# Nuclear Factor-Kappa-B Signaling in Lung Development and Disease: One Pathway, Numerous Functions

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In contrast to other organs, the lung completes a significant portion of its development after term birth. During this stage of alveolarization, division of the alveolar ducts into alveolar sacs by secondary septation, and expansion of the pulmonary vasculature by means of angiogenesis markedly increase the gas exchange surface area of the lung. However, postnatal completion of growth renders the lung highly susceptible to environmental insults such as inflammation that disrupt this developmental program. This is particularly evident in the setting of preterm birth, where impairment of alveolarization causes bronchopulmonary dysplasia, a chronic lung disease associated with significant morbidity. The nuclear factor $\kappa$-B (NF$\kappa$B) family of transcription factors are ubiquitously expressed, and function to regulate diverse cellular processes including proliferation, survival, and immunity. Extensive evidence suggests that activation of NF$\kappa$B is important in the regulation of inflammation and in the control of angiogenesis. Therefore, NF$\kappa$B-mediated downstream effects likely influence the lung response to injury and may also mediate normal alveolar development. This review summarizes the main biologic functions of NF$\kappa$B, and highlights the regulatory mechanisms that allow for diversity and specificity in downstream gene activation. This is followed by a description of the pro and anti-inflammatory functions of NF$\kappa$B in the lung, and of NF$\kappa$B-mediated angiogenic effects. Finally, this review summarizes the clinical and experimental data that support a role for NF$\kappa$B in mediating postnatal angiogenesis and alveolarization, and discusses the challenges that remain in developing therapies that can selectively block the detrimental functions of NF$\kappa$B yet preserve the beneficial effects.

Birth Defects Research (Part A) 100:202–216, 2014.


**Key words:** bronchopulmonary dysplasia; angiogenesis; inflammation; mouse models; endothelial cells

## Introduction

During the final stage of lung development, the formation of the alveoli by secondary septation results in a 20-fold increase in the gas-exchange surface area of the lung (Burri, 2006). Disruption of alveolarization during infancy results in bronchopulmonary dysplasia (BPD), the most common complication of premature birth (Jobe, 2011). While advances in the medical care of preterm infants have reduced mortality, the incidence of BPD has not decreased in the past 10 years (Kinsella et al., 2006). Infants with BPD require significant respiratory support early in life, and many demonstrate long-term deficits in pulmonary function (Northway et al., 1990; Filippone et al., 2003; Doyle et al., 2006; Fakhoury et al., 2010) and delayed distal lung growth (Balinotti et al., 2010).

Data suggest that angiogenesis is essential for alveolarization, and that disrupted angiogenesis is key to the pathogenesis of BPD. Angiogenic factors belonging to the vascular endothelial growth factor (VEGF) family increase during alveolarization and are suppressed by hyperoxia, an injury that disrupts alveolar development (Hosford and Olson, 2003). Blocking postnatal angiogenesis impairs alveolarization in animal models (Jakkula et al., 2000; Le Cras et al., 2002), while promoting angiogenesis by enhancing VEGF rescues the disruption of secondary septation induced by hyperoxia (Thébaud et al., 2005). Both VEGF levels and pulmonary capillary density are decreased in the lungs of infants dying from BPD (Bhatt et al., 2001).

While the pathogenesis of BPD is multifactorial, inflammation appears to be one factor that disrupts alveolarization. Chorioamnionitis increases the risk of BPD (Watterberg et al., 1996), and levels of pro-inflammatory cytokines are elevated in the amniotic fluid of premature infants who develop BPD as compared to those without lung disease (Munshi et al., 1997). Intrauterine administration of endotoxin in animal models induces alveolar simplification similar to that observed in patients with BPD (Moss et al., 2002). Similarly, epithelial overexpression of the inflammatory cytokine interleukin-1$\beta$ during embryonic development impairs alveolarization in mice (Bry et al., 2007). Both hyperoxia and mechanical ventilation, injuries that impair distal lung growth, induce the expression of pro-inflammatory mediators in the lung.

As an important regulator of cellular proliferation, differentiation, inflammation, and angiogenesis, the nuclear factor kappa B (NF$\kappa$B) family of transcription factors likely regulates many of the biologic processes that are essential for alveolarization. In this review, the main functions of this complex pathway will be summarized, and the regulatory mechanisms that allow for the diverse effects of NF$\kappa$B activation described. Given that a fully comprehensive review of the vast literature surrounding NF$\kappa$B is not possible, a specific emphasis will be placed on reviewing the role of NF$\kappa$B in modulating inflammation and

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Published online 17 March 2014 in Wiley Online Library (wileyonlinelibrary.com). Doi: 10.1002/bdra.23233

