REVIEW

Epidermal growth factor receptor-mediated innate immune responses and their roles in airway diseases

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ABSTRACT: Inhaled air is contaminated with pathogens and particulates that may deposit in the airways and damage the host. In response to these invaders, the airway epithelium has developed innate immune responses that provide a defence against the invaders and protect the airway structure and function. Thus, the epithelium of conducting airways becomes the ''battleground'' between the invaders and the host.

Recent evidence suggests that airway epithelial surface signalling through the epidermal growth factor receptor (EGFR) is a convergent pathway producing innate immune responses to a variety of infectious and noninfectious noxious stimuli. In the present review, the EGFR signalling pathways leading to airway mucin production, neutrophil recruitment (*via* interleukin-8 production) and airway epithelial repair were examined.

The importance of these findings in human airway diseases was also investigated. The current authors suggest that the exaggerated innate immune responses found in chronic inflammatory airway diseases (*e.g.* chronic obstructive pulmonary disease, cystic fibrosis and severe asthma) contribute to the pathogenesis or the aggravation of these diseases. Potential therapies include inhibition of the various elements of the described epidermal growth factor receptor cascade. In considering each therapeutic intervention, the potential benefits must be considered in relation to potential deleterious effects.

KEYWORDS: Asthma, chronic obstructive pulmonary disease, cystic fibrosis, interleukin-8, mucins, neutrophils

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Water-based animals utilise gills for the exchange of oxygen and carbon dioxide. As animals migrated to land during evolution, the expanded respiratory exchange apparatus was moved deep within the thoracic cavity and the lungs (the organs for gas exchange) required ''air tubes'' (the conducting airways) to communicate with atmospheric air. Inhaled air is contaminated with potential invaders, *e.g.* bacteria, viruses and other particulate irritants, such as allergens and cigarette smoke. Thus, the airway epithelium has become the ''battleground'' between invaders and host. An early system for immediate responses to the invaders was developed as a first line of defence; these local defences have been called ''innate immune responses''. They are ''innate'' because they do not require prior exposure to the invading microbe and they have the capability (*i.e.* receptors) to intercept microbial signals constitutively. They are ''immune'' in that they protect against infectious diseases.

Innate immunity in the lungs is an area of research that is evolving rapidly. This includes the mechanisms utilised by the airway epithelial cells to detect individual microbial and other products and the effector molecules produced for defence by the epithelium [1, 2]. During the past decade, receptors called pattern-recognition receptors (PRRs) have been identified, which recognise the presence of inhaled pathogens. The airway epithelium expresses PRRs, such as Toll-like receptors (TLRs), which recognise specific pathogen motifs. The PRRs play major roles in innate immune responses because their recognition of pathogens triggers host responses that are mediated by various effectors. Among the effector molecules are antimicrobial peptides that are produced and secreted by the epithelial cells and

that display microbicidal activity or inhibit growth of inhaled microorganisms [3, 4]. Mucins secreted by airway cells are recognised to be important in the adhesion to [5, 6] and clearance of microbes by mucociliary transport and cough [7, 8]. Extravasation of leukocytes in the airways and their phagocytosis of inhaled particulates are important in the normal eradication of the invaders [2]. Damage to epithelial tissue is also repaired by epithelial innate defence mechanisms. Normally, local responses are adequate to destroy the pathogens without obvious inflammation, and normal structure and function are maintained without symptoms. In contrast, exaggerated or inappropriate innate immune responses have been hypothesised to result in abnormal structural and functional outcomes and, thus, in disease [2].

In the present review, the focus is on the epidermal growth factor receptor (EGFR) signalling pathway, which produces specific protective responses by the epithelium, with special emphasis placed on mucin production and secretion (for mucociliary clearance of invading organisms), neutrophil recruitment (for bacterial killing) and epithelial wound healing (for repair of injured tissue). EGFR is the prototypical member of the ErbB family, which comprises of four receptors: EGFR/ErbB1/human epidermal growth factor receptor (HER)1, ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4 [9]. These are transmembrane receptors containing a ligand-binding site in the extracellular domain, a single membrane-spanning region and a cytoplasmic protein tyrosine kinase-containing domain. Upon activation by their cognate ligands, ErbB receptors form dimers (either homodimers or heterodimers), leading to activation of the intrinsic kinase domain, which results in phosphorylation of specific tyrosine residues in the intracellular domain. In the human airway epithelium it has been reported that EGFR, HER2 and HER3 are expressed, whereas expression of HER4 is absent or barely detectable [10, 11]. Thus, EGFR homodimers and EGFR-HER2 or EGFR-HER3 heterodimers may form when EGFRs are stimulated with their ligands. The formation of heterodimers between EGFR and other members of the ErbB family are likely to be important in determining cellular fate and responses. Since little data exists on the roles of EGFR heterodimers in innate immune responses of the airway epithelium, the present review focuses on EGFR. Emphasis is placed on potential roles of this complex signalling pathway in airway epithelial defences. Finally, relevant studies in human subjects with airway diseases are reviewed.

