Blocking NF- κ B

An Inflammatory Issue

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The nuclear factor (NF)- κ B is considered the master regulator of inflammatory responses. Studies in mouse models have established this transcription factor as an important mediator of many inflammatory disease states, including pulmonary diseases such as acute lung injury and acute respiratory distress syndrome. Endothelial cells provide the first barrier for leukocytes migrating to the inflamed sites and hence offer an attractive cellular context for targeting NF- κ B for treatment of these diseases. However, recent studies showing that NF- κ B also plays an important role in resolution phase of inflammation and in tissue repair and homeostasis have challenged the view of therapeutic inhibition of NF- κ B. This article reviews the regulation of NF- κ B in the context of endothelial cell signaling and provides a perspective on why “dampening” rather than “abolishing” NF- κ B activation may be a safe and effective treatment strategy for inflammation-associated pulmonary and other inflammatory diseases.

Keywords: endothelium; transcription factors; signal transduction; lung inflammation; injury

Inflammation has been known to mankind since ancient times; the first description of inflammation came from Aulus Cornelius Celsus, a Roman encyclopedist in the first century, who documented four cardinal signs of inflammation: rubor, calor, dolor, and tumor (redness, heat, pain, and swelling). Inflammation is basically a host defense response to tissue injury occurring from infection, wounding, or chemical exposure, causing redness, heat, pain, and swelling in the affected area. It serves to orchestrate a multifactorial network of chemical signals to remove the invading microbial pathogen and facilitate the healing of the injured tissue. Inflammation is usually self-limiting, but in many disease states it becomes exuberant and persistent, causing detrimental effects and further injury to the host tissues (Figures 1A and 1B). Therefore, the efficacy of treating inflammation-associated diseases may lie in strategies aimed at dampening inflammation rather than abolishing it (Figure 1C). The development of such a therapeutic strategy depends on uncovering the intricate signaling network in control of the initiation, maintenance, and resolution phase of inflammation, which may lead to identification of suitable targets whose inhibition selectively suppresses the detrimental inflammation without compromising the host defense response.

A characteristic feature of pulmonary inflammation is the massive infiltration of leukocytes, particularly neutrophils (i.e., polymorphonuclear leukocytes [PMNs]) in the lung that ultimately leads to disruption of capillary-alveolar barriers and the development of

pulmonary edema with severe consequences for pulmonary gas exchange (1, 2). To reach an inflammatory site in the pulmonary tissues and alveolar spaces, circulating PMNs must first traverse the endothelial barrier (the inner lining of blood vessel), a process referred to as diapedesis or transendothelial migration (TEM). TEM of PMNs is mediated by a cascade of cellular events initiated by infectious agents or noninfectious inflammatory stimuli. The sequence of events is initiated with local activation of rapidly responding resident cells, primarily macrophages, in the lung interstitium and alveolus, resulting in the release of several proinflammatory cytokines, including tumor necrosis factor (TNF)- α , IL-1 β , and chemokines such as IL-8 and macrophage inflammatory protein 2- α (3, 4). These soluble mediators serve to establish autocrine and paracrine loops to further activate the macrophages and endothelial cells (ECs). Activated endothelium produces numerous molecules, including adhesion molecules (vascular cell adhesion molecule-1, intercellular adhesion molecule-1 [ICAM-1]), cytokines (TNF- α , IL-1 β , IL-6), and chemokines (IL-8/CXCL8). The coordinate action of these proteins promotes lung PMN recruitment by facilitating adhesion and TEM of PMNs (5, 6). An essential event mediating the expression of genes encoding cytokines, chemokines, and adhesion molecules involves activation of the transcription factor nuclear factor kappa B (NF- κ B) (4, 7).

NF- κ B: A MASTER REGULATOR OF INFLAMMATORY RESPONSES

NF- κ B is viewed as a master regulator of inflammatory responses because it plays an essential role in the evolution as well as the resolution phase of inflammation (8). NF- κ B controls a wide spectrum of biological effects ranging from immune and stress-induced responses to cell fate decisions such as proliferation, differentiation, tumorigenesis, apoptosis, and tissue remodeling (9, 10). Inappropriate activation of NF- κ B is associated with many inflammatory disease states such as cancer, atherosclerosis, arthritis, inflammatory bowel disease, and Alzheimer's disease (7, 10, 11). In the context of lung inflammatory diseases, NF- κ B is implicated in the pathogenesis of acute lung injury/acute respiratory distress syndrome, asthma, idiopathic pulmonary fibrosis, bronchoalveolar dysplasia, and chronic obstructive pulmonary disease (12–17). Recent studies indicate that proinflammatory transcriptional programs that are activated by NF- κ B to shape the inflammatory response vary depending upon the stimulus and the cell type involved (8). For instance, NF- κ B target genes that are activated in ECs upon stimulation with LPS can be different from those induced by the proinflammatory cytokine TNF- α . Thus, understanding how information from various inputs is relayed to NF- κ B in pulmonary vascular cells, particularly ECs, because of its essential role in the recruitment of circulating leukocytes to the inflamed sites is of fundamental importance in controlling specific proinflammatory transcriptional programs associated with a particular inflammatory disease not only in the lung but in other organs as well. The following sections briefly review the current state of knowledge about NF- κ B and discuss the regulation of this fascinating family of transcription factors in the endothelium.