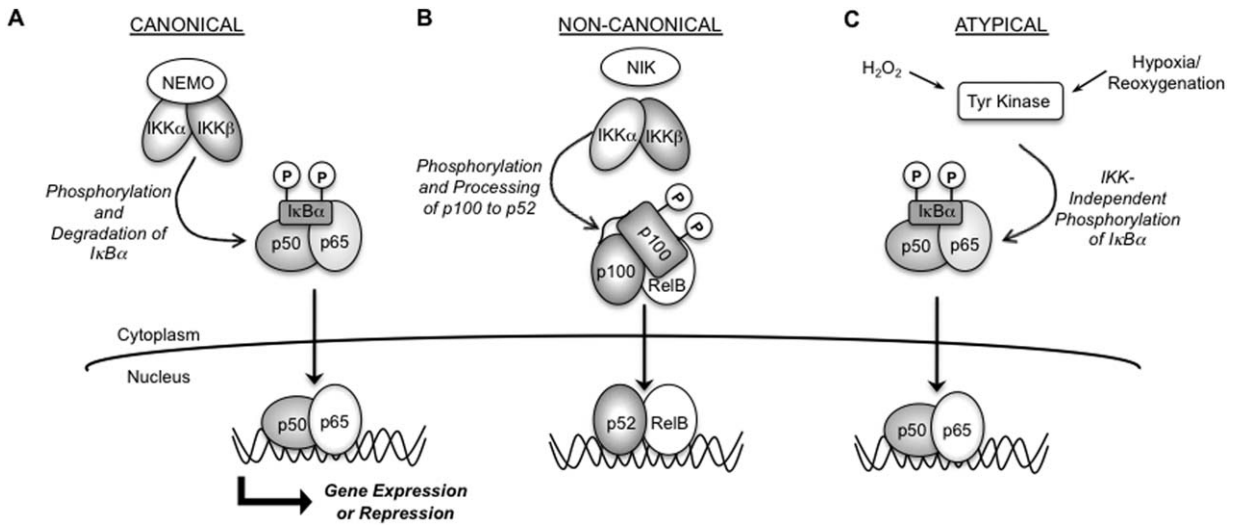


**FIGURE 1.** *Pathways Leading to Activation of NFκB.* **A:** *In the canonical pathway of activation, the IKK kinase complex, consisting of kinases IKKα and IKKβ, and the regulatory subunit IKKγ, phosphorylate IκBα on serines 32 and 36. This results in the rapid ubiquitination and degradation of IκBα, which unmasks nuclear localization sequences present on the NFκB subunits, resulting in the rapid translocation of active NFκB complexes into the nucleus, where they bind to κB binding sites in the promoters of target genes and promote gene expression or repression.* **B:** *In the noncanonical pathway, IKKα is activated by the NFκB-inducing kinase (NIK), resulting in the processing of p100 to p52, and the nuclear translocation of RelBp52 dimers, which bind to unique κB elements.* **C:** *IKK-independent atypical activation of NFκB (observed in response to hypoxia/reoxygenation, or $H_2O_2$ stimulation) can occur by means of tyrosine kinase-mediated phosphorylation of IκBα on tyrosine 42, and subsequent dissociation or degradation of IκBα.*

angiogenesis, and in highlighting the data most relevant to lung development and disease.

## BACKGROUND OF NFκB

The NFκB family of transcription factors consists of five evolutionarily conserved members including Rel (cRel), RelA (p65), RelB, NFκB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) (Ghosh and Karin, 2002). NFκB proteins share a highly conserved, N-terminal, Rel homology domain that allows for DNA binding, dimerization with other NFκB family members, and association with inhibitory, IκB proteins. The subunits cRel, RelA, and RelB possess C-terminal transactivation domains that confer transcriptional activity, and although p50 and p52 lack transactivation domains, they can promote transcription by dimerizing with NFκB subunits containing transactivation domains (Hayden and Ghosh, 2012).

In most cells, NFκB dimers are sequestered in the cytoplasm by IκB proteins. In the canonical pathway of activation (Fig. 1A), stimulus-induced degradation of IκBs occurs by means of the phosphorylation of IκB proteins by the IκB kinase complex consisting of two catalytically active kinases, IKKα and IKKβ, and a regulatory subunit, IKKγ. Degradation of IκB proteins unmasks nuclear localization sequences present on the individual NFκB subunits, resulting in the rapid translocation of active NFκB complexes into the nucleus, where they bind to κB binding sites in the promoters of target genes. Activation of NFκB results in the downstream gene regulation of an expansive and diverse group of target genes, many of which are involved in essential cell functions including survival, adhesion, proliferation, the cellular-stress response, and in the regulation of inflammation. An alternative pathway of NFκB activation also exists. In this noncanonical pathway (Fig. 1B), IKKα mediated processing of p100 results in the nuclear translocation of RelB-p52 heterodimers, and plays a key role in B-cell mediated adaptive immunity (Senftleben et al., 2001). In addition, atypical activation can occur by means of non-IKK dependent phosphorylation of the IκBs (Fig. 1C), and in the absence of IKK-mediated post-translational modifications of NFκB subunits (described below), potentially resulting in the nuclear translocation of NFκB dimers with distinct functional effects (Perkins, 2006, 2007).

## MECHANISMS ALLOWING FOR SPECIFICITY IN NFκB SIGNALING

Given the ubiquitous expression of NFκB molecules in most cell types, several mechanisms must exist to allow for specificity in downstream target gene expression depending on the nature and cellular context of NFκB activation. The degenerate nature of the κB binding sequence, the ability of NFκB subunits to form different homo- and heterodimers, posttranslational modifications of NFκB subunits that either positively or negatively regulate transcriptional activity, and cross-talk and interactions with other

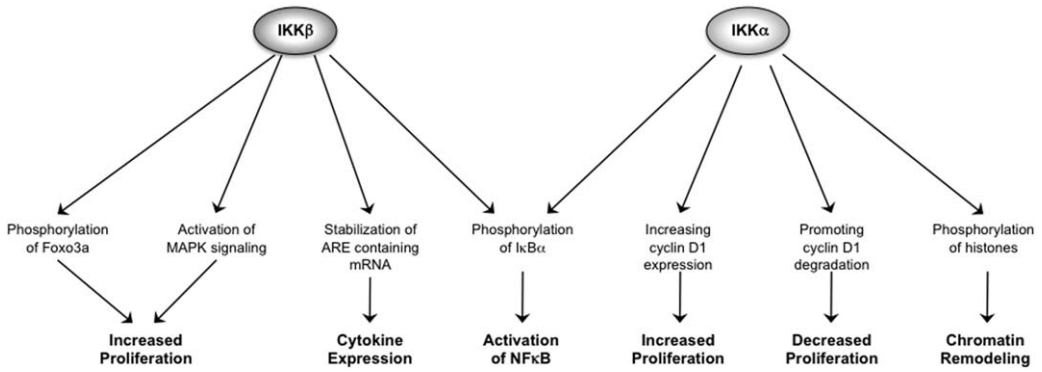


**FIGURE 2.** *NFκB Dependent and Independent Actions of the IKKs. Both IKKα and -β phosphorylate IκB leading to NFκB mediated gene regulation. IKKβ can promote cell proliferation by inhibiting the anti-proliferative effects of FOXO3a, and activating the pro-proliferative MAP kinase pathway. IKKβ can also enhance gene transcription in an NFκB dependent manner by stabilizing mRNA transcripts containing AU-rich element (ARE) motifs. IKKα can either promote proliferation by increasing β-catenin-mediated transcription of cyclin D1, or inhibit proliferation by increasing cyclin D1 degradation. IKKα can also broadly affect gene transcription, independent of NFκB, by regulating chromatin remodeling.*

transcription factors all result in tremendous diversity in the downstream effects of NFκB activation (Hayden and Ghosh, 2012).

