Targeting the phosphoinositide-3-kinase/protein kinase B pathway in airway innate immunity

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Abstract

The airway innate immune system maintains the first line of defense against respiratory infections. The airway epithelium and associated immune cells protect the respiratory system from inhaled foreign organisms. These cells sense pathogens via activation of receptors like toll-like receptors and taste family 2 receptors (T2Rs) and respond by producing antimicrobials, inflammatory cytokines, and chemokines. Coordinated regulation of fluid secretion and ciliary beating facilitates clearance of pathogens via mucociliary transport. Airway cells also secrete antimicrobial peptides and radicals to directly kill microorganisms and inactivate viruses. The phosphoinositide-3-kinase/protein kinase B (Akt) kinase pathway regulates multiple cellular targets that modulate cell survival and proliferation. Akt also regulates proteins involved in innate immune pathways. Akt phosphorylates endothelial nitric oxide synthase (eNOS) enzymes expressed in airway epithelial cells. Activation of eNOS can have anti-inflammatory, antibacterial, and anti-viral roles. Moreover, Akt can increase the activity of the transcription factor nuclear factor erythroid 2 related factor-2 (Nrf2) that protects cells from oxidative stress and may limit inflammation. In this review, we summarize the recent findings of non-cancerous functions of Akt signaling in airway innate host defense mechanisms, including an overview of several known downstream targets of Akt involved in innate immunity.

Key Words: Lung; Nitric oxide synthase; Nuclear factor erythroid 2 related factor-2; Respiratory infections; Cystic fibrosis
INTRODUCTION

The respiratory epithelium is the first-point of defense in the respiratory system, which is continuously exposed to a wide variety of inhaled pathogens. The classic role of the respiratory epithelium in airway defense is clearance of inhaled microbes via ciliary beating and mucociliary clearance (MCC). However, the respiratory epithelium is complex and contains not only epithelial cells but also resident macrophages, dendritic cells, and other leukocytes. All of these cell types sense infection through protein receptors like toll-like receptors (TLRs) and taste family 2 receptors (T2R) bitter taste receptors which activate production of a wide variety of antimicrobial peptides and radicals as well as inflammatory cytokines and chemokines. For example, stimulation of TLRs on airway epithelial cells regulates the expression of genes encoding multiple cytokines and antimicrobial peptides via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling. Activation of the innate immune system thus stimulates adaptive immunity and is also associated with apoptosis and other signal transduction pathways.

One understudied protein in airway innate immunity is protein kinase B, also known as Akt, a widely expressed serine/threonine kinase. Activation of Akt by upstream kinases such as phosphoinositide-3-kinase (PI3K) stimulates phosphorylation of downstream targets involved in cell proliferation, apoptosis, and/or cell growth depending on the signaling context. Akt has also recently been suggested to play a role in innate immunity by regulating immune cell development, survival, and function. Akt also has other targets important for innate immune responses of the airway epithelial cells themselves. Akt phosphorylates and activates endothelial (e) nitric oxide synthase (NOS), an enzyme that produces nitric oxide (NO). NO has many biological functions in the airway, including activating smooth muscle relaxation, increasing ciliary beat frequency, and having direct bactericidal and anti-viral effects. Another target activated by Akt is nuclear factor erythroid 2 related factor-2 (Nrf-2), a transcriptional factor that drives the expression of antioxidant genes that can protect against oxidative stress-induced by microbes as well as over-active inflammatory pathways.

Abnormalities of innate immunity are linked with numerous airway diseases including chronic rhinosinusitis and cystic fibrosis (CF). This review focuses on the Akt-dependent regulation of innate immunity in the lung and the potential role of Akt in protecting the airways against infection. Emphasis will be placed on novel directions for drug development. Furthermore, we summarize the current understanding of the role of the airway epithelium in Akt-dependent innate immunity and host defenses against bacterial infections in CF.

OVERVIEW OF THE PHOSPHOINOSITIDE-3-KINASE / PROTEIN KINASE B PATHWAY

Akt was identified almost 30 years ago by its homology to the v-akt retroviral oncogene and subsequently found to be a 57 kDa serine and threonine protein kinase that plays a pivotal role in cell proliferation, survival, and death. (Figure 1). There are three conserved mammalian Akt isoforms: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ). Some Akt isoform knockout studies conducted in mice have suggested there...
Figure 1  Protein kinase B signaling pathway. Stimulation of receptor tyrosine kinases or G-protein-coupled-receptors activates phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$) which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP$_2$) at the plasma membrane to generate PIP$_3$. Inactive Akt in the cytosol gets recruited to the plasma membrane where it gets phosphorylated at T308 in the kinase domain by phosphoinositide dependent kinase 1 and S473 in the regulatory domain by mTORC2 resulting in full activation; Signal termination is achieved by PTEN where it dephosphorylates PIP$_3$ to PIP$_2$; Additionally, PP2A and PHLPP have shown to regulate Akt kinase activity by direct dephosphorylation; Activation of Akt is known to regulate crucial transcription factors such as nuclear factor-kB (NF-kB), CREB, FOXO, and Nrf-2, each of which regulates a variety of target genes that regulate cell survival, proliferation, differentiation, migration, and metabolism; Akt is known to phosphorylate IkB kinase which phosphorylates IkB-a releasing NF-kB to translocate into the nucleus and transcribe genes; Activation of Akt might increase or suppress NF-kB regulated genes (IL-8, IL-18) depending on the stimulus. Both FOXO and CREB are known to regulate apoptosis and phosphorylation of these transcription factors by Akt, has shown to control cell survival. Nrf-2 activation by Akt increases the production of antioxidant genes such as HO-1 and NQO-1 that counteracts oxidative stress and inflammation. RTK: Receptor tyrosine kinase; GPCR: G-protein-coupled receptor; PI3K: Phosphoinositide-3-kinase; PIP$_2$: Phosphatidylinositol 4,5-bisphosphate; Akt: Protein kinase B; PDK1: Phosphoinositide dependent kinase 1; mTORC2: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; PHLPP: PH domain and leucine rich repeat protein phosphatases; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; CREB: cAMP response element binding protein; FOXO: Forkhead family of transcription factors; Nrf-2: Nuclear factor erythroid 2 related factor-2; PP2A: Protein phosphatase 2; HO-1: Heme oxygenase; NQO-1: NADPH quinone dehydrogenase 1.

