describes the mechanism of action of two well-known inhibitors of NF-κB, A20 and IκBβ.

6.1: Regulatory ubiquitination and A20

It is well-appreciated that almost all NF-κB signaling pathways proceed via the activation of the IKK complex as NF-κB activity is absent in mice deficient in both IKKα and IKKβ [107]. Phosphorylation of Ser-177 and Ser-181 in the 'active loop'' of IKKβ is required for the activation of IKK [71]. However, how this phosphorylation happens is unknown [108]. There are two obvious possibilities: IKK could either autophosphorylate itself or be phosphorylated by some other kinase (like TAK1). The biggest issue is that the receptors leading to IKK activation lack kinase activity or other known enzymatic activity. Hence, either kinase activity needs to be recruited to the pathway or conformational changes upon receptor ligation must somehow lead to IKK phosphorylation.

It is conceivable that these conformational changes might be caused by the oligomerization of adaptor proteins upon receptor ligation. Another hypothesis is that the adaptor proteins may be ubiquitinated with K63-linked, non-degradative, ubiquitin chains; and it is these ubiquitin chains that activate TAK1/IKK. The first part of the theses broadly tests the second hypothesis to determine if regulatory ubiquitination might lead to NF-κB activation.

Initially, it was thought that these ubiquitin chains provide the oligomeric structure for stabilizing the signaling complexes or recruits the kinase complexes, like the IKK complex (via NBD of NEMO) or TAK1 complex (via the NBD of the TAB adaptor proteins), bringing these kinases closer together for trans-phosphorylation. Another non-exclusive model holds that

ubiquitination of upstream adapters may directly activate the downstream kinase complexes. Recently though, it was shown that even ubiquitin chains that are not anchored to any substrate can directly activate IKK or TAK1 [168]. If direct activation of kinase complexes by substrate-linked ubiquitin or free-ubiquitin were to occur, it would be expected that short ubiquitin chains would be as effective as long ubiquitin chain in activating the kinase complex. However, this was not found to be the case. Moreover, it is unclear as to how the IKK complex or the TAK/TAB complex would differentiate between regulatory polyubiquitin chains linked to one substrate from those linked to a different substrate (or differentiate the substrate-linked chains from unanchored ubiquitin chains).

Crucially, if ubiquitin chains were to directly activate the IKK complex, it would mean that IKKβ or NEMO would have to be ubiquitinated. In tissue-culture based experiments and knock-down models, regulatory ubiquitination of NEMO at K-399 has been shown to be essential for NF-κB activation [165]. However, subsequently knock-in mice generated with a single mutation at this residue was found to have normal NF-κB activation. Hence, the genetic murine model has conclusive demonstrated that ubiquitination of NEMO at K-399 is not essential for NF-κB activation.

There is however a plethora of biochemical evidence to suggest that upstream adaptor molecules like RIP1 indeed get ubiquitinated in response to TNFα stimulation. Western-blot analysis shows the slower-migrating forms of RIP1, or ubiquitinated-RIP1 [286]. The slower-migrating forms of RIP1 are augmented if only RIP1 that is recruited to TNF-receptor complex is analyzed [164], [244]. The ubiquitin linkage on RIP1 may be K63-linked [163, 164, 230, 514] or linear [244]. Moreover, RIP1 is essential for activation of NF-κB as concluded from mice

deficient in RIP1 [241]. In spite of this, for reasons outlined below (in addition to my findings), it is doubtful if the ubiquitination of RIP1 is actually essential for RIP1 mediated activation of NF-κB.

RIP1 has been shown to be ubiquitinated on Lys-377 and reconstitution studies using the K377R mutant in cells deficient in RIP1 showed the importance of this residue in ubiquitination of RIP1. However, the K377R mutant is also not recruited to the activated TNF receptor [163, 230]. Hence, the implications of ubiquitination at this residue remain unclear. Ubc5 (E2) is important for RIP1 ubiquitination, and Ubc5 knock-down virtually abrogates RIP1 ubiquitination [231] in cells. The cells lacking UbC5 though have only modest impairment in IKK activation. Similarly TRAF2/5 (E3-ligase) is important for RIP1 ubiquitination. But reconstitution of cells deficient in both TRAF2 and TRAF5 with TRAF2 RING mutants abolishes RIP1 ubiquitination without affecting IKK activation [515]. Perhaps the cleanest in vitro experiment, replacing endogenous ubiquitin with K63R-ubiquitin (genome knock-in at the ubiquitin locus), has showed that K63-linked ubiquitin is not required for TNFR-mediated IKK activation [231].

The greatest support for regulatory ubiquitination actually comes from the well-established roles of deubiquitinases as negative regulators of IKK activation [211]. A20 plays an essential role in downregulation NF-κB activation and consequent inflammation, as mice lacking A20 die from persistent inflammation in multiple organs and cachexia [134]. In humans, dysregulation of A20 has been associated with multiple autoimmune disorders. However, what was not known till now was if the deubiquitinase function of A20 (directed mainly towards RIP1) was actually essential for downregulation of NF-κB.