The NFκB subunits, RelA, RelB, and cRel contain transactivation domains capable of inducing transcription activation, while the subunits p50 and p52 lack these domains. However, in some situations, p50 and p52 homodimers can activate transcription, by interacting with proteins that function as transcriptional coactivators (Smale, 2012). More often, p50 and p52 homodimers function as transcriptional repressors by competing for DNA binding with NFκB dimers that are able to activate transcription (Bohuslav et al., 1998), and by modifying chromatin remodeling by interacting with histone deactylases (Zhong et al., 2002). The repression of NFκB transcription by p50 homodimers appears to be one mechanism to limit NFκB target gene activation, with the induction of p50 dimers implicated in the development of endotoxin tolerance (Kastenbauer and Ziegler-Heitbrock, 1999). In addition, individual dimer combinations may activate distinct panels of target genes as a result from specificity in the interactions with a specific transcription factors and coregulatory proteins (Smale, 2012), or binding affinity to select consensus sequences (Chen and Ghosh, 1999; Kunsch et al., 1992).

In addition to IκBα, there are two additional "typical" IκBs: IκBβ and IκBε. All three share similar structures, containing six ankyrin repeats (Hinz et al., 2012). However, knockout studies demonstrated non redundant functions for the IκBs, as mice lacking IκBα have a more, severe and distinct phenotype than mice lacking either IκBβ and IκBε (reviewed by Hinz et al., 2012). In addition, distinct IκB molecules appear to preferentially bind select combinations of NFκB dimers (Tran et al., 1997; Whiteside et al., 1997), thus increasing the specificity of IκB-mediated regulation of NFκB-activation. In their unprocessed forms, p100 and p105 contain C-terminal ankryin like repeats, similar to those found in the IκB molecules. Thus, p100 and p105 can also hold their NFκB-subunit partners inactive in the cytoplasm (Hayden and Ghosh, 2004), and perhaps partially compensate for the loss of function of the "typical" IκBs (Tergaonkar et al., 2005).

Recently, NFκB independent functions for both IKKα and IKKβ have been identified (Fig. 2). In addition to phosphorylating the IκBs, IKKs can also phosphorylate NFκB subunits such as RelA, modifying transcriptional activity and lending further specificity of target gene expression (Zhong et al., 2002). IKKβ plays an important role in mediating cell survival and proliferation. IKKβ can phosphorylate and inhibit the activity of the transcription factor FOXO3a (Hu et al., 2004), a factor that inhibits proliferation of lung myofibroblasts (McGowan and McCoy, 2013). IKKβ also activates the pro-proliferative MAP kinase pathway by inducing p105 processing, thereby removing p105-mediated inhibition of the MAPK pathway (Beinke et al., 2004). In addition, IKKβ modulates the mRNA stability of transcripts containing AU-rich element (ARE) motifs (Gringhuis et al., 2005), thus potentially influencing the expression of numerous cytokine, chemokine, and growth factors, many of which contain ARE motifs. IKKα can also regulate cell proliferation in NFκB-independent manner, either promoting proliferation by stabilizing β-catenin-mediated cyclin D1 transcription (Lamberti et al., 2001; Albanese et al., 2003), or alternatively, inhibiting proliferation by phosphorylating cyclin D1, leading to cyclin D1 degradation (Kwak et al., 2005). In addition, IKKα can affect gene expression more broadly by acting as a histone H3 serine10 kinase, thus regulating chromatin structure (Anest et al., 2003; Yamamoto et al., 2003). These numerous NFκB-independent functions for both IKKα and IKKβ likely contribute to the seemingly

**TABLE 1.** *Developmental Phenotypes in Mice Containing Genetic Modifications in NFκB Family Members*

| Genetic modification | Phenotype | References |
|---|---|---|
| *IKKα−/−* | Perinatal lethality<br>Defects in epidermal differentiation<br>Skeletal and craniofacial defects | Li and others, 1999a; Winston and others, 1999 |
| *IKKβ−/−* | Embryonic lethality between E12.5 and E14<br>Severe liver degeneration and widespread hepatic apoptosis | Li and others, 1999b; Li and others, 1999c |
| *IκBα −/−* | Lethality by postnatal day 10<br>Widespread dermatitis and granulopoiesis | Beg and others, 1995a; Klement and others, 1996 |
| *RelA (p65) −/−* | Embryonic lethality at E15.5<br>Widespread TNF-α mediated hepatic apoptosis | Beg and Baltimore, 1996; Beg and others, 1995b |
| *cRel −/−* | Impaired B cell survival and isotype switching<br>Impaired T and dendritic cell function | Kontgen and others, 1995 |
| *RelB −/−* | Chronic multi-organ inflammation<br>Inability to clear autoreactive T cells<br>Increased susceptibility to viral and bacterial infections | Burkly and others, 1995; Weih and others, 1995;<br>Weih and others, 1997b |
| *RelB−/−p50−/−* | Lethality by 4 weeks of age<br>Exaggeration of inflammatory phenotype observed in RelB −/− mice | Weih and others, 1997a |

contradictory results frequently obtained in experimental studies that use inhibitory strategies that target NFκB nuclear translocation versus studies using strategies to specifically block IKK activity.