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Component of NF- κ B Complexes

NF- κ B was first identified in 1986 by Sen and Baltimore (18) while investigating the identity of proteins that bind to the Ig heavy-chain and the κ light-chain enhancers in the nucleus of B cells. Because of its essential role in the transcription of Ig κ light chain in B cells, the identified nuclear factor was named NF- κ B. The mammalian family of NF- κ B has five members: NF- κ B1 (p50 and its precursor p105), NF- κ B2 (p52 and its precursor p100), RelA (p65), RelB, and c-Rel. These proteins exist as dimers of distinct composition, and the prototype of these dimeric forms is the p50/RelA heterodimer. A unique feature of these proteins is the presence of a highly conserved N-terminal, 300-amino acid Rel homology domain (RHD) composed of two immunoglobulin-like structures. The RHD harbors nuclear localization signal and is responsible for dimerization, DNA binding, and interaction with inhibitory I κ B proteins. A distinguishing feature of RelA, RelB, and c-Rel is the presence of a transactivation domain within the carboxy-terminal region that renders these proteins transcriptionally active. The proteins p105 and p100 are synthesized as precursors to p50 and p52, respectively, and contain ankyrin repeats and a glycine-rich region. The presence of ankyrin repeats renders these proteins inactive, whereas the glycine-rich region serves to facilitate cotranslational processing of p105 to p50 and posttranslational processing of p100 to p52 (7–10, 19).

The activity of NF- κ B is regulated primarily by the I κ B family of inhibitory proteins. The I κ B family is composed of three groups: (1) the typical I κ B proteins I κ B α , I κ B β , and I κ B ϵ , which are present in the cytoplasm of unstimulated cells and undergo signal-induced degradation and resynthesis; (2) the precursor proteins NF- κ B2/p100 and NF- κ B1/p105, which undergo processing to yield NF- κ B family members p50 and p52, respectively; and (3) the atypical I κ B proteins I κ B ζ , BCL-3, and I κ BNS, which generally are not present constitutively but can be induced upon stimulation and exert their effects in the nucleus (8, 9, 19). All I κ B proteins contain five to seven ankyrin repeats that mediate association of I κ B with NF- κ B dimers, and this interaction masks the nuclear localization signal of NF- κ B proteins and thus retains them in the cytoplasm (7–9, 19). The prototypical member of the I κ B family is I κ B α .

Regulation of IKK/NF- κ B activation

Activation of NF- κ B is contingent upon its release after degradation or in some cases dissociation of I κ B α (7–10, 20). I κ B α degradation-dependent release of NF- κ B represents a more common mechanism of NF- κ B activation and is initiated through serine (Ser³² and Ser³⁶) phosphorylation of I κ B α by the I κ B kinase (IKK) complex. Phosphorylation leads to I κ B α ubiquitylation and subsequent degradation by the 26 S proteasome. This causes the release of cytoplasmic NF- κ B dimer, which undergoes nuclear translocation and activates the transcription of target genes including I κ B α . The resynthesized I κ B α contributes to the termination of the transcriptional response by associating with and retaining the NF- κ B dimers in the cytoplasm (8, 10, 19).

The IKK complex mediating the phosphorylation of I κ B α is composed of three core subunits: IKK α , IKK β , and IKK γ . IKK α and IKK β (also known as IKK1 and IKK2) are the catalytic subunits, and IKK γ (also known as NF- κ B essential modifier [NEMO]) is a regulatory subunit (8–10, 19). Activation of IKK β is an essential event in most canonical NF- κ B signaling pathways, including those that are activated by TNF- α , IL-1, thrombin, and LPS (Figure 2). Genetic studies indicate that IKK β is the predominant kinase responsible for phosphorylation of I κ B α , although emerging evidence indicates a role for

IKK α in this response (9, 19). In contrast, the noncanonical or alternate NF- κ B pathway does not require participation of IKK β and IKK γ and is strictly dependent on IKK α , which selectively phosphorylates the two C-terminal serines of NF- κ B2/p100 associated with RelB (Figure 2). Phosphorylation induces the processing of p100, resulting in the release of p52-containing transcriptionally active dimers, predominantly p52/RelB heterodimer. The released p52/RelB dimer translocates to the nucleus, where it activates the transcription of genes involved in development and maintenance of lymphoid organs (9, 19). This pathway is activated mainly through stimulation of the CD40 and lymphotoxin- β receptors, the B cell-activating factor of the TNF family, and LPS (Figure 2).

An important regulatory pathway that can be activated in addition to the cascade inducing I κ B α degradation and can thus control the transactivation potential of NF- κ B involves phosphorylation of RelA/p65. Phosphorylation of RelA/p65 at Ser²⁷⁶ or Ser³¹¹ in the RHD, or Ser⁵²⁹ or Ser⁵³⁶ in the transactivation domain, increases the transcriptional capacity of NF- κ B (7, 9, 21). Unlike I κ B α phosphorylation, the RelA/p65 phosphorylation site and the kinase involved vary in a stimulus- and cell type-specific manner. For instance, TNF- α engages mitogen- and stress-activated kinase-1 (MSK-1) to catalyze Ser²⁷⁶ phosphorylation of RelA/p65, whereas the LPS-induced phosphorylation is mediated by the catalytic subunit of protein kinase A (PKA_c). PKA_c phosphorylates RelA/p65 in the cytoplasm, whereas MSK-1 functions in the nucleus. TNF- α also promotes phosphorylation of RelA/p65 at Ser³¹¹, but this is mediated by another kinase, PKC ζ . Phosphorylation of Ser⁵²⁹ is mediated by casein kinase 2 (CK2), whereas Ser⁵³⁶ is phosphorylated by IKK α and IKK β (7, 9, 21). The multiplicity of phosphorylation sites and the kinases involved raises the possibility that concurrent phosphorylation of multiple sites have cooperative functional effects on the transcriptional activity of NF- κ B.