may be specific functions for certain isoforms in growth, metabolism, and development, though this may be in large part due to differences in the tissue distributions of the isoforms$^{[15,16]}$. All three isoforms of Akt contain an N-terminal Pleckstrin Homology (PH) domain, a kinase domain, and a C-terminal regulatory domain. Activation of some receptor tyrosine kinases (RTKs) and/or some G-protein-coupled-receptors (GPCRs) by growth factors such as insulin-like growth factor-1 can activate the Akt pathway via plasma membrane recruitment and activation of class I PI3K isoforms$^{[15,17]}$. Activated PI3K can phosphorylate phosphatidylinositol 4,5-bisphosphate (PI$_4$, 5P$_2$) to generate phosphatidylinositolinositol (3,4,5)-trisphosphate (PIP$_3$). Inactive cytosolic Akt gets subsequently recruited to the membrane via the interaction of PIP$_3$ with the Akt PH domain. Akt can also be recruited to the membrane by PI3, 4P$_2$ produced by class II PI3K phosphorylation of PI4P$^{[18]}$. Akt localization to the plasma membrane induces conformational changes that allow phosphoinositide-dependent protein kinase-1 (PDK-1) to phosphorylate threonine (T) 308 within the activation loop of the Akt1 kinase domain (corresponding to T309 and T305 in Akt2 and Akt3, respectively) and mTOR Complex 2 (mTORC2) to phosphorylate serine (S) 473 within the hydrophobic C-terminal Akt regulatory domain (corresponding to S474 and S471 in Akt2 and Akt3, respectively)$^{[19]}$. Maximal activation of the kinase requires phosphorylation of both residues$^{[20]}$. Multiple other phosphorylation sites exist in Akt that can be phosphorylated by kinase complexes like mammalian target of rapamycin (mTORC2) (T450 in Akt1), CK2 (S129 in Akt1) (GSK-3a (T312 in Akt1), cyclin A-CDK2 (S477 and T479 in Akt1), though they how they modulate Akt activity is less clear$^{[1]}$.

Once Akt is activated, it can phosphorylate multiple downstream targets and/or redistribute to many cellular compartments, including the nucleus$^{[20]}$. A study done
with a genetically-encoded fluorescent biosensor for Akt activity showed that its activity in the nucleus was less rapid but more sustained compared with cytosolic Akt activity\(^1\), suggesting that Akt can be regulated differently in the cytosol vs nucleus or other organelles. Activation of Akt is also negatively regulated by phosphatases, including phosphatase and tensin homolog (PTEN) that antagonizes PI3K signaling by dephosphorylating PIP, and converting it back to PI4, 5P. Protein phosphatase 2 (PP2A) and PH domain and Leucine-rich repeat Protein Phosphatases (PHLP2) also reduce Akt activation by dephosphorylation at T308 and S473, respectively\(^19\).

Dysregulation of the PI3K/Akt pathway is associated with diabetes, cancer neurological disorders, and cardiovascular diseases\(^20\). Numerous studies have reported that components of the Akt signaling pathway are frequently mutated in multiple types of cancer; in some cases, this is associated with tumor aggressiveness\(^10\). In many tumors, Akt activity is upregulated via one or more mechanisms including loss of PTEN, mutations in the PI3K catalytic subunit, or loss of expression of phosphatases such as leucine rich repeat protein phosphatases (PHLPP)\(^1\)1 and PHLPP2 that dephosphorylate Akt\(^20\).

Beyond the well-known functions of Akt in cell proliferation and survival and, consequently, the pathophysiology of cancer, the Akt pathway has several roles in the immune system. Akt signaling is important for the maturation and survival of dedicated immune cells. Activation of the Akt pathway is a necessity for the development of human dendritic cells (DCs)\(^15\)2 and survival of activated B cells\(^23\). Furthermore, within airway epithelial cells, there are many known downstream targets of Akt that have important innate immune functions. The below sections will describe the innate immune system of the respiratory epithelium and importance of some known Akt targets in airway innate immunity.

**OVERVIEW OF RESPIRATORY INNATE IMMUNITY**

The innate immune system is the first line of defense against potentially dangerous microbes, and its main role is to recognize pathogens and initiate fast defensive responses. Because the human respiratory tracts are exposed to a myriad of pathogens daily, the immune system needs to recognize and initiate host defenses against these pathogens\(^11\). Akt may regulate multiple points of the airway innate immune system as well as the airway’s ability to detect pathogens.

**Mucociliary clearance: The physical defense of the airways**

The primary physical innate defense mechanism of the airways is mucociliary clearance (MCC) (Figure 2). The main functional components of MCC are mucus production by airway secretory cells\(^15\)2 and mucus transport by airway ciliated cells\(^13\)4-20. Cilia are specialized organelles lining airway epithelial cells. Mucus traps inhaled particulates and pathogens, and coordinated ciliary beating drives debris-laden mucus toward the pharynx, where it is swallowed or expectorated\(^27\). The airway surface liquid (ASL) is composed of the mucus layer that rides on top of the periciliary liquid (PCL) that surrounds the cilia. The composition of the PCL (volume, viscosity, and pH) mainly depends on epithelial ion channels\(^28\). Dysregulation of epithelial ion channels in CF is associated with increased mucus viscosity and PCL depletion\(^29\) that impairs MCC. Direct cilia motor protein defects in primary ciliary dyskinesia (PCD) also impair MCC. Both CF and PCD patients are more susceptible to airway infections\(^30\)-\(^33\), supporting the importance of effective MCC to airway defense. A reduction of ciliated cells is also observed in patients with inflammatory diseases like chronic rhinosinusitis\(^32\)-\(^35\) as well as after exposure to compounds in cigarette smoke\(^36\).