Additional specificity in downstream target gene activation is also mediated by the interaction of NFκB with other cell signaling pathways. NFκB can interact with additional transcription factors, including c-Jun/AP-1 and early growth response-1 (EGR-1), two pathways that have been implicated in the pathogenesis of emphysema (Zhang et al., 2000;Reddy et al., 2012). In some cases, binding of NFκB to heterologous transcription factors allows for the recruitment of NFκB to promoters not containing κB binding sites, as is the case for NFκB-mediated repression of kruppel like factor-2 (Kumar et al., 2005), a zinc-finger transcription factor with an essential role in early lung development (Wani et al., 1999). Furthermore, while the interactions with some transcription factors increases the DNA binding of NFκB complexes to target genes, as is observed with STAT3 (Nadiminty et al., 2006), interactions with similar factors (i.e. STAT1) can have the opposite effect, and impair NFκB DNA binding (Kramer et al., 2006).

## INSIGHT INTO THE BIOLOGIC FUNCTIONS OF NFκB: GENETIC STUDIES IN MICE

Important insight into the specific and redundant functions of various members of the NFκB family has been gained through the study of knockout mice containing targeted deletions of NFκB proteins (Table 1). Severe developmental and lethal phenotypes are observed upon deletion of NFκB family members that are widely expressed. Disruption of RelA causes embryonic lethality at embryonic day (E) 15.5 secondary to widespread hepatocyte apoptosis, and identified an essential role for NκB in preventing tumor necrosis factor-alpha (TNF-α) induced cell death (Beg et al., 1995b; Beg and Baltimore, 1996). Mice lacking IKKβ phenocopy the *RelA−/−* mice, dying in utero between E12.5 and E14 as a result of hepatic apoptosis and necrosis, and display impaired activation of NFκB in response to TNF-α and IL-1 (Li et al., 1999b, 1999c). In contrast, mice lacking IKKα have a distinct phenotype. IKKα null mice survive embryonic development but die soon after birth and display limb and craniofacial defects (Li et al., 1999a; Winston et al., 1999). Furthermore, the IKK complex lacking IKKα is still able to phosphorylate IκB in vitro. These data, combined with the embryonic lethality of the *IKKβ−/−* mice, demonstrated that IKKα and β possess distinct functions and a limited ability for each to compensate for the loss of the other. Loss of IκBα permits normal development, but knockout mice die within 10 days after birth, and display widespread dermatitis, granulopoiesis, and histologic abnormalities in the liver and spleen. Interestingly, while IκB-α deficient hematopoietic cells demonstrate increased NFκB activity and target gene expression, this effect is not observed in IκB-α null embryonic fibroblasts, suggesting

compensation by other IκB proteins in the latter cell type (Beg et al., 1995a; Klement et al., 1996).

In contrast, less severe developmental defects are observed upon targeted disruption of NFκB family members that display more limited tissue expression. Mice with targeted deletion of RelB develop chronic inflammation of numerous organs secondary to an impaired ability to clear autoreactive T-cells. In addition to this inflammatory phenotype, these mice also have functional immune defects, demonstrating increased susceptibility to viral and bacterial infections (Burkly et al., 1995; Weih et al., 1995, 1997b). The loss of p50 markedly exaggerates this inflammatory phenotype observed in the *relb−/−* mice, with *relb−/−p50−/−* mice dying within the first month of life, suggesting that p50 complexes partially compensate for the absence of RelB (Weih et al., 1997a). Mice deficient in cRel develop normally, but demonstrate impaired humoral immunity and increased susceptibility to intracellular parasites (Kontgen et al., 1995).

The severe developmental and immune defects observed in these models hindered the ability to fully assess the role of many of these molecules outside of early embryonic development. However, with the development and increasing availability of Cre-Lox technology to permit tissue-specific, conditional deletions of NFκB pathway members, accumulating evidence suggests that NFκB may play a much broader role in later organ development and disease. Those transgenic and conditional knock-out models relevant to lung injury, inflammation and development will be discussed in more detail in the sections below.

## NFκB AS A KEY REGULATOR OF LUNG INJURY AND INFLAMMATION

*Pro-inflammatory effects of NFκB signaling.* Soon after its initial description, growing evidence suggested that the NFκB pathway was important in the pathogenesis of inflammatory diseases. NFκB is activated by numerous pro-inflammatory stimuli including cytokines, pattern recognition receptors (e.g., TLRs), oxidative stress, and UV radiation (Li and Karin, 1998). Furthermore, NFκB downstream target genes include a diverse group of factors that are important for the innate and adaptive immune response, including cytokines, chemokines, and cell adhesion molecules (Lawrence and Fong, 2010). Enhanced activation of NFκB has been implicated in the pathogenesis of several inflammatory diseases including rheumatoid arthritis (Foxwell et al., 1998; Gregersen et al., 2009), inflammatory bowel disease (Hollenbach et al., 2004), atherosclerosis (Sun et al., 2013), and cancer (Karin and Greten, 2005). In the lung specifically, increased activation of NFκB has been observed in experimental models of lung injury induced by hyperoxia (Yang et al., 2004; Wright et al., 2009), oxidative stress (Moodie et al., 2004), mechanical ventilation (Ko et al., 2013), and endotoxin (Everhart et al., 2006; Alvira et al., 2007).

Data from murine models containing genetic deletions of NFκB proteins have provided additional evidence for a pro-inflammatory function for NFκB in the lung (Table 2). Overexpression of a constitutively active form of IKKβ in lung epithelial cells increases pro-inflammatory gene expression, and results in neutrophilic infiltration of the lung and high protein pulmonary edema (Cheng et al., 2007). Selective inhibition of NFκB in the distal airway epithelium by overexpressing a dominant negative form of IκB-α that cannot be degraded (IκBαSR), diminishes lung inflammation and reduces pro-inflammatory cytokine expression in response to inhaled lipopolysaccharide (LPS) (Skerrett et al., 2004). Similarly, overexpressing IκBαSR in the respiratory epithelium under the control of the CC10 promoter, limits neutrophilic infiltration and TNF-α and MIP-2 expression in response to intranasal LPS. Of note, the protection against LPS-induced lung inflammation in those transgenic mice was observed despite the retained ability of the alveolar macrophages to activate NFκB in response to TNF-α stimulation (Poynter et al., 2003), thus highlighting a key role for the respiratory epithelium in propagating the inflammatory response in the lung.

