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# Translational Review

# A20 Regulation of Nuclear Factor–κB

## Perspectives for Inflammatory Lung Disease

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**Persistent activation of NF-κB is central to the pathogenesis of many inflammatory lung disorders, including cystic fibrosis, asthma, and chronic obstructive pulmonary disease. A20 is an endogenous negative regulator of NF-κB signaling, which has been widely described in autoimmune and inflammatory disorders, including diabetes and Crohn's disease, but which has received little attention in terms of chronic lung disorders. This review examines the existing body of research on A20 regulation of NF-κB signaling and details the mechanism and regulation of A20 action focusing, where possible, on pulmonary inflammation. A20 and its associated signaling molecules are highlighted as being of potential therapeutic interest for the treatment of inflammatory disorders, and a proposed model of A20 activity in inflammatory lung disease is provided.**

**Keywords:** A20; NF-κB signaling; inflammatory lung disease

The transcription factor NF-κB regulates the expression of a large number of genes in response to infection, inflammation, and other endogenous and exogenous stressors (1). Under resting conditions, NF-κB is normally inactive as a result of binding to inhibitors of κB (IκB) (e.g., IκBα). NF-κB activation is triggered by stimulation of various receptors, including TNF receptor, Toll-like receptors (TLRs), and T-cell receptor (TCR), and in response to a wide variety of stimuli (e.g., TNF-α, IL-1, bacterial LPS, and antigen). These receptors induce signaling cascades, leading to the degradation of IκBα and subsequent release of NF-κB. NF-κB translocates to the nucleus and activates transcription of proinflammatory genes. Enhanced or prolonged NF-κB activation has been described in chronic inflammatory lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) (2, 3. Up-regulation of NF-κB has been reported in asthma, COPD, and CF after exacerbations induced by bacterial or viral infection. However, inflammation persists in asthma and CF even during times of "being well" (3).

Persistent inflammation and airway remodeling are common to asthma, CF, and COPD, although debate exists as to whether remodeling occurs as a consequence of or in parallel with uncontrolled inflammation (4). In asthma, remodeling is characterized by airway narrowing and smooth muscle hypertrophy (5), whereas patients with CF and COPD show extensive bronchiectasis (6, 7). Inhaled corticosteroids are commonly prescribed in asthma to control persistent airway inflammation, but in many patients treated with standard doses, their effect is insufficient to provide proper control. In a study of 180 children with uncontrolled asthma, the implementation of step-up therapy (inhaled corticosteroids, long-acting β agonists, and leukotriene receptor antagonists) could not completely prevent asthma exacerbations (8). Corticosteroids recruit histone deacetylase (HDAC)-2 to tighten the chromatin structure of DNA, making it more difficult for NF-κB to bind and initiate gene transcription (9, 10). In asthma and COPD, HDAC-2 activity is reduced, particularly in severe disease, and the degree of HDAC-2 reduction is inversely correlated with steroid resistance (11). Meanwhile, the removal of steroid therapy from patients with CF was found to have little effect on their inflammatory state, suggesting that these compounds have limited impact on the chronically inflamed CF lung or that these patients may be corticosteroid resistant (12).

The regulation of NF-κB signaling is an important step in controlling and terminating the innate and adaptive immune response. New therapies that target the cellular defects leading to chronic inflammatory lung disorders rather than conventional treatments that act on existing inflammation while seeking to prevent further exacerbations may provide an alternative treatment regime for many patients. The current review focuses on A20, an endogenous and inducible inhibitor of TNFR, TLR, and TCR activation of the NF-κB pathway and of other pathways involving TRAF6 (e.g., IL-17 signaling) (Figure 1).

### A20 AND INFLAMMATORY LUNG DISEASES

The zinc finger protein A20 (also known as TNF-α–induced protein 3) exerts its actions on the NF-κB pathway immediately after activation of the TNF-receptor and TLR/IL-1R pathways (13), and, in a negative feedback loop, the A20 gene is itself induced by NF-κB, resulting in the cyclical control of cellular responses to inflammation (14).