Phosphorylation of RelA/p65 has an important function in the recruitment of transcriptional coactivators p300 and its homolog CBP (cAMP-response-element-binding protein-binding protein) to induce acetylation of RelA/p65 (21, 22). Acetylation of RelA/p65 by p300/CBP is implicated in regulating the nuclear function of NF- κ B (21, 22). Acetylation at Lys²²¹ stabilizes RelA/p65 binding to the κ B site and, together with the acetylation of Lys²¹⁸, prevents the association of RelA/p65 with I κ B α , whereas acetylation at Lys³¹⁰ enhances transcriptional activity without influencing binding to DNA or I κ B (21).

Activation of IKK is the converging point in the mechanism of NF- κ B activation in response to the majority of extracellular signals (Figure 2). Phosphorylation of two serine residues in the activation loop within the kinase domain of IKK α (Ser¹⁷⁶ and Ser¹⁸⁰) or IKK β (Ser¹⁷⁷ and Ser¹⁸¹) is essential to the activation of its kinase activity. However, the mechanisms by which the signals are transmitted from cell surface receptors to IKK phosphorylation are poorly understood. It is now becoming increasingly clear that each receptor system engages a distinct set of adaptor molecules and signaling enzymes to construct a unique pathway that leads to IKK activation, and the cellular context also influences the signaling to IKK.

SIGNALING MECHANISMS OF IKK/NF- κ B ACTIVATION IN THE ENDOTHELIUM

The IKK and NF- κ B complexes are present in the endothelium, and a role for NF- κ B has been implicated in a number of EC functions. Activation of NF- κ B in ECs renders the otherwise “antiadhesive, anticoagulant, and relatively restrictive (semipermeable)” microvasculature into a “proadhesive, procoagulant, and leaky” one (6, 12, 23–27). Indeed, EC-selective

Figure 1. (A) Inflammation is usually self-limiting; it is activated in response to injury or infection and resolves with elimination of the invading pathogen or healing of the injured tissue. However, in many disease conditions, inflammation becomes excessive, fails to resolve, and exerts detrimental effects on the host tissues, leading to (B) many inflammatory disease states. (C) Therapeutic strategy for inflammatory diseases (blocking versus dampening inflammation). The key to an effective treatment of inflammatory diseases may lie selectively targeting the detrimental inflammation. ALI/ARDS = acute lung injury/acute respiratory distress syndrome; BPD = bronchoalveolar dysplasia; COPD = chronic obstructive pulmonary disease.

Figure 3. Thrombin regulation of NF- κ B signaling in endothelial cells. Ligation of protease-activated receptor-1 (PAR-1) by thrombin causes activation of $G_{\alpha q}$ and dissociation of $G_{\beta \gamma}$ complex, which in turn lead to parallel activation of PKC δ - and PI3 kinase and the downstream kinase Akt-dependent pathways. These pathways converge to stimulate IKK, which in turn promotes RelA/p65 homodimer binding to the promoter of target genes secondary to phosphorylation and degradation of I κ B α . PKC δ also engages p38 MAP kinase to induce phosphorylation and thereby transcriptional capacity of RelA/p65. The resultant expression of proinflammatory genes such as ICAM-1 promotes adhesion and transendothelial migration of leukocytes.

Figure 2. Nuclear factor (NF)- κ B activation pathways. The canonical pathway is activated by proinflammatory cytokines (TNF- α , IL-1 β), G-protein coupled receptors (GPCR) (thrombin), Toll-like receptors (TLR) (LPS), and many other stimuli, including viruses and antigen receptors. This pathway is dependent on the activation of IKK β , which phosphorylates I κ B α at Ser³² and Ser³⁶, leading to its ubiquitylation and degradation by the 26 proteasome. NF- κ B complexes, typically a heterodimer of p50 and RelA/p65, migrate to the nucleus and activate expression of multiple inflammatory, innate immune, and antiapoptotic genes. The noncanonical pathway is induced by cytokines such as lymphotoxin- β receptor (LT β R), B-cell-activating factor (BAFF), CD40L, and LPS and requires NF- κ B-inducing kinase (NIK)-dependent activation of IKK α . Activated IKK α phosphorylates p100 NF- κ B subunit for its proteasome-dependent processing to p52, generating p52/RelB heterodimers for translocation to the nucleus. The nuclear p52/RelB activates distinct transcriptional programs, mainly the ones involved in the development and maintenance of secondary lymphoid organs.

blockade of NF- κ B activation attenuates lung inflammation, prevents vascular leak, and improves survival in murine models of sepsis (12). The key signaling events regulating IKK/NF- κ B activation, particularly in the context of EC stimulation with the proinflammatory cytokine TNF- α and the procoagulant thrombin, are summarized below. The reason for focusing on these mediators is that they serve to establish a close interaction and bidirectional cooperation between inflammation and coagulation whereby inflammation not only leads to activation of intravascular coagulation but the latter also promotes inflammatory activity (28). An implication of these interactions is that they may lead to a vicious cycle of feed-forward mechanisms to amplify the inflammation/coagulation axis and thus prevent them from resolving.