MECHANISMS OF CELL SURFACE SIGNALLING AND EGFR ACTIVATION
Mucin production via EGFR signalling
Description of mucins and stimuli inducing mucins
It has long been known that some chronic airway diseases (e.g. asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF)) are associated with mucous hypersecretion. Among the major components of human mucus are large-sized glycoproteins, the gel-forming mucins [12]. Understanding of mucous hypersecretion began with the cloning of these mucin genes [13–16]. During the 1990s, several studies reported that multiple stimuli upregulate mucin gene expression and protein production [17–30].

Involvement of EGFR activation in mucin production
LI et al. [31] reported that the intracellular downstream pathway for mucin production in response to Pseudomonas aeruginosa bacteria included a Src-dependent Ras–mitogen-activated protein kinase–pp90rsk pathway, but no surface receptor or surface signalling pathway was described. In 1999, TAKEYAMA et al. [32] discovered that EGFR activation results in mucin production, an effect that was prevented by selective EGFR tyrosine kinase inhibitors. EGFR is a 170-kDa membrane glycoprotein, which is expressed in various epithelial cells, including airways. In studies of rats in vivo, it was also shown that ovalbumin-induced sensitisation of rat airways increased goblet cell production via an EGFR pathway [32]. Treatment with EGFR inhibitors also prevented interleukin (IL)-13-induced mucin production in rat airway epithelium in vivo [33]. Thereafter, a profusion of studies reported that various stimuli cause mucin production via EGFR activation in human airway epithelial cell lines, normal human bronchial epithelial cells and in animals in vivo [34].

Ligands involved in EGFR activation
EGFR ligands, including epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding (HB)-EGF, amphiregulin, betacellulin and epiregulin, bind to EGFR, leading to direct activation of the receptors [35]. Many inflammatory cells produce these ligands, including eosinophils [36, 37], neutrophils [38], mast cells and macrophages [38, 39]. Thus, both activated eosinophils [37] and activated mast cells [40] have been shown to induce mucin synthesis in airway epithelial cells via the release of EGFR ligands. The roles of bone marrow-derived cells in EGFR signalling in disease deserve further investigation.

Ligand-dependent EGFR activation
Many stimuli do not activate EGFR directly. For example, ligands for G-protein-coupled receptors (GPCRs), such as endothelin-1, lysophosphatidic acid, thrombin [41] and IL-8 [42], induce epithelial EGFR activation. Airway epithelial cells produce EGFR proligands [10], synthesised as transmembrane precursors that require cleavage by metalloproteases to release the soluble mature ligands [43]. Originally, no soluble ligands were detected in the culture medium when epithelial cells were treated with GPCR activators [41, 44], suggesting that this EGFR activation does not involve EGFR-ligand binding. The activation was presumed to occur solely via intracellular mechanisms (so-called ''ligand-independent'' EGFR phosphorylation). Subsequently, PRENZEL et al. [45] showed that GPCR activation releases EGFR ligands into the cell culture medium, implicating ligand release from the epithelial cells in GPCR-induced EGFR activation. In that study, batimastat, a general metalloprotease inhibitor, prevented GPCR-induced EGFR ligand release, EGFR phosphorylation and subsequent cell proliferation, implicating an epithelium-derived metalloprotease in GPCR-induced EGFR signalling.

Lipopolysaccharide (LPS), a secreted product of Gram-negative bacteria, was reported to induce mucin production by unknown innate surface signalling mechanisms [30, 31]. Subsequently, KOHRI et al. [46] showed that P. aeruginosa bacterial supernatant induces mucin production in human airway epithelial (NCI-H292) cells via EGFR activation. Additionally, KOHRI et al. [47] reported that stimulation with

human neutrophil elastase induces mucin production in cultured airway epithelial cells *via* EGFR activation. In the latter study, the authors showed that elastase-induced mucin production occurs *via* proteolytic activation of an EGFR signalling cascade involving the cleavage of TGF-α (a ''ligand-dependent'' process) [47]. The metalloprotease responsible for ligand cleavage was not identified.