A20 has been implicated in the pathogenesis of various inflammatory diseases and is associated with the progression or development of diseases, including breast cancer, Crohn's disease, and diabetes mellitus, and was initially investigated as a tumor suppressor gene (15). In particular, A20 somatic mutations are reported in as many as 44% of Hodgkin lymphoma cells (15). Despite this, the role of A20 in inflammatory lung disease has remains poorly defined. Gon and colleagues found that A20 prevented the production of the proinflammatory cytokine IL-8 by negatively regulating TLR2- and TLR4-induced inflammation in healthy human bronchial airway epithelial cells (16). Follow-up studies examining the events behind recurrent airway obstruction in bronchial epithelial cells of affected horses show that A20 mRNA expression was negatively associated with the number of inflammatory cells present (17) and IL-8 produced (18). The authors concluded that increased TLR4 signaling in combination with deregulated A20 activity contributes to the pathogenesis of recurrent airway obstruction.

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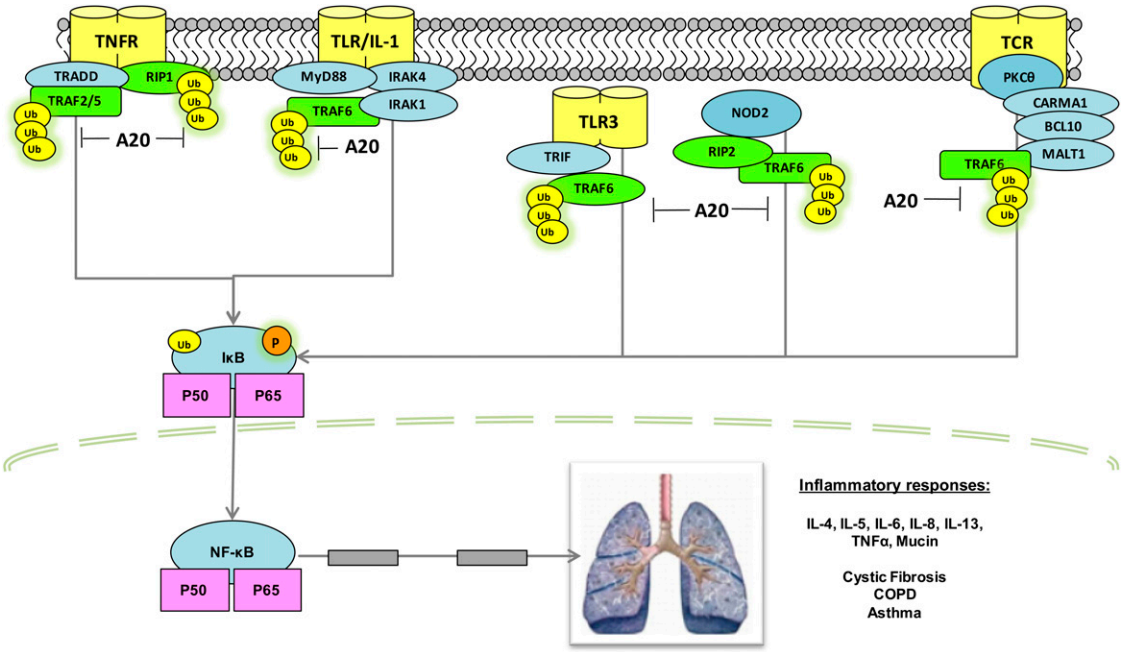


***Figure 1.*** A20 regulation of NF-κB signaling in inflammatory lung disease. Activation of the NF-κB pathways is tightly regulated. The process begins with receptor–ligand association with the cell membrane. This triggers binding and activation of membrane tethered proteins responsible for inflammatory signaling. A20 inhibits further downstream signaling at the sites shown. This inhibits the phosphorylation of IκB, thereby preventing the translocation of NF-κB subunits (i.e. p50 and p65) to the nucleus. This prevents the transcription of proinflammatory cytokines including IL-8, IL-13, and TNF-α.