PKC Regulation of IKK/NF- κ B

Protein kinase C (PKC) has emerged as an important mediator of IKK/NF- κ B activation in ECs. Clues about the role of PKC in the mechanism of IKK/NF- κ B activation came from studies showing activation of NF- κ B and expression of its target genes in response to PKC activators, phorbol esters, and phorbol dibutyrate. Subsequent studies using isoform-specific pharmacological and genetic approaches implicated a role for PKC isoforms in IKK/NF- κ B activation induced by a number of other inflammatory mediators. For example, the novel PKC δ isoform was identified as a key kinase in transmitting thrombin signaling to IKK/NF- κ B in ECs and in vascular smooth muscle cells (24, 25) (Figure 3). The signaling cascade that links thrombin stimulation of its receptor PAR-1 (protease-activated receptor 1) and the heterotrimeric G-protein $G_{\alpha q}$ to PKC δ include TRPC/Ca²⁺/CAMMK β /AMPK (29). Analysis of NF- κ B signaling revealed that PKC δ serves two functions in this response; one causes activation of IKK β to mediate the release of RelA/p65 secondary to phosphorylation and degradation of

Figure 4. TNF- α regulation of NF- κ B signaling in endothelial cells. Activation of TNF receptor (TNFR) stimulates PI3K γ , which in turn activates PKC ζ . Activated PKC ζ triggers the activation of NADPH oxidase, resulting in ROS generation, which in turn induces RelA/p65 homodimer binding to the promoter of target genes secondary to phosphorylation and degradation of I κ B α . Additionally, PKC ζ phosphorylates and thereby increases the transcriptional activity of the RelA/p65. The expression of proinflammatory genes such as ICAM-1 by this mechanism mediates adhesion and transendothelial migration of leukocytes.

I κ B α , and the other involves activation of p38 MAP kinase, which increases the transcriptional capacity of the liberated NF- κ B through Ser⁵³⁶ phosphorylation of RelA/p65. Together, these events serve to induce the transcription of target genes including adhesion molecules (24, 25, 29) (Figure 3). Another PKC isoform that participates in PAR-1 signaling of NF- κ B activation involves Ca²⁺-dependent PKC α , which likely engages PKC δ via Rho signaling to mediate this response (30). Similarly, studies have shown that atypical PKC ζ occupies a central position in the signaling pathway activated by TNF- α to promote activation of NF- κ B and expression of its target gene ICAM-1 (31) (Figure 4). Further analysis showed that PKC ζ mediates this response by promoting I κ B α degradation-dependent nuclear translocation of RelA/p65. Another study reported that PKC ζ also enhances the transcriptional activity of NF- κ B via phosphorylation of RelA/p65 (32). Collectively, these findings indicate that PKC ζ controls TNF- α -induced NF- κ B signaling by inducing the DNA binding and transactivation potential of the bound RelA/p65.

PI3K/Akt Regulation of IKK/NF- κ B

PI3 kinase and its downstream effector Akt play a critical role in the mechanism of IKK/NF- κ B regulation in ECs. In the case of thrombin activation of IKK/NF- κ B, PI3K mediates an independent arm of signaling cascade (in parallel to G α q-PKC δ pathway) that also converges to IKK/NF- κ B (Figure 3). In this cascade, G β γ and Akt function as upstream and downstream effectors of PI3K, respectively, to link the signaling from PAR-1 to IKK/NF- κ B. These studies relied on genetic approaches involving a combination of dominant negative and constitutively active mutants of PI3K and Akt (33). In the case of TNF- α stimulation, PI3K γ is recruited as a critical mediator of NF- κ B activation and expression of its target gene ICAM-1 in ECs (34) (Figure 4). A recent study in which lung microvascular ECs from mice lacking

Figure 5. Regulation of thrombin-induced RelA/p65 nuclear translocation by the Rho-actin pathway. Activation of RhoA/ROCK in endothelial cells after thrombin stimulation of PAR-1 leads to two signaling events; one involves activation of IKK, and the other causes phosphorylation and thereby inactivation of cofilin-1, an actin-binding protein that promotes actin depolymerization. Activated IKK catalyzes phosphorylation and thereby degradation of I κ B α to mediate the release of RelA/p65. Inactivation of cofilin results in dynamic reorganization of the actin cytoskeleton. These events mediate the interaction of the released RelA/p65 with actin, and this interaction in turn promotes, by an undetermined mechanism, the nuclear import of RelA/p65. The nuclear RelA/p65 binds to the promoter and induces the expression of target genes such as ICAM-1.

p110 γ catalytic subunit of PI3K γ were used showed a defect in PKC ζ activation by TNF- α in PI3K γ ^{-/-} ECs. These results place PI3K γ upstream of PKC ζ in the mechanism of TNF- α -induced

Figure 6. An effective and safe therapeutic strategy for inflammation-associated diseases may lie in the identification of target(s) whose inhibition may lead to “dampening” rather than complete blockade of NF- κ B signaling. (A) Direct inhibition of the receptor or NF- κ B may lead to impaired host defense response, tissue repair, and homeostasis. (B) Inhibition of a given arm of signaling to NF- κ B (alone or in combination) may allow appropriate inhibition of inflammation such that the host defense and repair mechanisms are least affected.

activation of NF- κ B (34). These findings implicate a role of PI3K γ /PKC ζ in mediating TNF- α -induced NF- κ B activation and its target gene expression.