However, activation of NFκB in the alveolar macrophages appears to be important in initiating lung inflammation. In some cases, alveolar macrophages function as the "first responder," with activation of NFκB in alveolar macrophages resulting in the production of cytokines that then activate NFκB in other lung cell types. Depletion of alveolar macrophages from rats using liposomal clodonate significantly blunts NFκB activation in the whole lung and decreases inflammation in a model of lung injury induced by immunoglobulin immune complexes (Lentsch et al., 1999). In this study, NFκB activation in the lung could be restored by direct instillation of TNF-α, suggesting that alveolar macrophage derived TNF-α was required for pulmonary NFκB activation. Depletion of alveolar macrophages by the administration of intratracheal clodronate in addition to intravaneous clodronate effectively decreases neutophilic lung inflammation induced by either inhaled or systemic LPS, while IV clodronate alone has no appreciable effect (Koay et al., 2002).

Evidence from clinical studies also support a role for NFκB in inflammatory lung diseases. Alveolar macrophages (AM) obtained from adult patients with acute respiratory distress syndrome have lower cytoplasmic levels of NFκB subunits than AM from control patients, consistent with increased nuclear translocation of NFκB dimers (Moine et al., 2000). Increased active NFκB is found in the induced sputum and bronchial biopsies of asthmatic patients as compared to control patients (Hart et al., 1998). NFκB is constitutively activate in sinus biopsies taken from patients with cystic fibrosis, and in that study the ΔF508-CFTR mutation was found to induce NFκB activation by eliciting an endoplasmic reticulum stress response (Knorre et al., 2002). Enhanced activation

**TABLE 2.** *Pro- and Anti-Inflammatory Effects of NFκB in Genetically Modified Mouse Models*

| Modulation of NFκB signaling | Target cell | Physiologic effects | References |
|---|---|---|---|
| Pro-inflammatory effects | | | |
| Constitutive activation of NFκB by inducible over-expression of IKKβ via the CC10 promoter | Airway epithelial cells | Neutrophilic lung inflammation, high protein pulmonary edema, and increased pro-inflammatory gene expression | Cheng and others, 2007 |
| Inhibition of NFκB activation by over-expression of IκBαSR via the SP-C promoter | Distal airway epithelial cells | Decreases lung inflammation and inflammatory cytokine expression in response to inhaled LPS | Skerrett and others, 2004 |
| Inhibition of NFκB activation by over-expression of IκBαSR via the CC10 promoter | Airway epithelial cells | Decreased lung neutrophilic infiltration and TNF-α and MIP-2 expression in response to intranasal LPS | Poynter and others, 2003 |
| Cre-Lox mediated deletion of *IKKβ* using mice expressing Cre–recombinase under control of the CC10 promoter | Airway epithelial cells | Reduced neutrophilic lung inflammation after intranasal GBS infection. Delayed clearance of bacteria from the lung. | Fong and others, 2008 |
| Anti-inflammatory effects | | | |
| Homozygous deletion of *p50* and heterozygous deletion of *p65 (p50−/− p65+/− mice)* | All cells | Increased susceptibility to LPS-induced septic shock. Increased susceptibility to experimental colitis. Increased IL-12p40 and decreased IL-10 expression | Gadjeva and others, 2004; Tomczak and others, 2006; Tomczak and others, 2003 |
| *Deletion of p50 (p50−/− mice)* | All cells | Increased sensitivity to cigarette smoke induced emphysema, increased lung inflammation. Neonatal mice with increased sensitivity to hyperoxic lung injury, including decreased survival and increased lung cell apoptosis. | Rajendrasozhan and others, 2010; Yang and others, 2004 |
| Homozygous deletion of *cRel* and *p50* and heterozygous deletion of *p65 (cRel−/− p50−/− p65+/− mice)* | All cells | Development of spontaneous dermal and intestinal inflammation and chronic neutrophilia. Enhances mobilization of activated neutrophils from the bone marrow. | von Vietinghoff and others, 2010 |
| Cre-Lox mediated deletion of *IKKβ* using mice expressing Cre–recombinase under control of the LysM promoter | Myeloid cells | Increased sensitivity to LPS-induced septic shock.Enhanced IL-1β production. Decreased neutrophil apoptosis. Increased lung inflammation upon GBS exposure with increased activation of M1 macrophages. | Greten and others, 2007; Fong and others, 2008 |

of NFκB has also been implicated in the pathogenesis of the pulmonary vascular disease observed in some patients with end-stage cystic fibrosis (Henno et al., 2009). High levels of nuclear NFκB are observed in lung cancer tissue, with increased NFκB activity correlating with more advanced disease in lung adenocarcinoma (Tang et al., 2006).

New studies demonstrate that genetic differences in the regulation of the NFκB pathway may play a role in the development and progression of lung disease. Polymorphisms in the promoter for *NFKBIA*, the gene that encodes IκBα, result in decreased IκBα gene and protein expression, leading to enhanced NFκB mediated TNF-α production (Ali et al., 2013). The minor allele explored in that study was associated with an increased risk of severe RSV infection and airway hyperresponsiveness in infants and children. An additional variant in the *NFKBIA* promoter is also associated with an increased risk of ARDS, although in that study functional assays were not performed to determine if this polymorphism increases or decreases the expression of IκBα (Zhai et al., 2007).