The lungs of mice challenged with *Pseudomonas aeruginosa* showed enhanced expression of A20 mRNA 1 hour after infection (19), whereas A20 has been shown to inhibit NF-κB activation in bronchial epithelial cells after infection with H3N2 and H1N1 strains of influenza virus (20). These data suggest that A20 plays a protective role against bacterial and viral host infections. Consistent with this, A20-deficient cells are unable to terminate TNF- or LPS-induced NF-κB signaling (21). Adenoviral transfer of A20 has been shown to attenuate ovalbumin-induced allergic asthma in mice, inhibiting the production of mucin, the inflammatory cytokines IL-4, IL-5, IL-13, and TNF-α and preventing airway hyperresponsiveness (22). These findings highlight A20 as a potential therapeutic target for the treatment of chronic inflammatory lung diseases.

## A20 STRUCTURE AND BIOLOGICAL ACTIVITY

A20 is a 790–amino acid protein originally identified in human umbilical vein endothelial cells as a TNF-inducible gene (23). The original work by Dixit and colleagues reports that TNF-α induction of A20 is rapid and transient, with maximal expression evident after 1 hour (23). Similarly, LPS was found to upregulate the expression of A20 in endothelial cells (24). A20 induction was subsequently confirmed in numerous cell types and in response to various stimuli, including phorbol esters (24) and Epstein-Barr virus latent membrane protein 1 (25, 26), highlighting the protein as a central regulator of inflammation. A20 is found in low levels in unstimulated or unchallenged tissues but is inducible after bacterial or viral infections. Consistent with this, treatment with α-amanitin, a RNA polymerase II inhibitor, prevented LPS induction of A20 in enterocytes (27). However, A20 has also been shown to regulate TLR signaling initiated by commensal bacteria. After TNF-α treatment of mice specifically lacking enterocyte A20, commensal bacteria infiltrate the intestine, leading to systemic inflammation (28). In the absence of A20, the inflammatory signaling initiated by commensal bacteria cannot be down-regulated, leading to a breakdown of tolerance of the innate immune system to the commensal intestinal microflora (28, 29).

The amino acid sequence of A20 comprises an N-terminal ovarian tumor (OTU) domain (residues 1–370) and seven C-terminal A20-like zinc finger domains (30). For a schematic representation of the structure of A20, see the report by Verstrepen and colleagues (31) The OTU domain of A20 is characteristic of a superfamily of cysteine proteases, termed "otubains," that contain ubiquitin-associated and interacting motifs common to deubiquitylating enzymes (DUBs) (32). A20 does not possess general DUB activity but specifically targets polyubiquitinated substrates regulating NF-κB activation (33). The surface structure of A20 surrounding the active site is expansive, making it suitable for the cleavage of substrate-specific polyubiquitin chains. This structure is unique to A20, differing significantly from other members of the OTU superfamily, and may explain A20 specificity for NF-κB activators (33).

The C-terminal domain comprises six Cys-X4-Cys-X11-Cys-X2-Cys zinc finger motifs and a further single motif with the structure Cys-X2-Cys-X11-Cys-X2-Cys. A20 is not known to share this structure with other proteins and, as such, forms a new class of zinc finger protein (34). The ubiquitin ligase activity of A20 has been attributed to zinc finger 4 (31), whereas interactions with lysosomal compartments through the C-terminus have been shown to target associated signaling molecules for lysosomal degradation (35). Various classes of A20 binding proteins exist that facilitate the action of A20 and are reviewed in more detail by Beyaert (36) and Verstrepen (37).