The normal mucus layer is composed of mainly water, mucins, proteins, lipids, and salts. However, the gel properties of mucus are produced by mucins, large cross-linked glycoproteins, including mucin 5AC (MUC5AC) produced by surface goblet cells\(^3\) and MUC5B produced by mucus cells of submucosal glands\(^2\). Elevated MUC5AC levels are linked to asthma and may contribute to airway obstruction\(^2\)-\(^8\). Akt has been suggested to be linked to MUC5AC production, though the data are conflicting. In human bronchial epithelial cells, direct inhibition of Akt upregulates MUC5AC production\(^10\). Activation of the PI3K/Akt pathway may also significantly reduced influenza-induced MUC5AC overproduction via negative cross-talk with the mitogen-activated protein kinase (MAPK) pathway\(^37\). In contrast, other studies showed that inhibition of Akt reduces MUC5AC levels\(^40\)-\(^41\). The discrepancies in these studies might be due to different experimental models used. However, because Akt...
Figure 2  Mucociliary clearance and innate immunity in the lung. A: Trachea, bronchi, and conducting bronchioles comprise the conducting zone of the airways; B: The conducting airway epithelium is lined with columnar motile ciliated cells and secretory goblet cells. Goblet cells secrete mucins like MUC5AC that polymerize to form mucus, which traps inhaled pathogens and debris; The mucus layer rides on top of a less viscous PCL composed of salt, water, and antimicrobials; Together, the mucus and PCL comprise the airway surface liquid; Coordinated metachronal beating of the motile cilia within the PCL layer pushes the sticky mucus layer up to respiratory tree to the oropharynx, where it is expectorated or swallowed; This process is termed MCC, and is the physical defense of the airway against infection; Epithelial cells also secrete antimicrobial peptide and radicals (NO, H₂O₂) to directly kill pathogens and produce cytokines and chemokines to activate inflammation; Shown is a representative diagram of tracheal or bronchial epithelium; In lower conducting airways (non-cartilaginous bronchioles < approximately 1 mm in diameter), secretory club cells (also known as bronchiolar exocrine cells) are found instead of goblet cells; As described in the text, there are several potential mechanisms by which protein kinase B may regulate MCC and other innate immune pathways. Figure made using Biorender.com. PCL: Periciliary layer; MCC: Mucociliary clearance; MUC5AC: Mucin 5AC.

may play a role in regulating MCC by controlling MUC5AC levels, Akt inhibitors or activators may be a novel therapeutic strategy to manipulate MUC5AC levels to reduce mucus hypersecretion in asthma or chronic obstructive pulmonary disease (COPD).

Immune surveillance receptors in the airway

Beyond the airway’s physical defenses, Akt is also involved in immune surveillance in the airway. The airway utilizes a gamut of receptors such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and T2R bitter receptors to detect invading pathogens[46]. TLRs are pattern recognition receptors (PRRs) initially discovered based on homology to Drosophila toll receptors[46]. TLRs recognize pathogen-associated microbial patterns (PAMPs) and activate signaling pathways that can lead to increased transcription of cytokines as well as production of antimicrobial peptides and iNOS[47]. Dysfunction of TLR signaling has been linked to COPD, acute lung injury, CF, and CRS[48-50].

In humans, 11 TLRs have been identified and are involved in the innate sensing of microbial products[50]. These TLRs are found in dedicated immune cells such as macrophages and dendritic cells. TLRs are also found in fibroblasts, epithelial cells in the lung, intestine, and many other cell types[51,52]. Primary and immortalized airway cells express TLRs 2 through 10 at varying expression levels[53,54]. Lung epithelial cell TLRs respond to a variety of factors such as Pseudomonas aeruginosa flagellin (via TLR5), gram-negative bacterial lipopolysaccharide (LPS; via TLR4), unmethylated CpG from prokaryotic DNA (via TLR9), bacterial peptidoglycan (via TLR2), gram-positive bacterial lipoteichoic acid (via TLR2), viral double-stranded RNA (via TLR3), and fungal zymosan/beta-glucan (via TLR2)[43,55].

The broad principles of TLR signaling are already described by several excellent reviews[42,56]. Briefly, binding of PAMPs to TLRs activate their intracellular Toll/IL-1 receptor (TIR) domains[57] and recruits one or more TIR domain-containing adaptor proteins, leading to activation of downstream effectors such as nuclear factor-κB (NF-κB) and the transcription factor interferon regulatory factor (IRF) family.
proteins, including myeloid differentiation primary response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM)\(^8\). Signaling then proceeds through a series of adapter proteins. Association of MyD88 recruits IL-1R-associated kinase (IRAK)\(^8\) through interactions of N-terminal death domains in both proteins\(^8\). Phosphorylation of IRAK activates tumor necrosis factor receptor-associated factor-6 (TRAF6) which in turn activates transcription factors such as NF-κB and JNK to promote the production of cytokines or initiate apoptosis signaling pathways, respectively\(^8\). Some TLRs, like TLR3, can also activate MyD88-independent signaling pathways leading to NF-κB activation\(^8\).