The large number of clinical and experimental studies linking NFκB pathway activation to inflammation has led to interest in the development of therapeutic strategies to target NFκB signaling for the treatment of inflammatory diseases and cancer (Karin et al., 2004; Luo et al., 2005). The complex and intricate nature of the mechanisms regulating NFκB activation and transcriptional allows for several distinct strategies for inhibition of NFκB function including the disruption of NFκB-DNA binding, prevention of NFκB nuclear translocation, and direct inhibition of the IKKs. Nonsteroidal anti-inflammatory drugs act, in part, by inhibiting the NFκB signaling pathway (Kopp and Ghosh, 1994). Both aspirin and sulindac, a compound similar to indomethacin, competitively bind IKKβ and inhibit its catalytic activity (Yin et al., 1998, Yamamoto et al., 1999). The anti-inflammatory and anti-cancer agent thalidomide also blocks NFκB activation by inhibiting IKKβ (Keifer et al., 2001; Dredge et al., 2003). Anti-oxidant compounds such as N-acetyl-L-cysteine and vitamin C impair NFκB activity (Blackwell et al., 1996; Carcamo et al., 2002), both through direct effects on the IKK complex, and by affecting the proteasome pathway (Hayakawa et al., 2003). While the potential for such inhibitors in the treatment of chronic inflammatory diseases is high, recent studies have identified, novel anti-inflammatory functions for NFκB that may have important implications for therapeutic strategies that broadly block NFκB activation (Lawrence and Fong, 2010).

*Anti-inflammatory effects of NFκB signaling.* Some of the earliest evidence that NFκB also posses anti-inflammatory effects was derived from studying mouse models with targeted deletions of NFκB family members (Table 2). Experiments on mice lacking p50 and heterozygous for *p65 (p50−/− p65+/−)* demonstrated that these mice are more susceptible to LPS-induced septic shock than WT mice, suggesting novel anti-inflammatory roles for canonical NFκB signaling (Gadjeva et al., 2004). In further studies, these *p50−/−p65+/−* mice were also found to be more sensitive to experimental colitis in association with increased expression of the pro-inflammatory cytokine IL-12p40 and decreased expression of the anti-inflammatory cytokine IL-10 (Tomczak et al., 2003, 2006). Mice with targeted deletion of *p50 (p50−/−)* are more sensitive to cigarette smoke induced emphysema, demonstrating enhanced lung inflammation, and enhanced DNA binding and activity of the transcriptionally active p65/p50 dimers in association with alterations in chromatin remodeling (Rajendrasozhan et al., 2010). Neonatal *p50−/−* mice are also more sensitive to hyperoxic lung injury, displaying increased lung apoptosis and decreased survival as compared to WT mice (Yang et al., 2004). Mice that lack both cRel and p50, and are heterozygous for the *p65* subunit (*cRel−/−p50−/−p65+/−*), develop spontaneous dermal and intestinal inflammation in association with chronic neutrophilia (von Vietinghoff et al., 2010).

Apoptosis of inflammatory cells is a key mechanism that promotes the resolution of inflammation (Lawrence and Fong, 2010). In contrast to the anti-apoptotic role for NFκB observed in many cell types (Barkett and Gilmore, 1999; Romashkova and Makarov, 1999), NFκB appears to promote apoptosis of T cells by increasing FasL expression (Kasibhatla et al., 1999). Inhibiting NFκB activation during the resolution of inflammation prolongs the inflammatory response and impairs leukocyte apoptosis (Lawrence et al., 2001). Deletion of IKKβ in myeloid cells enhances IL-1β production and increases sensitivity to endotoxic shock, in association with neutrophilia resulting from impaired neutrophil apoptosis (Greten et al., 2007).

Additional anti-inflammatory effects of NFκB appear to result from the ability of NFκB to modulate macrophage polarization. Classical macrophage activation to the M1 phenotype by bacterial products such as LPS induces the expression of inflammatory mediators (Benoit et al., 2008), and angiostatic chemokines (Owen and Mohamadzadeh, 2013). In contrast, M2 macrophages promote tissue repair by producing enzymes that induce cell growth, proteases that remodel the extracellular matrix (ECM) (Varin and Gordon, 2009), and angiogenic factors that promote endothelial cell (EC) survival and proliferation such as VEGF-A. The NFκB pathway regulates both the switch from the M1 to the M2 phenotype, and also the pro-angiogenic function of M2 macrophages. Epithelial-specific deletion of IKKβ limits lung inflammation in adult mice infected with group B streptococcus (GBS), yet monocyte specific deletion of IKKβ augments inflammation and increases M1 polarization by abrogating IKKβ-mediated inhibition of STAT1, the transcriptional regulator of M1 cytokines (Fong et al., 2008). Similarly, survival of M1 activated macrophages is prolonged in mice with an inactive form of IKKα, where IKKα serves to increase the turnover

of NF$\kappa$B subunits, RelA and cRel, and to remove the NF$\kappa$B dimers from target gene promoters (Lawrence et al., 2005).

Whether NF$\kappa$B plays pro or anti-inflammatory effects is influenced in part by the timing and degree of inhibition. In a rat model of carrageenin-induced pleurisy, early activation of NF$\kappa$B in leukocytes induces the transcription of pro-inflammatory genes, while later activation results in the expression of anti-inflammatory genes. In that model, inhibiting NF$\kappa$B before injury improves inflammation, while inhibiting NF$\kappa$B after the onset of inflammation protracts the inflammatory response in part due to impaired leukocyte apoptosis (Lawrence et al., 2001). In a model of transplantation mediated ischemia-reperfusion lung injury, partial inhibition of IKK$\beta$ activity was protective, allowing for improved graft function, decreased pulmonary edema, and diminished stromal cell apoptosis. In contrast, complete abrogation of IKK$\beta$ activity had the opposite effect, markedly increasing markers of lung injury and worsening graft function (Huang et al., 2011). Maturational differences in NF$\kappa$B activation also appear to be important, particularly in the lung. In response to hyperoxia, NF$\kappa$B is activated in the lungs of neonatal but not adult mice. In this model, NF$\kappa$B activation in the neonatal lung protects against hyperoxia-mediated lung injury by suppressing lung apoptosis (Yang et al., 2004). In response to systemic LPS, the pattern of NF$\kappa$B activation in neonatal and adult mice is distinct. Moreover, inhibition of NF$\kappa$B in adult mice decreases lung inflammation, while the same treatment in neonatal mice exaggerates the lung inflammatory response (Alvira et al., 2007). Given these contrasting functions, greater clarity is needed in understanding and predicting when NF$\kappa$B is playing a beneficial versus pathologic role in inflammatory lung diseases.