## A20 REGULATION OF NF-κB SIGNALING

A20 regulation of NF-κB has received considerable attention. Binding of TNF-α to the receptor results in the recruitment of TNF-receptor–associated death domain, which further recruits receptor-interacting protein (RIP) or TNF-receptor associated factor (TRAF)2 (Figure 1). It is thought that A20 blocks further activation of the NF-κB pathway at this point by interfering with

RIP or TRAF signaling (38). Wertz and coworkers reported that A20 inhibited TNF-signaling through dual ubiquitin-editing actions on RIP1 with the N-terminal acting as an E3 ubiquitin ligase and the C-terminal exerting DUB activity. Ordinarily, RIP1 is bound to downstream signaling proteins such as NF-κB essential modifier, an IKK adaptor protein, resulting in phosphorylation of IκB and NF-κB translocation to the nucleus. The C-terminal zinc finger of A20 interrupts this sequence by removing the $Lys^{63}$ polyubiquitin chain of RIP1 and in so doing marks RIP1 for proteasomal degradation (30). In addition to removal of the polyubiquitin chain from RIP1, A20 acts as an E3 ligase and recruits an E2 protein ubiquitin-conjugating enzyme E2N to promote the polyubiquitination of RIP1 (30).

A20 null mice display signs of runting from 1 week and often do not survive beyond this point (39). Histological examination of these animals showed severe inflammation and tissue damage in multiple organs, and their spleens and livers showed increased numbers of activated macrophages, lymphocytes, and granulocytes even in the absence of TNF or LPS challenge (39). It was subsequently shown that lymphocyte activation was not essential to the inflammatory response in A20 null mice, indicating a role for A20 in preventing spontaneous innate immune cell–mediated inflammation and tissue destruction (39).

A recent genome-wide association study into Celiac disease in British, Irish, and Dutch populations identified two novel single nucleotide polymorphisms (SNPs) as risk regions, TNF-α–induced protein 3 (A20 at protein levels) and REL (NF-κB p50/p65) (40). However, there were no changes in gene expressions in biopsies and whole blood in these patients (40), suggesting that patients did not experience acute inflammation at the time of tissue and blood sampling. The study did not investigate the ubiquitination or deubiquitination activity of A20. However, the study suggests a role for heritable variations in A20, which can alter the NF-κB pathway and predispose individuals to inflammatory diseases such as Celiac disease (40).

Mice deficient in A20 are particularly sensitive to exogenous administration of TNF and LPS, suggesting that A20 plays a critical role in protection from chronic inflammation (39). Indeed, A20 is essential in promoting tolerance to LPS in enterocytes (27). However, A20 and TNF double-deficient mice developed spontaneous inflammation leading to premature death, indicating that in addition to regulating TNF-dependent mechanisms of NF-κB activation, A20 is also likely to be involved in TNF-independent activation of NF-κB (21).

TLR2 and TLR4 are PAMP recognition molecules, which, when activated at the cell membrane, recruit the adapter molecule myeloid differentiation primary response gene 88 (MyD88) and IL-1 receptor–associated kinase to the receptor complex (41–43). IL-1 receptor–associated kinase interacts with TRAF6, which activates NF-κB through stimulation of the IκB kinases IKKα and IKKβ. This triggers NF-κB translocation to the nucleus, where several genes responsible for inflammation and immune responses are transcribed (38). The role of A20 in TLR-induced NF-κB activation was recently investigated by Turer and colleagues using mice deficient in A20 and MyD88 (29). Although mice expressing MyD88, but not A20, displayed uncontrolled inflammatory responses and release of IL-1β and TNF-α, which led to premature death (29), A20/MyD88 double null mice no longer showed premature lethality and cachexia, suggesting that the spontaneous inflammation in A20-deficient mice can be mainly assigned to TLR signaling (29). The study also showed that A20 inhibits TRAF6 activity and may restrict TIR-domain–containing adapter-inducing IFN-β–dependent actions by modulating proteins in this signaling complex (29). TRAF6 has subsequently been shown to recruit A20 after 45 minutes of IL-1β treatment (13). A20 binds to TRAF6, which prevents TRAF6 interactions with Ubc13 (13), an E2-conjugating enzyme, which catalytically activates IKK and therefore NF-κB in response to IL-1β but not TNF-α (44). The A20-TRAF6 axis has been highlighted as an essential component in preventing LPS-stimulated NF-κB activation. Silencing of A20 was shown to restore TRAF6 action and NF-κB activation after LPS stimulation (45).