In epithelial and immune cells, experimental studies have identified both positive and negative cross-talk between TLR activation and the PI3K/Akt pathway\(^8\)\(^\text{-}\)\(^8\). It is not yet fully understood how Akt is linked to TLR signaling, and these links maybe cell type-dependent or even TLR-isotype-dependent. PI3K, upstream of Akt, is often activated by TLRs in many cells\(^8\), with Akt phosphorylation peaking at approximately 20 min and decreasing by approximately 1 h after stimulation\(^8\). Activation of Akt via TLR stimulation may increase NF-κB signaling and cytokine expression in macrophages\(^8\), while other studies showed that the PI3K/Akt pathway suppresses TLR-induced cytokine secretion in monocytes via inhibition of NF-κB\(^8\)\(^\text{-}\)\(^8\). One study suggested that binding of vasoactive intestinal peptide (VIP) to GPCRs reduced TLR4 expression via Akt in macrophages and regulatory T cells\(^8\)\(^\text{-}\)\(^8\). Another group demonstrated the activation of PI3K/Akt after stimulation of TLR4 is crucial for β cell survival\(^8\). The role of Akt in airway TLR signaling is relatively unexplored, but data suggest that pharmacological manipulation of PI3K or Akt signaling may be a mechanism by which NF-κB activity could be controlled during bacterial or viral infection and the resulting activation of TLRs in the airway.

Cross-talk between TLRs and Akt maybe particularly important during cellular hyperoxia in the lung. Oxygen therapy is commonly used to reduce tissue hypoxia in patients with pulmonary disease. However, hyperoxia can induce lung damage that may be tied to a reduction of Akt signaling. Expression of a constitutively active form of Akt protected mouse lungs from hyperoxic injury\(^8\). In a rat model of bronchopulmonary dysplasia (BPD), exposure of neonatal lungs to high (95%) oxygen reduced the expression of Akt, while overexpression of Akt was protective against lung damage\(^8\). TLR4-deficient mice showed increased lung injury, higher mortality, and reduced levels of phospho (p)-Akt after hyperoxia. Expression of anti-apoptotic BCL-2 and activation of p-Akt significantly attenuated hyperoxia-induced lung injury in these TLR4-deficient mice\(^8\). Thus, activating the Akt pathway with receptor ligands or direct activators like SC-79\(^8\) may be useful for treatment of lung injury during hyperoxia.

Other PRRs exist beyond TLRs. NLRs are PRRs that activate signaling pathways leading to activation of the inflammasome. Unlike the transmembrane TLRs, NLRs are cytosolic. NLRs can respond to microbial pathogens and stimulate the production of cytokines. Depending on the domains that are expressed, NLRs can be categorized as NOD receptors, NLRP, NLRC, or NLRB, and have been extensively reviewed\(^8\). NOD1 and NOD2 are expressed in lung epithelial cells, endothelial cells, alveolar macrophages, and airway smooth muscle cells\(^8\). Binding of NOD1 and NOD2 to secreted bacterial moieties results in activation of NF-κB, and polymorphisms of these receptors may increase susceptibility to respiratory infections\(^8\). NLRP3 may play a major role in recruiting neutrophils and dendritic cells during *Mycoplasma pneumoniae* lung infection in mice\(^8\). Because NLRs are relatively novel compared with TLRs, the knowledge of NLRs/Akt/PI3K/NF-κB in the lung immunity field is still rapidly developing.

Two decades ago, the GPCRs for bitter taste (known as taste family 2 receptors or T2Rs) were discovered in taste bud type II cells on the tongue\(^8\). There are 25 T2R isoforms in humans\(^8\)\(^,\)\(^8\) that detect bitter compounds in food. However, in recent years, the discovery of the T2Rs in extraoral tissues has suggested other roles for these receptors beyond taste, including immune surveillance\(^8\). A variety of bitter receptors are expressed in the motile cilia in human airway epithelial cells\(^8\) and macrophages\(^8\) which are stimulated by bitter molecules such as denatonium benzoate\(^8\), thujone from the wormwood plant\(^8\), sodium thiocyanate\(^8\), phenylthiocarbamide (PTC)\(^8\), and bitter plant flavonoids\(^8\). These T2Rs also recognize gram-negative bacterial products such as acyl-homoserine lactone (AHL)\(^8\) and quinolone\(^8\) quorum-sensing molecules, suggesting they may play a role in sensing developing biofilms.

Stimulation of bitter receptors in sinonasal epithelial cell cilia activates Ca\(^2+\)-dependent nitric oxide (NO) production which is bactericidal\(^8\). Additionally, NO can act as a second messenger to stimulate soluble guanylyl cyclase (sGC) and protein
kinase G (PKG) to phosphorylate downstream effector proteins within the cilia and increase the ciliary beat frequency and thereby MCC\(^{10}\). One T2R isoform expressed in respiratory cilia is T2R38. Common polymorphisms in the TASSR38 gene that render the T2R38 receptor nonfunctional are associated with increased susceptibility to upper respiratory infection\(^{11}\), susceptibility to chronic rhinosinusitis\(^{89,93}\), and surgical outcomes after functional endoscopic sinus surgery\(^{10}\).

T2Rs also play other roles in the airway. A different subset of T2R isoforms in non-ciliated solitary chemosensory cells (SCCs), sometimes called tuft cells\(^{10,11}\), leads to the propagation of Ca\(^{2+}\) to neighboring ciliated cells via gap junctions, triggering the neighboring cells to release anti-microbial peptides such as beta-defensin 1 and 2\(^{12,13}\), which can permeabilize fungi and both gram-positive and negative bacteria\(^{14}\). Moreover, in mouse asthma models, bitter receptor agonists are effective in reducing airway smooth muscle contraction by modulating Ca\(^{2+}\) signaling\(^{15,16}\).

Such studies of primary cells in vitro and patients in vivo suggest that T2Rs may contribute to the recognition of bacterial products similarly to TLR signaling\(^{17}\). Since T2Rs activate endothelial nitric oxide synthase (eNOS) to acutely produce NO in ciliated cells, targeting this pathway through Akt, which phosphorylates and activates eNOS\(^{18,19}\) independently of Ca\(^{2+}\), as described below, is possibly a way to activate these innate immune responses in patients with polymorphisms that render specific T2Rs like T2R38 nonfunctional. Akt also has many other downstream targets, including Nrf-2\(^{10,11}\) that play a role in the above innate immune processes. Several of these targets are reviewed below.