Oxidant Regulation of IKK/NF- κ B

Oxidants serve an important signaling function in regulating NF- κ B activation. The role of oxidant signaling in NF- κ B-dependent gene expression in ECs has been studied in response to a host of stimuli. In particular, studies in the context of TNF- α stimulation have yielded important information concerning the source and mechanism of oxidant generation and its involvement in NF- κ B activation and its target gene expression. It was found that TNF- α induces oxidant generation in ECs via activation of NADPH oxidase and that this event is critical for NF- κ B activation and ICAM-1 expression (34). Evidence in support of this conclusion came from experiments in which p47^{phox}-/- ECs and gp91^{phox}-/- were isolated from the lungs of mice deficient in p47^{phox} or the gp91^{phox} subunit of NADPH oxidase (35). Further investigations revealed a role of PKC ζ in activating NADPH oxidase via its ability to phosphorylate p47^{phox}, which in turn targets it to the plasma membrane for functional assembly of the NADPH oxidase complex. Consistent with a role of PI3K γ as the upstream kinase of PKC ζ , NADPH oxidase activation failed to occur in PI3K γ -/- ECs (34). Considered together, these results indicate the involvement of PI3K γ /PKC ζ /NADPH in oxidant generation required for TNF- α -induced NF- κ B signaling in ECs (Figure 4). However, thrombin-induced NF- κ B activation in ECs appears to be NADPH oxidase-independent and instead occurs via a pathway in which thrombin induces inositol triphosphate receptor-dependent Ca²⁺ signals, which are in turn transmitted to mitochondria to stimulate mitochondrial ROS (mROS) generation in mouse lung microvascular ECs. The production of mROS by this pathway induces NF- κ B activation and ICAM-1 expression and promotes leukocyte adhesion to the vascular endothelium (36). These findings demonstrate a role of stimulus-specific (thrombin versus TNF- α) involvement of mitochondrial versus NADPH oxidase-derived ROS in activating NF- κ B signaling in ECs and show a fundamental difference between G protein-coupled receptors and TNFr signaling.

MAP Kinase Regulation of IKK/NF- κ B

Several studies have implicated a role of MAP kinases, particularly p38 MAP kinase, in regulating NF- κ B signaling in ECs. Thrombin and TNF- α activate p38 MAP kinase in ECs, and inhibition of this kinase impairs NF- κ B signaling (6, 24). For example, expression of dominant negative of p38 MAP kinase inhibited thrombin- and TNF- α -induced NF- κ B-dependent reporter activity and expression of target genes such as vascular cell adhesion molecule-1, ICAM-1, MCP-1, and IL-8 (6, 7, 24). Additionally, a number of agents inhibit NF- κ B signaling by targeting p38 MAP kinase, consistent with the requirement of this kinase in the response. There are, however, some conflicting reports concerning the involvement of p38 MAP kinase in the mechanism of NF- κ B-dependent gene expression. Although some studies demonstrate a role for p38 MAPK in NF- κ B activation, others argue against it (6, 24, 37). The reason for this discrepancy is not clear but may reflect the differences in source and culture conditions of cells and the use of pharmacological versus genetic approaches to address the role of p38 MAP kinase.

Small GTPase and Actin Cytoskeleton Regulation of IKK/NF- κ B

The small GTPases- and actin cytoskeleton-dependent activation of NF- κ B activation provides another example of the

fundamental differences between G-protein coupled receptors and TNFr signaling in ECs. Our studies showed a critical requirement of the RhoA/Rho-associated kinase (ROCK) pathway in thrombin-induced NF- κ B signaling. Inhibition of the RhoA/ROCK pathway impaired thrombin-induced NF- κ B activation and ICAM-1 gene expression but had no effect on these responses induced by TNF- α (38), indicating the stimulus-specific regulation of NF- κ B by RhoA/ROCK. RhoA/ROCK controls NF- κ B activation by virtue of promoting I κ B α phosphorylation and degradation, leading to nuclear uptake and DNA binding of RelA/p65 and inducing Ser⁵³⁶ phosphorylation, which enhances the transcriptional activity of the bound RelA/p65 (38). Other studies also showed that the RhoA/ROCK pathway does not participate in TNF- α -induced NF- κ B-dependent gene expression (39). In contrast, Rac1 has been shown to play a role in NF- κ B regulation by TNF- α (40). Chen and colleagues (40) showed that adenoviral-mediated expression of a dominant negative mutant of Rac1 (N17Rac1) was effective in attenuating TNF- α -induced adhesion molecule expression by impairing the transcriptional activity of NF- κ B.

The lack of involvement of Rac1 in thrombin response can be ascribed to the inability of thrombin to activate Rac1; however, given the ability of TNF- α to activate RhoA (39), a similar argument cannot be advanced to explain the RhoA/ROCK-independent regulation of NF- κ B by TNF- α (38). One plausible explanation could be that thrombin and TNF- α may engage distinct mechanisms of RhoA/ROCK activation as well as a specialized set of downstream effectors activated by each agonist. Consistent with this possibility, Fazal and colleagues (41) recently identified an important contribution of actin cytoskeleton downstream of RhoA/ROCK in thrombin- but not TNF- α -induced NF- κ B-dependent gene expression. This study showed that stabilization or destabilization of the actin cytoskeleton each selectively inhibited thrombin-induced nuclear translocation of RelA/p65 without affecting the I κ B α degradation-dependent release of RelA/p65. Further investigations (42) revealed a central role of cofilin-1, an actin-binding protein that causes actin depolymerization, in linking the RhoA/ROCK pathway to dynamic changes in the actin cytoskeleton necessary for nuclear translocation of RelA/p65 and expression of ICAM-1. These findings are consistent with a model wherein RhoA/ROCK serves a dual role in mediating NF- κ B signaling by coordinating IKK-mediated release and cofilin-dependent alterations in actin cytoskeleton required for RelA/p65 translocation to the nucleus (Figure 5).