## ENDOGENOUS REGULATION OF A20 ACTIVITY

A20 is inducible via NF-κB and other stimuli, including LPS, TNF-α, and IL-1β (for a recent overview, *see* Ref. 31). Once induced, A20 activity is endogenously regulated by adapter proteins and E3 ligases. Shembade and colleagues report that A20 requires Ring finger protein 11 (RNF11) to down-regulate NF-κB and JNK activity in THP-1 cells (46). RNF11 is essential for inactivation of RIP1 by A20, and, together with the E3 ligase Itch and the adapter protein TAX1BP1, is a vital element of the "A20 ubiquitin-editing complex," which leads to transient activation of inflammation (46). Earlier work revealed that TAX1BP1 required zinc finger domains and an amino-acid motif termed PPXY (Pro-Pro-X-Try, where "X" is any amino acid) to interrupt NF-κB signaling. The zinc finger bound RIP1, whereas the PPXY motif recruited Itch, which was found to be essential for the termination of TNF-induced NF-κB signaling (47).

In addition to facilitating the action of A20, RNF11 was shown to independently inhibit NF-κB at the level of IKK (46). Moreover, RNF11 prevents the ubiquitination of RIP1 and TRAF6, which may prove crucial in the inhibition of TLR-, TCR-, and IL-1β–induced NF-κB signaling (46). Shembade and colleagues showed that A20, in a complex with RNF11, Itch, and TAX1BP1, promotes deubiquitination of TRAF6 and disrupts TRAF6 association with Ubc13 (or indeed the E2 enzyme UbcH5c), terminating IKK and NF-κB activation (13).

RNF11 also regulates the activity of TGF-β (48). Although TGF-β is frequently cited as having antiinflammatory effects on a variety of cell types and tissues (49), it may, under certain circumstances, induce NF-κB. There is a growing body of evidence showing that higher levels of TGF-β contribute to airways remodeling in chronic lung disorders and to the NF-κB–driven release of proinflammatory cytokines. In particular, in human airway cells, exogenous administration of TGF-β induces IL-8 release (50). In addition, Bonniaud and colleagues suggest that IL-1β–induced NF-κB–driven inflammation is likely linked to fibrotic progression in mouse lungs via TGF-β regulation of Smad signaling (51). In mice it has been shown that silencing of the TGF-β receptor is associated with an increase in NF-κB activation and a reduction in pancreatic fibrosis (52). It has also been shown that TGF-β directly activates NF-κB via TGF-β–activated kinase 1 (Figure 2) (53) and enhances bacterial-induced NF-κB by activating the acetylation of p65 (54). Although TGF-β may also be antiinflammatory, in the lung at least, the literature suggests that an increase in TGF-β levels or signaling leads predominantly to fibrotic progression and airways remodeling, which leads to an increase in the release of NF-κB–driven inflammatory cytokines.

TGF-β signaling may occur in a Smad-dependent or -independent manner. Although this is a complex process, a simplified and nonexhaustive schematic is given in Figure 2. RNF11 regulates Smad-dependent signaling by binding directly to Smad4, thereby activating TGF-β signaling, or may interact with Smurf2 to abolish Smurf2 ubiquitination of the TGF-β receptor (48). Alternatively, RNF11 may cooperate with Smurf2 to degrade AMSH, a deubiquinating enzyme that enhances TGF-β signaling (55). Although RNF11 may act on Smad-independent signaling through its interaction with