### NF$\kappa$B AS A KEY REGULATOR OF ANGIOGENESIS

While perhaps most well known for its role in promoting inflammation, accumulating evidence suggests that NF$\kappa$B is a key regulator of angiogenesis during development and disease. Conditional deletion of the NF$\kappa$B activator, IKK$\beta$, in Tie2 expressing cells, causes embryonic lethality between E13.5 and E15.5 in the majority of affected pups, in association with a disruption of fetal liver vasculature and hepatocyte apoptosis (Hou et al., 2008). In a similar model, a comparably high rate of embryonic mortality was observed after homozygous deletion of IKK$\beta$ in ECs, in association with disrupted placental vascularization, and impairments in EC survival and migration. In that same study, adult mice with heterozygous loss of IKK$\beta$ demonstrated impaired postischemia neovascularization (Ashida et al., 2011). Unfortunately, in both models, the significant amount of embryonic lethality hindered a comprehensive assessment of the role of IKK$\beta$ in the formation and function of other vascular beds at later stages of development. In contrast, inhibiting NF$\kappa$B by endothelial overexpression of the I$\kappa$B$\alpha$SR mutant repressor does not induce embryonic mortality. However, these transgenic mice demonstrate increased vascular permeability, a loss of tight junction formation, and enhanced sensitivity to LPS-induced septic shock (Kisseleva et al., 2006). Taken together, these three studies suggest an important role for NF$\kappa$B/IKK signaling in preserving endothelial function and homeostasis, yet highlight that the functions of IKK and NF$\kappa$B in the endothelium are not completely overlapping.

Extensive experimental data also support the notion that NF$\kappa$B is a key regulator of angiogenesis in cancer. In lung adenocarcinoma, IKK$\alpha$ is increased in the tumor endothelium, and overexpression of IKK$\alpha$ increases tumor vascularization and growth in a murine model of lung cancer (DeBusk et al., 2008). Activated IKK$\beta$ is observed in multiple tumor types, and recent data identified that IKK$\beta$ inhibits the tumor suppressor, tuberous sclerosis 1, and enhances VEGF expression and angiogenesis (Lee et al., 2007). Thalidomide was recently recognized to have therapeutic potential as an anti-oncogenic agent given its ability to inhibit angiogenesis by directly blocking IKK activation and NF$\kappa$B-mediated expression of proangiogenic factors such as IL-8 (Keifer et al., 2001).

As indicated above, a key mechanism by which NF$\kappa$B promotes angiogenesis is through the direct regulation of angiogenic cytokines and growth factors. LPS directly stimulates sprouting of human microvascular endothelial cells by means of TNF receptor associated factor 6 (TRAF6)-mediated activation of NF$\kappa$B (Pollet et al., 2003), and inhibition of NF$\kappa$B blocks FGF-induced angiogenesis in this model. NF$\kappa$B increases the transcription of the AP-1 family member JunB, a key mediator of hypoxia-induced VEGF expression and angiogenesis (Schmidt et al., 2007). In an experimental model of melanoma, oncogenic activation of NF$\kappa$B enhances tumor angiogenesis by increasing the production of angiogenic cytokines such as angiogenin, and blocking NF$\kappa$B prevents tumor angiogenesis and leads to the regression of established tumor vasculature (Schaafhausen et al., 2013). In human ovarian cancer cells, inhibiting NF$\kappa$B activation with the I$\kappa$B$\alpha$SR diminishes the expression of VEGF and the proangiogenic cytokine, interleukin-8, and decreased tumorigenicity and valscularization of the lesions (Huang et al., 2000).

In contrast to these reports, a smaller number of studies have suggested that NF$\kappa$B possesses angiostatic functions. Endothelial specific over-expression of the I$\kappa$B$\alpha$SR mutant repressor increases tumor growth in a murine model of metastatic melanoma (Kisseleva et al., 2006). Of note, in these mice the increased tumor burden was associated with increased, but markedly disorganized tumor vasculature. While NF$\kappa$B promotes the expression of matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9 that promote EC migration (Ko et al., 2005; Mountain et al., 2007), NF$\kappa$B can also induce the expression of tissue inhibitors of metalloproteinase-1, an effect predicted

to inhibit migration (Wilczynska et al., 2006; Tabruyn and Griffioen, 2008). Furthermore, while the expression of both VEGF and VEGFR2 in EC are induced by hypoxia-induced mitogenic factor by means of an NFκB-dependent pathway (Tong et al., 2006a, 2006b), NFκB can also regulate the expression of the vascular endothelial growth factor inhibitor (Xiao et al., 2005). Therefore, additional data will be important to clarify when and how NFκB/IKK plays pro- versus anti-angiogenic functions to effectively exploit these pathways for therapeutic benefit.

Cross-talk also occurs between the NFκB/IKK pathway and additional angiogenic signaling pathways. In pulmonary artery smooth muscle cells, NFκB increases HIF-1α transcription, and HIF-1α target gene transactivation (Bonello et al., 2007). IKK-$\beta$ regulates HIF-1α expression in the tissues of hypoxic mice, and loss of IKK$\beta$ causes defective induction of HIF-1α target genes, including VEGF (Rius et al., 2008). In turn, HIF-1α can also activate NFκB by enhancing the phosphorylation and degradation of IκB, and increase NFκB nuclear localization and transcriptional activity (Scortegagna et al., 2008). The NFκB pathway also modulates angiogenesis by regulating Notch signaling, a pathway important for vascular patterning during development. The proangiogenic cytokine TNF-α induces a tip cell phenotype in EC by increasing the expression of the notch ligand, jagged-1, by means of an NFκB-dependent mechanism (Sainson et al., 2008; Johnston et al., 2009). The importance of the interactions between the NFκB, HIF, and Notch pathways is further evidenced by the phenotype of mice that express the IκBαSR under control of an endothelial specific promoter. When exposed to femoral artery ligation, these transgenic mice demonstrate: a significant impairment in blood flow recovery; extensive, disorganized, and excessively branched, collateral vessels; decreased HIF-1α expression; and marked reductions in the notch ligands, Jagged-1 and delta-like ligand 4 (Tirziu et al., 2012).