### DOWNSTREAM TARGETS OF AKT INVOLVED IN INNATE IMMUNITY AND INFLAMMATION

#### Nitric oxide synthases

Nitric oxide synthase (NOS) enzymes catalyze the production of NO. L-arginine and NAD(P)H are converted to NO, NAD(P), and L-citrulline, requiring tetrahydrobiopterin (BH\(_4\)) as a co-factor. NO is an important physiological signaling molecule that regulates processes like pulmonary vascular tone. NO also stimulates sGC to produce cyclic GMP (cGMP), which then activates PKG\(^{20}\). PKG increases ciliary beat frequency to enhance MCC of detrimental pathogens, as described above\(^{21}\). Enhancing airway cell NO production by addition of L-arginine, adding artificial NO donors, introducing cell-permeant cGMP analogues, or using cGMP phosphodiesterase inhibitors to increase cGMP can all enhance ciliary beat frequency in rat, mouse, bovine, and human airway ciliated cells\(^{22,23,24}\); conversely, inhibition of NOS reduces the ciliary beat frequency in cultured ciliated airway epithelial cells\(^{25}\).

There are three mammalian NOS isoforms; endothelial (eNOS, or NOS3), neuronal (nNOS or NOS1), and inducible (iNOS or NOS2) isoforms, named after the tissue where they were originally discovered. The main NOS isoform in neurons is nNOS\(^{26}\), but nNOS has also been detected in epithelial cells of various organs, pancreatic islets, and vascular smooth muscle, and exocrine acinar cells\(^{27}\). The dominant isoform in endothelial cells that maintains vascular tone and blood pressure is eNOS\(^{28}\). The airway epithelium generally expresses eNOS at baseline, while iNOS expression can be up-regulated during inflammation\(^{29,30,31,32,33}\). Pollutants and cigarette smoke can downregulate eNOS expression and subsequently reduce the production of NO\(^{34}\).

Both eNOS (NOS3) and nNOS (NOS1) are generally constitutively expressed and are regulated acutely via binding of Ca\(^{2+}\)-bound calmodulin as well as phosphorylation, described below. Activation of airway epithelial cells or immune cells by inflammatory mediators can cause transcription of iNOS (NOS2) via NFkB\(^{129,130}\). Like eNOS and nNOS, iNOS requires Ca\(^{2+}\) for function, but the affinity is so high that iNOS is maximally activated at resting Ca\(^{2+}\) levels, and iNOS can output high levels of NO in the cell microenvironment, ranging from 10 nmol/L to \(\mu\)mol/L amounts\(^{17}\). These high levels of NO can be involved in immune cell killing of bacteria\(^{13}\). In contrast, nNOS and eNOS produce lower levels of NO often associated with cellular signaling pathways that regulate a variety of physiological endpoints like ciliary beating and vascular tone, as described above, and also macrophage phagocytosis\(^{14}\). However, Ca\(^{2+}\)-dependent activation of eNOS in sinonasal airway epithelial cells can directly kill bacteria like P. aeruginosa in the airway surface liquid\(^{12,13}\). The sinuses are thought to be sites of high NO production, important for immune function in the airways, and reduced fractional exhaled NO (FeNO) is correlated with several airway diseases\(^{35,36}\).

Beyond Ca\(^{2+}\)-calmodulin binding to eNOS, it can also be activated by Ca\(^{2+}\)-
independent mechanisms. Akt is an important regulator of eNOS function. Akt increases eNOS-mediated NO production by phosphorylation at Ser-1177 in humans and S1176 in mice\[^{127}\]. Akt inhibitors such as wortmannin and LY294002 reduce NO production and PKG activity in platelets\[^{128}\], while Akt co-immunoprecipitates with eNOS, suggesting the two proteins physically interact\[^{129}\]. In mice in vivo, defective angiogenesis in Akt1 knockout mice can be rescued by a phospho-mimetic (SI176D) mutation in eNOS rendering the enzyme constitutively active\[^{130}\]. This demonstrates the physiological importance of the regulation of eNOS by Akt.

Other proteins such as heat shock protein 90 (HSP90) can also associate with eNOS and modify its activity. Biochemical studies have shown a synergetic activation of eNOS by HSP90 and Akt in a calcium-independent manner in response to physiological agonist like insulin\[^{128-130}\]. However, HSP90 also enhances Ca\(^2+\)-calmodulin activation of eNOS\[^{131}\].

An important role for eNOS has been demonstrated in various models of lung injury. In the lungs of male C57BL/J6 wild-type or eNOS knockout mice exposed to mechanical ventilation, reduced phospho-Akt, phospho-eNOS, and NO leads to increased epithelial permeability. The authors concluded that the PI3K/Akt/eNOS pathway exerts significant protective effects against ventilation-induced lung injury\[^{132}\]. Production of NO by eNOS may also be important for protection against neonatal hypoxia in mice\[^{133}\]. Thus, data presented above suggest that activating eNOS by directly targeting PI3K/Akt signaling may have several beneficial effects in lung disease, including protection against bacterial infections, reduced damage during mechanical ventilation, or reduced inflammation.

**The nuclear factor erythroid 2 related factor-2 transcription factor**

Another downstream target of Akt is Nrf-2, a transcription factor that serves as a master regulator of cellular responses against oxidative stress. Nrf-2 belongs to the cap “n” collar (CNC) family of transcription factors. Nrf-2 counteracts oxidative stress and inflammation by initiating transcription of genes encoding antioxidant proteins such as NADPH: quinone oxidoreductase (NQO1) and heme oxygenase (HO-1)\[^{134}\]. Nrf-2 binds to a specific approximately 41 base pair consensus enhancer sequence known as the antioxidant response element (ARE) to promote transcription of antioxidant and other genes\[^{135}\]. Nrf-2 is regulated by Kelch-like ECH-associated protein-1 (Keap1), which binds to and sequesters Nrf-2 in the cytosol and targets Nrf-2 for ubiquitination and proteasomal degradation\[^{136}\]. Nrf-2 is rapidly turned over, with a half-life of approximately 20 min in many cells\[^{137,138}\]. Keap-1 facilitates the interaction of Nrf-2 with its E3 ubiquitin ligase. However, when the Nrf-2-Keap1 interaction is disrupted, Nrf-2 can escape ubiquitination and translocate to the nucleus\[^{139}\]. Disruption of the Nrf-2-Keap1 interaction can occur by oxidative modification of cysteine thiols on Keap-1, binding of heavy metal oxidants like Cd\(^{2+}\) or Cr\(^{6+}\) to Keap-1, or activation of Akt signaling\[^{140,139}\].