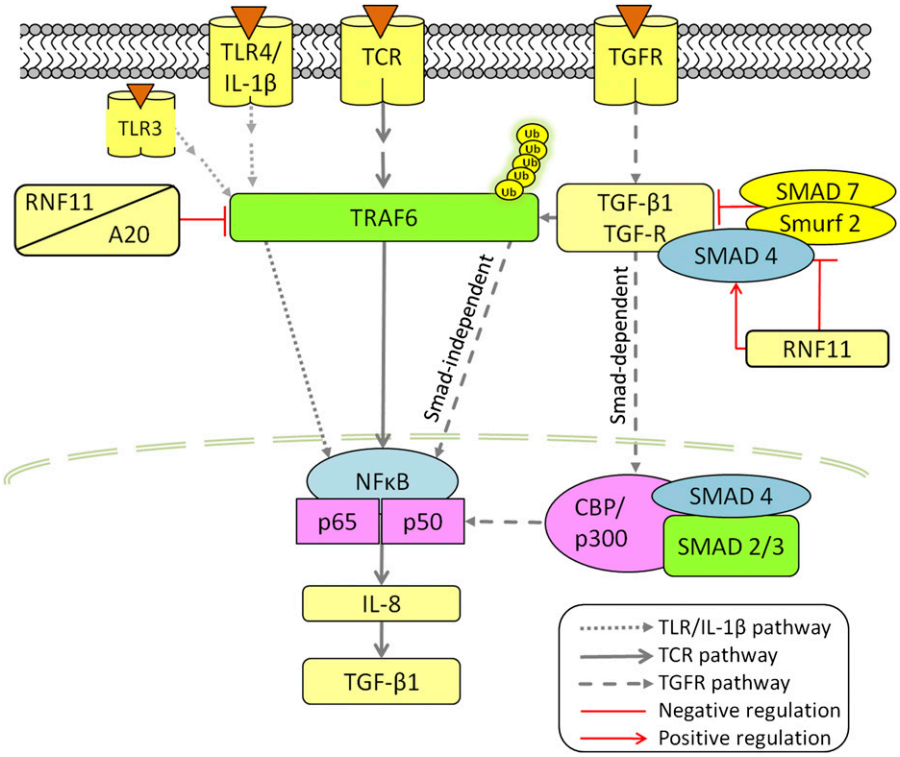


***Figure 2.*** Regulation of NF-κB–driven inflammatory signaling pathways by A20 and RNF11. A20, in a complex with RNF11, Itch, and TAX1BP1, promotes deubiquitination of TRAF6, thereby terminating IKK and NF-κB activation. The effective down-regulation of NF-κB and the ubiquitin-editing action of A20 is dependent on the presence of RNF11. RNF11 also modulates TGF-β signaling in several ways. RNF11 may bind directly to Smad4, which is responsible for activating TGF-β signaling. However, RNF11 may also interact with Smurf2 to abolish Smurf2 ubiquitination of the TGF-β receptor. Alternatively, RNF11 may cooperate with Smurf2 to degrade a deubiquinating enzyme that enhances TGF-β signaling.

TRAF6 and regulation of TRAF6 ubiquitination, the ubiquitination of TRAF results in activation of TGF-β–activated kinase 1 and NF-κB (56). These findings indicate a role for RNF11 in maintaining an appropriate balance between activation and silencing of TGF-β signaling.

Given these findings, the interaction of A20 with RNF11, or the "A20 ubiquitin editing complex," may provide an interesting target for the treatment of inflammatory lung diseases characterized by persistent activation of NF-κB and airway remodeling.

## A20 DYSREGULATION IN CHRONIC LUNG DISEASE

The described findings suggest that A20 and the "A20 ubiquitin editing complex" play an essential role in regulating and terminating NF-κB–induced inflammation. Investigations of A20 in chronic lung disorders have been sparse. However, the cell culture and animal model work described above highlight the potential of A20 in reducing inflammation and, potentially, in modulating remodeling. Indeed, constitutive overexpression of A20 in the murine heart reduces fibrotic transition and inflammation by blocking TGF-β1–dependent signaling, including the activation of Smad2, -3, and -4 (57). Therapies that target A20 or RNF11 action may also provide an alternative means of modulating NF-κB activation in individuals who are corticosteroid resistant.