The first part of my theses shows that "the deubiquitinase activity of A20 is dispensable for its role in NF-κB signaling". The knock-in mouse that we have generated selectively abrogates the deubiquitinase function of A20 *in vivo*. These mice display normal NF-κB activation without showing any inflammatory phenotype. Our results clearly demonstrate that the well-characterized role of A20 in limiting inflammatory responses is due to effects other than deubiquitination of K63-ubiquitin chains. Given that the presumed role of A20 as a deubiquitinase has been used to support the importance of regulatory K63 ubiquitination in NF-κB signaling, we believe that our studies will help focus future research efforts into alternative target pathways that do not depend on K63 ubiquitination. As A20 clearly has an important role in many diseases, understanding the true molecular function of A20 remains an important and worthwhile goal for the future.

It is tempting to speculate that K63-linked ubiquitin chains may play an alternative role in stabilizing upstream signal complexes by preventing untimely and inadvertent K48-linked degradative ubiquitination of these signaling complexes. In this regard, both A20 and CYLD have been shown to work by not only disassembling K63-linked ubiquitin chains (deubiquitinase role) but also facilitating the assembly of K48-linked ubiquitin chains to their substrates [196], [516]. It might well be that the timely ligation of the well characterized, K48-linked degradative ubiquitin chains is the function of regulatory ubiquitination.

In the bigger context, the crucial issue is determining the mechanism for activation of kinases downstream of receptors that lack inherent kinase activity. Given the absence of receptor-enzymatic activity, ubiquitination of adaptor proteins provides an appealing mechanism

for the activation of IKK. However, extensive genetic experiments using knock-in models are required to show the importance of regulatory ubiquitination in the activation of NF-κB

6.2: GSK-3β and cross-regulation of NF-κB pathway

The first substrate of GSK-3β discovered in 1984 was glycogen synthase [454]. However, subsequently around 50 different substrates of GSK-3β have been discovered and GSK-3β has been determined to be the converging point of diverse pathways [455]. A reason for this might be that while most kinases are inducibly activated, GSK-3β has been found to be constitutively active in the quiescent state but inactivated in response to stimuli. In these theses, we report another substrate of GSK-3β, IκBβ, which is constitutively phosphorylated by GSK-3β at Ser-346 in unstimulated cells.

On a global scale, GSK-3β is clearly required for the upregulation of NF-κB, as mice deficient in GSK-3β are embryonic lethal, and die from liver degeneration [467]. It does seem however that the increased death observed in GSK-3β knockout mice is disproportionate to the observed decrease in NF-κB signaling. While luciferase-based reporter assays show that NF-κB cannot be activated in GSK-3$\beta^{-/-}$ MEFs [467], [506], results obtained from EMSA analysis are less convincing [506]. It is not clear if GSK-3β directly activates NF-κB and the phenotype of GSK-3$\beta^{-/-}$ mice might result from cross-regulation of other pathways. In any case, this is more due to the decreased expression of pro-survival genes during development.

The evidence that GSK-3β may play a profound role in regulating the inflammatory response in adult mice comes from studying the effects of pharmacological inhibitors. In this regard, lithium, a well-characterized pharmacological inhibitor of GSK-3β is used an anti-

depressant and has anti-inflammatory properties ameliorating experimental autoimmune encephalomyelitis [517]. However, there are conflicting reports where other pharmacological inhibitors of GSK-3β have also been shown to induce the production of pro-inflammatory cytokines. For example, certain pharmacological inhibitors have been show to increase IL-17 mediated IL-6 production [502], TNF-mediated IL-6 production in endothelial cells [500] and LPS-induced expression of TNFα in cardiomyocytes [501].

Hence, it seems that inhibition of GSK-3β would have different effects in different cell types and also depend on the stimuli and signaling context. This is not unique for GSK-3β and is indeed common for other kinases that regulate multiple pathways. For example, Protein Kinase A (PKA) is thought to have a global anti-inflammatory role [518] and downregulates NF-κB. However, multiple studies have also showed that PKA can also activate NF-κB by phosphorylating p65 at Ser-276 [519], [520], [521] It is now appreciated that PKA selectively modulates NF-κB activation in different conditions [518].

Our study shows that GSK-3β downregulates basal inflammation by constitutively phosphorylating IκBβ at Ser-346. This is profoundly important as mice succumb to endotoxin shock because of increased expression of TNFα, when the phosphorylation is abrogated by a peptide which selectively inhibits phosphorylation of IκBβ by GSK-3β at Ser-346. The work also highlights that a small-molecule mimic of S346D-IκBβ might be a useful therapeutic to reduce the side-effects of GSK-3β inhibitors.

We also show how the same protein can perform two opposite functions. Thus, IκBβ is anti-inflammatory when phosphorylated at Ser-346 but selectively increases the expression of TNFα in the unphosphorylated state upon stimulation with LPS. Hence, IκBβ acts as a chaperone

and stabilizes the p65:cRel dimer. Stabilization by phosphophorylated IκBβ results in sequestration of the dimer in the cytoplasm and mediates an anti-inflammatory role. In contrast, complexation with the unphosphorylated IκBβ stabilizes the p65:cRel dimer in the nucleus and mediates prolonged expression of TNFα.

It is tempting to speculate about the cross-talk of the IκBβ mediated pathway with the β-catenin mediated pathway. The absence of GSK-3β leads to the stabilization of β-catenin and the formation of unphosphorylated IκBβ. It is unknown but possible that these two molecules cooperate with each other to facilitate gene expression.

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