Activation of antioxidant gene transcription by Nrf-2 may be protective in multiple tissues against injury and inflammation in a variety of conditions such as autoimmune and neurodegenerative diseases\[^{141,142}\]. Nrf-2 induction may counterbalance excess mitochondrial production of ROS, and Nrf-2 levels may be decreased in mitochondria-related neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases\[^{143,144}\]. Nrf-2 activators are in clinical development for cancer, although, due to Nrf-2’s role in promoting cell survival, there is controversy over whether activating or inhibiting Nrf-2 will be useful in different types of cancer\[^{145,146}\]. In head and neck cancer, high levels of Nrf-2 may be associated with poorer patient outcomes\[^{147}\]. Multiple mechanisms for aberrant activation of Nrf-2 in cancer have been reported, including Keap-1 mutations, epigenetic factors, and genetic changes\[^{148}\]. Thus, while Nrf-2 is cytoprotective against oxidative stress, hyperactive Nrf-2 may be deleterious in some cancers.

Induction of Nrf-2 reduces the expression of pro-inflammatory cytokines such as IL6, IL1β, and COX2 in mice exposed to UV radiation; the same study showed that in healthy human subjects, Nrf-2 activator sulforaphane reduced solar-stimulated UV radiation-induced skin erythema\[^{147}\]. Nrf-2 may interfere with lipopolysaccharide (LPS)-induced production of IL6 and IL1β in murine macrophages\[^{148}\]. In the lung specifically, Nrf-2 activation may attenuate airway inflammation linked to allergy\[^{149,150}\] or COPD and emphysema\[^{151-153}\]. In most studies using mouse models of airway disease, the deletion of Nrf-2 results in increased inflammation and injury. Nrf-2 deficient mice are more susceptible to cigarette-smoke induced emphysema\[^{154,155}\], and when cigarette-smoke-exposed Nrf-2 deficient and Wt. mice were exposed to influenza virus, Nrf-2-deficient mice exhibited higher mortality\[^{156}\]. Nrf-2 may also directly modulate TLR4 signaling\[^{157}\], though most studies of inflammation point to downstream effects of Nrf-2 on NF-kB-induced cytokine secretion. Nrf-2 knockout
mice exhibit more lung inflammation in response to LPS or TNFα compared with WT mice\cite{184,185}, likely via enhanced NF-κB signaling.

Nrf-2 is also likely important during oxidative stress induced by airway hypoxia or hyperoxia. Nrf-2-dependent reduction of alveolar growth inhibition caused by hyperoxia increases survival in newborn mice\cite{192}. Pharmacological inhibition of Akt resulted in higher levels of inflammation and lower expression levels of antioxidant genes in mice exposed to hyperoxia, likely via reduced Nrf-2 signaling\cite{201}. However, this study also found that PI3K/Akt signaling promoted inflammation after hyperoxic injury in a Nrf-2-independent manner\cite{204}. These studies suggest that activating PI3K/Akt/Nrf-2 signaling may reduce inflammation in lung diseases where oxidative stress is an important component of the pathophysiology, though more work is needed to understand the relationship of Akt and Nrf-2 to initial injury and subsequent sustained inflammatory responses after injury.

Many experimental studies in the airway have focused on the beneficial effects of Nrf-2 activation against commonly seen oxidative stressors in lung diseases. Activation of Nrf-2 (either via endogenous receptors, overexpression, or activators like curcumin or sulforaphane) is protective against oxidative stress-induced lung damage caused by exposure to compounds in cigarette smoke\cite{174,118} or H₂O₂\cite{201}. While Nrf-2 activators have shown benefit in animal models, we hypothesize that activation of PI3K/Akt/Nrf-2 signaling may also be beneficial and requires more investigation, as it would combine Nrf-2 activation with the activation of other beneficial pathways, like eNOS.

Only a limited number of studies exist on protective effects of Akt-dependent Nrf-2 activation. In prostate cancer, increased Akt and Nrf-2 activity correlated with cell survival\cite{205}. Another study reported that raw garlic can reduce cardiac hypertrophy in fructose-fed type 2 diabetic mice through activation of the PI3K/Akt pathway; this study showed that activation of Akt increased Nrf-2 activity that protected mouse hearts from oxidative stress\cite{206}. Similarly, overexpression of constitutively active Akt increased Nrf-2 activation in retinal pigment epithelium\cite{207}. In this study, both the induced levels of Nrf-2 and basal levels were reduced by PI3K inhibitors wortmannin and LY294002, confirming the Nrf-2 is activated downstream of PI3K/Akt\cite{207}.

**Inositol trisphosphate receptors**

Inositol trisphosphate receptors (IP₃Rs) are endoplasmic reticulum (ER)-resident Ca²⁺ channels that contribute to Ca²⁺ release downstream of GPCR activation and other stimuli that activate phospholipase C\cite{208}. Phospholipase C catalyzes hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) to release inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. While diacylglycerol can activate protein kinase C, IP₃ can bind to the IP₃ receptor and sensitize it to resting cytosolic Ca²⁺ levels to cause the channel to open and promote Ca²⁺ release from endoplasmic reticulum stores\cite{208}. The IP₃ sensitivity and Ca²⁺ release activity of IP₃Rs can be regulated by IP₃ phosphorylation by multiple kinases\cite{209}. A consensus motif for Akt phosphorylation is contained within the C-terminal tail of all three IP₃R isoforms\cite{209}.