### NFκB AND LUNG DEVELOPMENT

Unfortunately, knowledge regarding the role of NFκB during late embryonic development in general, and lung development specifically, has been limited by the embryonic and perinatal lethality observed in many of murine models containing deletions of NFκB family members (Table 1). In addition, knockout studies targeting a single protein in a family of structurally related proteins can be limited by compensation by other members of the group. However, the development and increased application of genetic methods to create mice with tissue-specific deletions in NFκB molecules, and strategies to inhibit NFκB signaling at key time points in lung development, has resulted in new data to suggest that the NFκB/IKK pathway may play an essential role in late lung development.

NFκB is a key regulator of genes that mediate cellular proliferation and survival. NFκB promotes the transcription of several anti-apoptotic genes (Barkett and Gilmore, 1999) including the cellular inhibitors of apoptosis (cIAP1, cIAP2, and xIAP) (Wang et al., 1998), and Bcl-2 family members (Zong et al., 1999). NFκB also positively regulates proliferation by increasing the expression of cyclin D1, a protein that promotes progression from the G1 to the S phase of the cell cycle (Hinz et al., 1999). NFκB is constitutively active in the lungs of neonatal mice at the onset of the alveolar stage of lung development, but minimally active in adult lungs (Iosef et al., 2012). Inhibiting the NFκB/IKK pathway using BAY 11-7082, a potent inhibitor of IKK activity, has no effect on adult mice, but durably impairs alveolarization in neonatal mice, markedly decreasing lung cell proliferation and increasing apoptosis. In part, this effect appears to be related to NFκB-mediated effects in the pulmonary vasculature where constitutive NFκB activation promotes neonatal pulmonary endothelial cell survival, proliferation, and angiogenesis, and directly regulates the expression of VEGFR2.

However, the NFκB pathway also appears to play an important developmental and homeostatic role in other cell types within the lung. Overexpression of RelA in the distal pulmonary epithelium using the surfactant protein C promoter increases the number of alveolar epithelial type I and II cells and decreases epithelial cell apoptosis (Londhe et al., 2008). Furthermore, targeted deletion of IKK$\beta$ from the respiratory epithelium increases type II cell apoptosis, decreases epithelial VEGF expression, and delays alveolar formation (Londhe et al., 2011). Genetic ablation of the NFκB subunit p50 results in the development of spontaneous emphysema in mice at 4 months of age, which may be related to increases in the activation of matrix metalloproteases, MMP-9 and MMP-12 (Rajendrasozhan et al., 2010). Taken together, these studies suggest that NFκB plays an important role during late lung development by enhancing pulmonary endothelial survival, proliferation and angiogenesis; promoting epithelial proliferation and differentiation; and by preserving lung cell homeostasis by suppressing protease activation.

In contrast, studies performed at earlier stages of lung development suggest that activation of NFκB is detrimental to lung growth. Exposure of murine lung explants to LPS at the late canalicular/ early saccular stage of development impairs lung branching. This detrimental effect appears to result from activation of NFκB in lung macrophages, as both macrophage depletion and targeted inactivation of NFκB preserved airway branching, while macrophage specific NFκB activation was sufficient to disrupt airway branching (Blackwell et al., 2011). Whether these seemingly disparate roles for NFκB in mediating lung growth result from differences in the upstream activation (i.e., constitutive versus induced) or cell type of activation (i.e., endothelial and epithelial versus macrophage), or are secondary to distinct functions for NFκB at

different stages of lung development (i.e., canalicular/saccular versus alveolar), remains to be determined.

Clinical evidence linking NFκB to lung development or BPD is limited. A single report observed that premature infants who develop BPD have evidence of increased NFκB activity in tracheal lavage fluid (Bourbia et al., 2006), a finding that was interpreted by the authors to suggest that NFκB contributes to the pathogenesis of BPD. Recently, however, the same polymorphism in the *NFKBIA* gene that enhances NFκB activation and increases the risk of severe RSV infection, confers a decreased risk for the development of moderate to severe BPD in premature infants (Ali et al., 2013). These data provide the first clinical data supporting the notion that NFκB signaling may play an essential and previously unrecognized protective role in late lung development, and suggest perhaps, that the NFκB activation observed in the lungs of preterm infants may represent a compensatory, rather than pathologic response.

## Conclusions and Future Directions

Since its initial discovery by Ranjan Sen and David Baltimore in 1986 (Sen and Baltimore, 1986), more than 50,000 reports have been published related to NFκB activation and its downstream effects. The number of biologic functions and cellular processes attributed to NFκB signaling are expansive and constantly increasing. As new data emerges, it is becoming clear that the diversity of downstream functions observed result from extremely intricate and tightly regulated mechanisms that control NFκB activation in a cell-, stimulus-, and temporal-specific manner. Early studies using knock out mouse models demonstrated the importance of NFκB in mediating cell survival and controlling innate and adaptive immunity. This was followed by extensive evidence demonstrating a role for NFκB in activating and perpetuating the inflammatory response, and lead to great interest in the development of therapeutic strategies to block NFκB signaling. However, more recent data has demonstrated that NFκB serves both pro-and anti-inflammatory functions, allowing NFκB to function in both the initiation and in the resolution of inflammation. In addition, accumulating evidence supports the notion that NFκB is an important regulator of angiogenesis, and identifies a role for NFκB in acting upstream of well-known, angiogenic pathways such as VEGF and HIF. Finally, emerging experimental and clinical data suggest that NFκB may play a unique, beneficial and developmentally essential role in the late saccular/early alveolar lung. Moving forward, the challenge will be to further delineate the mechanisms that allow for these distinct and contrasting functions for NFκB to tailor the development of therapeutic strategies to selectively block or enhance discrete components of the pathway to effectively treat or prevent lung diseases such as BPD.

## Acknowledgment

The author has no conflicts of interest to disclose.

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