Tyrosine Kinase Regulation of IKK/NF- κ B

Tyrosine kinase represents another important family of signaling molecules that play a critical role in NF- κ B regulation. Initial studies implicating tyrosine kinases in NF- κ B regulation used pharmacological inhibitors and showed that inhibition of tyrosine kinases by this approach markedly attenuated TNF- α -induced NF- κ B-dependent gene expression by virtue of impairing NF- κ B binding to DNA (43). Recent studies by Bijli and colleagues (44, 45) have identified c-Src and Syk (spleen tyrosine kinase) as novel regulators of NF- κ B activation. These studies showed that inhibition or depletion (RNAi knockdown) of c-Src or Syk each reduced thrombin-induced transcriptional activity of NF- κ B and expression of ICAM-1. The suppressive effect of inhibiting or depleting c-Src or Syk on NF- κ B-dependent gene expression occurred without affecting I κ B α degradation and DNA binding function of NF- κ B and was not associated with the exchange of NF- κ B dimers. Inhibition or depletion of c-Src or Syk also failed to prevent phosphorylation of RelA/p65 at Ser⁵³⁶, an event mediating the transcriptional activity of NF- κ B (44, 45). Thrombin

induced tyrosine phosphorylation of RelA/p65, and this phosphorylation was lost upon inhibition and depletion of c-Src or Syk (44, 45). Further analysis showed that Syk signals downstream of c-Src to mediate this response (45). These data reveal a novel mechanism of NF- κ B transcriptional activity that relies on tyrosine phosphorylation of RelA/p65. Depletion of Syk also impaired TNF- α -induced tyrosine phosphorylation and the transcriptional activity of RelA/p65 (K. M. Bijli unpublished results), suggesting that Syk is a common regulator of NF- κ B activity. However, the precise mechanism by which tyrosine phosphorylated RelA/p65 confers transcriptional competency to the bound NF- κ B remains unclear and requires the identification of the tyrosine phosphorylation site in RelA/p65.

Mechanistic Target of Rapamycin Regulation of IKK/NF- κ B

Mechanistic target of rapamycin (MTOR) (formerly mammalian target of rapamycin [mTOR]) appears to function as an endogenous modulator of NF- κ B signaling in ECs. Our studies have shown that thrombin engages PKC δ - and PI3K/Akt-dependent pathways to activate MTOR in the endothelium, and this event in turn serves to limit NF- κ B signaling and proinflammatory response (46). Because PKC δ , PI3K, and Akt also promote IKK/NF- κ B signaling (46), the paradoxical role of PKC δ and PI3K/Akt in stimulating MTOR (which suppresses NF- κ B signaling) led us to determine how these events are coordinated by the same upstream signals. Time-course studies revealed that PKC δ and PI3K/Akt mediate thrombin-induced activation of IKK/NF- κ B and MTOR in a reciprocal manner such that when NF- κ B activation is maximal, MTOR activation is minimal and vice versa (46). Such a mechanism of MTOR activation serves to control a delayed and transient activation of NF- κ B, and, consistent with this, inhibition or depletion of MTOR results in rapid and persistent NF- κ B signaling (47). Thus, the temporally divergent regulation of MTOR and NF- κ B signaling by the same upstream signals may be a mechanism of ensuring the tight regulation of inflammatory responses. Moreover, impairing MTOR signaling also potentiated TNF- α -induced NF- κ B activation and ICAM-1 expression, indicating that MTOR is a general modifier of these responses. This modifier role of MTOR is not restricted to ECs; a similar role of MTOR in restricting inflammatory responses was observed in monocytes/macrophages and was attributed to its ability to inhibit NF- κ B-dependent proinflammatory genes and to promote STAT-3-dependent antiinflammatory genes (48). However, unlike ECs and monocytes/macrophages, LPS-induced NF- κ B signaling in PMN is impaired upon inhibition of MTOR by rapamycin (49). These data suggest a cell-specific regulation of NF- κ B by MTOR. Given that MTOR exists in two complexes in which MTORC1 is rapamycin-sensitive and MTORC2 is rapamycin-insensitive and that the relative abundance and crosstalk between these complexes may vary depending upon the cellular context (50), cell-specific regulation of NF- κ B by MTOR is possible.

CONCLUSIONS

Emerging evidence indicates that NF- κ B, in addition to its established role as an initiator of inflammation, plays an important role in the resolution phase of inflammation and in tissue repair (51–53). Consistent with this, studies in which NF- κ B was inhibited after the initiation and during the resolution phase of inflammation showed a persistent inflammation and delayed tissue repair (51). Recently, an unexpected role of NF- κ B in controlling tissue immune homeostasis has been identified (54). Studies in genetic mouse models have shown that loss of NF- κ B signaling in nonimmune epithelial or parenchymal cells causes spontaneous development of severe inflammatory conditions (54). It is also becoming clear

that, in addition to their role as a kinase for I κ B α and RelA/p65, IKK α and IKK β have several I κ B α - and RelA/p65-independent targets (55). In view of the complexity associated with NF- κ B functions and the specificity of the signaling events from cell surface receptors to IKK phosphorylation, therapeutic use of IKK inhibitors is less likely to yield the desired results. Similarly, direct inhibition of the cell surface receptors or NF- κ B may lead to complete blockade of NF- κ B signaling and is thus associated with the risk of impairing the host defense and repair mechanisms (Figure 6). Thus, an effective therapeutic strategy for inflammation-associated diseases may rely on dampening NF- κ B signaling and requires elucidation of the intricate signaling network of IKK/NF- κ B activation (Figure 6).