Phosphorylation of IP₃Rs by Akt has been suggested to reduce Ca²⁺ efflux from the ER in response to apoptotic stimuli, thus protecting cells from apoptosis\cite{210,211}. The activity of Akt2 in lymphocytes can reduce the duration of Ca²⁺ signaling and reduce activation of the NFAT transcription factor\cite{212,213}. However, one study suggested that the effects of Akt on IP₃Rs is specific to the type III IP₃R, while Akt activation does not affect type I IP₃R\cite{214}. As cytokine secretion can also be driven by Ca²⁺, reduction of Ca²⁺ signaling may reduce inflammation. However, the ability of Akt to inhibit apoptosis or inflammation may depend on the predominant subtype of IP₃R expressed in a specific cell type.

However, further research needs to be done on the role of Akt in Ca²⁺ release in airway cells. In some cells, Akt signaling may enhance Ca²⁺ release. In neurons, progesterone was shown to potentiate IP₃R-dependent Ca²⁺ release via Akt signaling\cite{215,216}. Akt activity may also regulate the expression of IP₃Rs through multiple pathways. Akt2 activation of the ETS1 transcription factor may increase the expression of type II IP₃R expression in dendritic cells\cite{217}. Additionally, Nrf-2 was shown to bind to the promoter of the gene encoding the type III IP₃R and reduce its expression in cholangiocytes, resulting in reduced Ca²⁺ signaling and reduced secretion from the bile duct\cite{218}. Pharmacological targeting of the Akt pathway may modulate airway cell cytokine release and apoptosis through alteration of Ca²⁺ signaling, but it remains to be determined if inhibition or activation of Akt would be more beneficial.
POTENTIAL ROLES OF THE PHOSPHOINOSITIDE-3-KINASE/AKT PATHWAY IN CYSTIC FIBROSIS

Cystic fibrosis (CF) is an autosomal recessive disease caused by nearly 2000 different known mutations in the CFTR gene, which encodes the CF transmembrane conductance regulator (CFTR) protein. Although the life expectancy of CF patients is increasing with current small molecule therapies\cite{189,190}, CF affects approximately 75000 people in North America, Australia, and Europe\cite{189,190}. The CFTR protein is expressed in the apical membranes of airway surface epithelial cells\cite{189,190}, airway submucosal gland serous cells\cite{21,192,199}, and a recently discovered rare cell type termed the ionocyte\cite{194,199}. CFTR functions as chloride (Cl\(^-\)) and bicarbonate (HCO\(_3\)-) anion channel\cite{190} to regulate salt and fluid homeostasis and control the volume and pH of the airway surface liquid\cite{189}. Dehydration of the ASL caused by defective CFTR function leads to thickened mucus that impairs mucociliary clearance and increases susceptibility to respiratory pathogens\cite{21}, particularly the gram-negative opportunistic bacterium *P. aeruginosa*\cite{190}. Respiratory failure is responsible for > 95% of CF patient deaths\cite{190}. However, the reduced flux of Cl\(^-\) and HCO\(_3\)- ions through CFTR also affects multiple other organs where CFTR is expressed, including the exocrine pancreas, male reproductive tract, and sweat glands\cite{21}.

CFTR belongs to ATP binding cassette (ABC) superfamily of proteins and consists of two membrane-spanning domains (MSD), two nucleotide-binding domains (NBD), and a regulatory (R) domain\cite{189,190}. The R domain consists of charged amino acids and several sites for phosphorylation by cAMP-dependent protein kinase A (PKA) as well as protein kinase C (PKC)\cite{200}. Phosphorylation of the R domain enhances the association of adenosine triphosphate (ATP) to the NBDs, allowing a conformational change that results in the opening of the CFTR channel pore\cite{200}. Subsequent hydrolysis of the ATP leads to channel closing\cite{200}. Maturation of CFTR protein requires proper domain folding, glycosylation, and trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus and eventually the plasma membrane. Dysregulation of any point in this complex multiple-step process can create a non-functional protein\cite{200}. The most common mutation occurring in CF patients is the deletion of phenylalanine at position 508 (termed ΔF508 or F508del)\cite{200}.

In the CF lung, numerous studies have suggested the thickened mucus that is the hallmark of CF is accompanied by increased inflammation. The accumulation of neutrophils may increase inflammation and damage bronchial walls\cite{190}, while increased levels of pro-inflammatory cytokines and chemokines such as IL8 and TNF-α may also contribute to the destruction of lung tissue\cite{190,200}. However, loss of CFTR may also confer hyper-inflammatory cellular properties, suggesting intrinsic cellular signaling defects caused by loss of CFTR function beyond the inflammation secondary to defective mucociliary clearance and bacterial infection\cite{191,201,202,203}. CFTR itself has been linked to TLR4\cite{202,203} and Akt\cite{204,205} signaling via its proposed role as a signaling scaffold\cite{206,207}. Thus, defective CFTR function may likely result in dysregulation of innate immunity beyond just loss of MCC\cite{208}. All of these mechanisms may contribute to airflow obstruction, increased risk for bacterial infection, and damage to the microenvironment of the lung. It is not yet fully clear how small molecule CFTR corrector and potentiator therapies may suppress hyper-inflammation phenotypes in CF lungs\cite{209}.

Exhaled air from CF patients also contains less NO compared to non-CF individuals, possibly via decreased production of NO, increased metabolism of NO, downregulation of NOS enzymes, or polymorphisms in NOS genes\cite{210,211,212,213}. Small molecule CFTR modulators have been shown to restore airway NO production, and the fraction of exhaled NO (F\(_\text{E}\text{NO}\)) has been proposed to be a biomarker of pharmacological restoration of CFTR\cite{214,215,216}. Moreover, boosting NO signaling may also increase the effect of corrector/potentiator modulator therapies\cite{217}, suggesting multiple levels of feedback may exist between CFTR and the NO signaling pathway.