It is well established that A20 is induced after bacterial or viral stimulation and NF-κB activation. However, work from our laboratory shows that A20 regulation is altered in chronic pulmonary disease (i.e., CF or asthma). Using airway epithelial cells from healthy control volunteers, we found maximal A20 induction 1 hour after LPS stimulation (58). This quickly returns to basal levels. These findings are consistent with the original observations of Dixit and colleagues who reported maximal A20 expression 1 hour after TNF challenge in human endothelial cells. However, in CF or asthmatic epithelial cells, maximal A20 expression is observed 4 hours after LPS stimulation, and by 12 or 24 hours after stimulation, A20 expression has fallen significantly below basal levels. The cause of this reduction is unclear; however, a reduction in A20 expression after LPS challenge to levels that are lower than basal expression only occurs in the diseased states (58).

Despite the induction of A20 in CF and asthmatic cells 4 hours after LPS challenge, our data show that A20 does not prevent or diminish NF-κB activation and subsequent release of proinflammatory cytokines (58). We investigated the colocalization of A20 with its target proteins and the expression of the other members of the A20 ubiquitin editing complex. These initial investigations indicate that in healthy non-CF cells (16HBE41o-) A20 colocalizes with TRAF6 but fails to do so in a CF bronchial epithelial cell line (CFBE41o-) (59). We also observed a time-dependent reduction in RNF11, Itch, and TAX1BP1 mRNA expression in CF cells. Therefore, it is unlikely that a simple induction of A20 is sufficient to restore the formation of the A20 ubiquitin editing complex in these cells. It is more likely that the up-regulation of all complex members is required to restore negative regulation of NF-κB by A20.

## A20: A NOVEL THERAPEUTIC TARGET FOR INFLAMMATORY LUNG DISEASE?

The role of A20 in down-regulating NF-κB–induced inflammation is well established. The reviewed body of research highlights A20 and the A20 "ubiquitin editing complex" as a potential therapeutic target to modulate chronic inflammatory diseases of the airways that are largely associated with exaggerated NF-κB activity. This approach may be hampered by mutations of the A20 gene, which have been associated with Crohn's disease and various lymphomas (60). However, work from our laboratory using an antibody against full-length human A20 show that it is induced in CF cells after stimulation (58). Modulation of A20 expression and activity may be able to

decelerate disease progression and could provide novel therapies for respiratory diseases. Therapies enhancing the expression or action of A20 may prove beneficial for a host of inflammatory lung disorders to promote the efficacy of existing therapies or to act directly on inflammatory signaling pathways. However, targeting negative regulators is difficult. Pharmaceutical induction or A20 or members of the A20 ubiquitin-editing complex is not possible to our knowledge and will require a significant financial and time investment. A potential means of restoring A20 regulation of NF-κB in chronic inflammatory lung disease may involve nonviral gene transfer of functional A20. However, this is limited by ethical issues.

Despite recent advances, understanding of the exact mechanism behind the dual ubiquitin activity of A20 remains fragmented. Shembade and colleagues recently showed that A20 may disrupt two key ubiquitin enzyme complexes (Ubc13 and UbcH5c) in TNFR and TLR signaling pathways and triggers their ubiquitination and subsequent proteasome-dependent degradation (13). More recently, Lin and colleagues suggested that the deubiquitination reaction did not simply transform higher-order to lower-order polyubiquitinated TRAF6 but rather removed the entire polyubiquitin chains by cleaving at the TRAF6–ubiquitin junction. This specificity may be due to specific recognition of polyubiquitinated substrates by A20 (33). Several SNPs have been identified across the A20 gene (61), which may support the hypothesis of a defect in the DUB function of A20 in chronic inflammatory disease. In light of potential therapeutic approaches, it may be interesting to examine the DUB activity of A20 in chronic lung disease and target the restoration of this function pharmaceutically.

There is a continuous and evolving interest in the role of A20 in inflammatory signaling, which is providing new results and summaries of its action (62, 63). Although direct inhibition of NF-κB may render the immune system unable to respond to pathogens, targeting individual TLRs or their signal transduction pathways has been suggested to provide a more specific way of treating inflammatory diseases without global suppression of the immune system (64). A better understanding of these processes and of the regulation of A20 itself will greatly improve the likelihood of A20-driven therapeutics for inflammatory lung diseases.

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