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References

- Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–1349.
- Maniatis NA, Orfanos SE. The endothelium in acute lung injury/acute respiratory distress syndrome. *Curr Opin Crit Care* 2008;14:22–30.
- Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maumder RJ, Kunkel SL, Walz A, Hudson LD, Martin TR. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1996;154:602–611.
- Fan J, Ye RD, Malik AB. Transcriptional mechanisms of acute lung injury. *Lung Cell Mol Physiol* 2001;281:L1037–L1050.
- Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994;76:301–314.
- Rahman A, Fazal F. Hug tightly and say goodbye: role of endothelial ICAM-1 in leukocyte transmigration. *Antioxid Redox Signal* 2009;11:823–829.
- Bijli KM, Rahman A. NF- κ B signaling in endothelium. In: Aird WC, editor. *Endothelial biomedicine*. Cambridge University Press; Cambridge, UK: 2007. pp. 784–795.
- Ghosh S, Hayden MS. New regulators of NF- κ B in inflammation. *Nat Rev Immunol* 2008;8:837–848.
- Hayden MS, Ghosh S. Signaling to NF- κ B. *Genes Dev* 2004;18:2195–2224.
- Karin M, Greten FR. NF- κ B: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005;5:749–759.
- Kumar A, Takada Y, Boriek AM, Aggarwal BB. Nuclear factor-kappaB: its role in health and disease. *J Mol Med* 2004;82:434–448.
- Ye X, Ding J, Zhou X, Chen G, Liu SF. Divergent roles of endothelial NF-kappaB in multiple organ injury and bacterial clearance in mouse models of sepsis. *J Exp Med* 2008;205:1303–1315.
- Janssen-Heininger YM, Poynter ME, Aesif SW, Pantano C, Ather JL, Reynaert NL, Ckless K, Anathy V, van der Velden J, Irvin CG, et al. Nuclear factor kappaB, airway epithelium, and asthma: avenues for redox control. *Proc Am Thorac Soc* 2009;6:249–255.
- Castranova V. Signaling pathways controlling the production of inflammatory mediators in response to crystalline silica exposure: role of reactive oxygen/nitrogen species. *Free Radic Biol Med* 2004;37:916–925.
- Rieger-Fackeldey E, Hentschel R. Bronchopulmonary dysplasia and early prophylactic inhaled nitric oxide in preterm infants: current concepts and future research strategies in animal models. *J Perinat Med* 2008;36:442–447.
- MacNee W, Tudor RM. New paradigms in the pathogenesis of chronic obstructive pulmonary disease I. *Proc Am Thorac Soc* 2009;6:527–531.
- Yoshida T, Mett I, Bhunia AK, Bowman J, Perez M, Zhang L, Gandjeva A, Zhen L, Chukwueke U, Mao T, et al. Rtp801, a suppressor of mTOR signaling, is an essential mediator of cigarette smoke-induced pulmonary injury and emphysema. *Nat Med* 2010;16:767–773.
- Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1986;46:705–716.

19. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004;25:280–288.
20. Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Diekmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, et al. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell* 1996;86:787–798.
21. Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* 2004;5:392–401.
22. Zhong H, May MJ, Jimi E, Ghosh S. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol Cell* 2002;9:625–636.
23. Rahman A, Anwar KN, True AL, Malik AB. Thrombin-induced p65 homodimer binding to downstream NF-kappa B site of the promoter mediates endothelial ICAM-1 expression and neutrophil adhesion. *J Immunol* 1999;162:5466–5476.
24. Rahman A, Anwar KN, Uddin S, Xu N, Ye RD, Platanias LC, Malik AB. Protein kinase C-delta regulates thrombin-induced ICAM-1 gene expression in endothelial cells via activation of p38 mitogen-activated protein kinase. *Mol Cell Biol* 2001;21:5554–5565.
25. Minami T, Abid MR, Zhang J, King G, Kodama T, Aird WC. Thrombin stimulation of vascular adhesion molecule-1 in endothelial cells is mediated by PKC δ -NF- κ B and PKC ζ -GATA signaling pathways. *J Biol Chem* 2003;278:6976–6984.
26. Tiruppathi C, Shimizu J, Miyawaki-Shimizu K, Vogel SM, Bair AM, Minshall RD, Predescu D, Malik AB. Role of NF-kappaB-dependent caveolin-1 expression in the mechanism of increased endothelial permeability induced by lipopolysaccharide. *J Biol Chem* 2008;283:4210–4218.
27. Song D, Ye X, Xu H, Liu SF. Activation of endothelial intrinsic NF- κ B pathway impairs protein C anticoagulation mechanism and promotes coagulation in endotoxemic mice. *Blood* 2009;114:2521–2529.
28. Levi M, van der Poll T. Two-way interactions between inflammation and coagulation. *Trends Cardiovasc Med* 2005;15:254–259.
29. Bair AM, Thippagowda PB, Freichel M, Cheng N, Ye RD, Vogel SM, Yu Y, Flockerzi V, Malik AB, Tiruppathi C. Ca²⁺ entry via TRPC channels is necessary for thrombin-induced NF-kappaB activation in endothelial cells through AMP-activated protein kinase and protein kinase Cdelta. *J Biol Chem* 2009;284:563–574.
30. Paria BC, Bair AM, Xue J, Yu Y, Malik AB, Tiruppathi C. Ca²⁺ influx induced by protease-activated receptor-1 activates a feed-forward mechanism of TRPC1 expression via nuclear factor-kappaB activation in endothelial cells. *J Biol Chem* 2006;281:20715–20727.
31. Rahman A, Anwar KN, Malik AB. Protein kinase C ζ mediates TNF α -induced ICAM-1 gene transcription in endothelial cells. *Am J Physiol Cell Physiol* 2000;279:C906–C914.
32. Anrather J, Csizmadia V, Soares MP, Winkler H. Regulation of NF-kappaB RelA phosphorylation and transcriptional activity by p21 (ras) and protein kinase C ζ in primary endothelial cells. *J Biol Chem* 1999;274:13594–13603.
33. Rahman A, True AL, Anwar KN, Ye RD, Voyno-Yasenetskaya TA, Malik ABG. α q and G β γ regulate PAR-1-induced NF- κ B activation and ICAM-1 transcription in endothelial cells. *Circ Res* 2002;91:398–405.
34. Frey RS, Gao XP, Javaid K, Siddiqui SS, Rahman A, Malik AB. Phosphatidylinositol 3-kinase γ signaling via PKC ζ induces NADPH oxidase-induced oxidant generation and NF- κ B activation in endothelial cells. *J Biol Chem* 2006;281:16128–16138.
35. Frey RS, Rahman A, Kefer J, Minshall RD, Malik AB. PKC- ζ regulates TNF α -induced activation of NADPH oxidase in endothelial cells. *Circ Res* 2002;90:1012–1019.
36. Hawkins BJ, Solt LA, Chowdhury I, Kazi AS, Abid MR, Aird WC, May MJ, Foskett JK, Madesh M. G protein-coupled receptor Ca²⁺-linked mitochondrial reactive oxygen species are essential for endothelial/leukocyte adherence. *Mol Cell Biol* 2007;27:7582–7593.
37. Zhou Z, Connel MC, MacEwan DJ. TNFR1-induced NF-kappaB, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. *Cell Signal* 2007;19:1238–1248.
38. Anwar KN, Fazal F, Malik AB, Rahman A. RhoA/ROCK pathway selectively regulates thrombin-induced ICAM-1 expression in endothelial cells via activation of IKK β and phosphorylation of RelA/p65. *J Immunol* 2004;173:6965–6972.
39. Cernuda-Morollon E, Ridley AJ. Rho GTPases and leukocyte adhesion receptor expression and function in endothelial cells. *Circ Res* 2006;98:757–767.
40. Chen XL, Zhang Q, Zhao R, Ding X, Tummala PE, Medford RM. Rac1 and superoxide are required for the expression of cell adhesion molecules induced by tumor necrosis factor-alpha in endothelial cells. *J Pharmacol Exp Ther* 2003;305:573–580.
41. Fazal F, Minhajuddin M, Bijli KM, McGrath JL, Rahman A. Evidence for actin cytoskeleton-dependent and -independent pathways for RelA/p65 nuclear translocation in endothelial cells. *J Biol Chem* 2007;282:3940–3950.
42. Fazal F, Bijli KM, Minhajuddin M, Rein T, Finkelstein JN, Rahman A. Essential role of cofilin-1 in regulating thrombin-induced RelA/p65 nuclear translocation and ICAM-1 expression in endothelial cells. *J Biol Chem* 2009;284:21047–21056.
43. Weber C, Negrescu E, Erl W, Pietsch A, Frankenberger M, Ziegler-Heitbrock HW, Siess W, Weber PC. Inhibitors of protein tyrosine kinase suppress TNF-stimulated induction of endothelial cell adhesion molecules. *J Immunol* 1995;155:445–451.
44. Bijli KM, Minhajuddin M, Fazal F, O'Reilly MA, Platanias LC, Rahman A. c-Src interacts with and phosphorylates RelA/p65 to promote thrombin-induced ICAM-1 expression in endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L396–L404.
45. Bijli KM, Fazal F, Minhajuddin M, Rahman A. Activation of Syk by PKC δ regulates thrombin-induced ICAM-1 expression in endothelial cells via tyrosine phosphorylation of RelA/p65. *J Biol Chem* 2008;283:14674–14684.
46. Minhajuddin M, Bijli KM, Fazal F, Sassano A, Nakayama KI, Hay N, Platanias LC, Rahman A. Protein kinase C δ g and gPI 3-kinase/Akt activate mammalian target of rapamycin to modulate NF- κ B activation and ICAM-1 expression in endothelial cells. *J Biol Chem* 2009;284:4052–4061.
47. Minhajuddin M, Fazal F, Bijli KM, Rahman A. Inhibition of mTOR potentiates thrombin-induced ICAM-1 expression by accelerating and stabilizing NF- κ B activation in endothelial cells. *J Immunol* 2005;174:5823–5829.
48. Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, Stuhlmeier KM, Kolbe T, Stulnig TM, Hörl WH, Hengstschläger M, et al. The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity* 2008;29:565–577.
49. Lorne E, Zhao X, Zmijewski JW, Liu G, Park YJ, Tsuruta Y, Abraham E. Participation of mammalian target of rapamycin complex 1 in Toll-like receptor 2- and 4-induced neutrophil activation and acute lung injury. *Am J Respir Cell Mol Biol* 2009;41:237–245.
50. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 2004;14:1296–1302.
51. Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA. Possible new role for NF- κ B in the resolution of inflammation. *Nat Med* 2001;7:1291–1297.
52. Chen LW, Egan L, Li ZW, Greten FR, Kagnoff MF, Karin M. The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nat Med* 2003;9:575–581.
53. Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, Garg HG, Quinn DA, et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat Med* 2003;11:1173–1179.
54. Pasparakis M. Regulation of tissue homeostasis NF- κ B signaling: implications for inflammatory diseases. *Nat Rev Immunol* 2009;9:778–788.
55. Perkins ND. Integrating cell-signaling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 2007;8:49–62.