As described above, eNOS is one target of Akt. Totani and colleagues showed that inhibition of CFTR in pulmonary endothelial cells reduced NO levels via reducing levels of activated phosphorylated Akt and activated phosphorylated eNOS\cite{218}. This was associated with an increase in IL8 levels. In mice, CFTR knockout macrophages had a significant reduction of Akt phosphorylation at S473 compared with control mice; this same study showed Celecoxib, an FDA-approved COX-2 inhibitor for osteoarthritis, activated the PI3K/Akt pathway and reduced inflammation in this mouse model\cite{219}. Thus, directly targeting Akt using small molecule activators or activating upstream PI3K may enhance NO production in CF lungs and alleviate inflammation. It may also have anti-bacterial effects similar to the activation of T2R
bitter taste receptors, which drive eNOS-mediated NO production via Ca$^{2+}$ rather than Akt. Of note, P. aeruginosa, the most common pathogen in CF lungs, is more susceptible to NO-induced killing than some other airway bacteria like Staphylococcus aureus\cite{104}. A lack of efficient NO production in CF cells, possibly due to intrinsic defects in Akt signaling, may partly contribute to why these bacteria are so prevalent in CF lungs while almost never causing infection in non-CF patients unless non-CF patients are otherwise immunocompromised\cite{109}.

NO itself has been suggested to activate CFTR via PKG in some studies\cite{129,215,217}, while NO has also been reported in other studies to have no effect on CFTR\cite{216}, inhibit CFTR trafficking and/or activation\cite{218,219}, or activate non-CFTR Cl$^{-}$ currents\cite{220}. Part of the discrepancy may be that most studies use different NO donor compounds at different concentrations, as well as occasionally more physiological ways to induce NO production (e.g., receptor activation). While no one has thoroughly examined the activation of CFTR downstream of specific Akt activation in the airway, targeting Akt would increase NO production to a more physiological level than NO donor compounds. Akt activation would stimulate endogenous eNOS, the major NOS isotype in uninflamed airway cells\cite{110}.

As indicated earlier, Nrf-2 is also a downstream target of Akt that plays a cytoprotective role against oxidative stress. Nrf-2 may convey resistance to pyocyanin, a bacterial product from P. aeruginosa that causes oxidative stress. The PI3K/Akt pathway is activated in lung epithelial cells during pyocyanin exposure, and the increased transcription of antioxidants may protect these cells from death\cite{221}. It has been suggested that defective Nrf-2 in CF cells causes enhanced oxidative stress that increases inflammatory cytokine production\cite{222}, and Nrf-2 function is restored when mutant CFTR function is enhanced by small molecule therapeutics\cite{223}. Alterations of Nrf-2 signaling in CF may also be tied to alterations in cAMP signaling and the CREB binding protein\cite{224}. Boosting Nrf-2 function by targeting the PI3K/Akt pathway may have beneficial effects in CF lungs.

Furthermore, Nrf-2 may also regulate expression of CFTR itself\cite{225}. We hypothesize that a direct Nrf-2 activator such as curcumin\cite{226}, dimethyl fumarate\cite{227} or andrographolide\cite{228} and/or activating Nrf-2 via Akt may be useful in combination with small molecule CFTR correctors and potentiators\cite{229}. Such a strategy may further increase the number of functional CFTR channels at the plasma membrane by boosting CFTR gene transcription. This may be useful in patients where specific CFTR mutations reduce the efficiency of small molecule correction or potentiation.

**CONCLUSION**

The respiratory epithelia are in constant contact with bacteria, viruses, and pathogens during every breath. Airway innate immunity is the first line of host defense against these challenges\cite{111}. Some of the strategies of the innate immune system of the airways include mucociliary-clearance, antimicrobial peptide secretion, NO production, cytokine secretion, and antioxidant gene production (Figure 3)\cite{229}. PI3K/Akt signaling is one of the major signaling pathways regulating multiple components of these processes. Akt signaling may be altered in airway diseases like CF. Together, the above studies discussed in this review suggest that therapeutic strategies to enhance the PI3K/Akt pathway and increase NO production, boost antioxidant transcription via Nrf-2, or activate other anti-inflammatory pathways might be particularly beneficial in CF patients. These strategies may also benefit patients with other inflammatory airway diseases like CRS, asthma, and/or COPD. Because pharmacological tools to inhibit PI3K\cite{230,231}, inhibit Akt\cite{232}, or even directly activate Akt\cite{77} are available, exploring the effects of Akt signaling in airway cells may yield druggable targets that can be translated to human therapeutics.
Toll-like receptors (TLR)

Nod-like receptors (NLR)

Bitter receptors (ciliated cell)

Goblet cells

Solitary chemosensory cell

Activation of NF-κB and release of cytokines

Inflammasome/cytokines

Nitric oxide

Mucus secretion

Anti-microbial peptide secretion (Defensins, lactoferrin, and lysozyme)

Figure 3: Summarization of non-specific immune strategies in lung innate immunity. Toll-like receptors are activated to recognize pathogen-associated microbial patterns/damage-associated molecular pattern (PAMPs/ DAMPs) and activate nuclear factor-κB to produce cytokines; nod-like receptors are cytosolic receptors that can recognize intracellular PAMPs/DAMPs and can form inflammasome to either produce cytokines such as interferons or induce pyroptosis (inflammation associated apoptosis); Stimulation of bitter receptors, expressed in ciliated cells in the airways, elevates Ca²⁺ that produces NO which activates protein kinase G and increase ciliary beat frequency; NO can directly kill bacteria; Goblet cells produce mucus that traps pathogens and bacteria which are then eliminated out of the upper airway system through MCC; Activation of bitter receptors in solitary chemosensory cells can increase intracellular Ca²⁺ that can produce antimicrobial peptides.

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