Tumour necrosis factor-α converting enzyme and other metalloproteases cleave EGFR proligands

Epithelial cells contain the metalloprotease tumour necrosis factor-α converting enzyme (TACE), a member of the a disintegrin and metalloprotease (ADAM) family, a group of zinc-dependent transmembrane metalloproteases [48, 49]. TACE cleaves EGFR proligands, such as pro-TGF-α, in epithelial tissues [50]. SHAO *et al.* [51] showed that the supernatant of *P. aeruginosa* and LPS increase mucin MUC5AC gene expression and protein production *via* an EGFR-dependent pathway: pre-incubation of the epithelial cells with an EGFR-neutralising antibody or with a TGF-α-neutralising antibody prevented MUC5AC synthesis, implicating ligand (TGF-α)-dependent EGFR phosphorylation. Knockdown of TACE inhibited the epithelial responses to LPS, including mucin production, thus establishing TACE as an important cell surface metalloprotease involved in airway epithelial defences against microbial presence [51]. Several members of the ADAM family of metalloproteases, including ADAM10, -12, -15 and -17, have been reported to cleave EGFR proligands in response to GPCR activation [52]. LEMJABBAR *et al.* [53] showed that stimulation of epithelial cells with lipoteichoic acid resulted in the activation of platelet-activating factor (PAF) receptor, a GPCR, leading to ADAM10-mediated shedding of pro-HB-EGF from the epithelial cell surface and subsequent mucin production. Thus, various ADAM family members may be involved in pro-EGFR ligand shedding and mucin production, depending on various features including the stimulus and the epithelial source.

Reactive oxygen species and Dual oxidase 1 activate TACE

Airway epithelial cells produce reactive oxygen species (ROS) [54], which are known to be involved in cell signalling [55]. In airway epithelial cells, ROS scavengers prevent cigarette smoke-induced TACE activation, EGFR phosphorylation and mucin production, implicating ROS in the activation of the TACE-EGFR pathway [56]. ROS are not only present in cigarette smoke and other inhaled substances, but are also generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [57]. Dual oxidase (Duox)1 is expressed in airway epithelial cells and generates ROS *via* NADPH oxidase activity [58, 59]. Stimulation of airway epithelial cells with neutrophil elastase induced TACE activation, TGF-α release and mucin production, effects that were prevented by ROS scavengers, implicating ROS in TACE activation [60]. Knockdown of Duox1 expression by small interfering RNA prevented the neutrophil elastase-induced responses, implicating Duox1 in TACE activation and mucin expression. The protein kinase C (PKC)δ/PKCθ inhibitor rottlerin also prevented these responses, implicating their role in Duox1 activation [61]. These results expand the epithelial surface signalling cascade that induces mucin production to include PKCδ/PKCθ-Duox1-ROS-TACE-proligand-EGFR. Generation of ROS can also be derived from stimuli, such as cigarette

smoke [62], and from cells recruited from the blood (*e.g.* neutrophils, eosinophils and macrophages) [63].

Activation of EGFR signalling by binding of environmental stimuli to cognate receptors

Inhaled ''invaders'' that deposit on the airway epithelium are recognised by airway epithelial cells *via* PRRs. Among these, TLRs are expressed in various immune cells (*e.g.* macrophages, dendritic cells, B-cells and specific types of T-cells) [64] and in airway epithelial cells. Thus, it has been shown that at least 10 TLRs (TLR1–10) are expressed in airway epithelial cells [65, 66]. Each TLR recognises specific ligands, resulting in cellular cascades that may produce different host responses [1]. For example, TLR2 recognises Gram-positive lipoteichoic acids and peptidoglycan, TLR3 recognises viral double-stranded RNA, TLR4 recognises Gram-negative LPS, TLR5 recognises flagellin and TLR9 recognises unmethylated bacterial DNA [1].

The airway epithelium is polarised and contains tight junctions that provide a physical barrier to particulates and large protein molecules, preventing their passive penetration through the epithelium [67]. The host response to inhaled pathogens can be initiated by extracellular microbial stimuli and does not necessitate invasion of the epithelial cells [68]. Thus, in airway epithelial cells, TLRs can be located at the apical membrane, where microbes or microbial products deposit. However, in phagocytes, some TLRs (TLR1, -2, -4, -5 and -6) are reported to be expressed at the cell surface, whereas other TLRs (TLR3, -7, -8 and -9) are reported to be expressed intracellularly [64]. Several investigators have studied the subcellular localisation of TLRs in airway epithelial cells. SOONG *et al.* [69] showed that only small amounts of TLR2 were present on the apical surface of airway epithelial cells under basal conditions, but bacterial stimulation caused TLR2 recruitment to the apical membrane of airway epithelial cells. TLR3 was not detected at the cell surface under basal conditions but was detected at the surface of cultured airway epithelial cells after stimulation with respiratory syncytial virus [70]. In each case, the recruitment of TLR2 or TLR3 at the cell membrane resulted in chemokine (*e.g.* IL-8) secretion. Similarly, GREENE *et al.* [66] reported that activation of TLR4 at the epithelial cell surface induces IL-8 secretion in CFTE29o- and 16HBE14o- cells. However, GUILLOT *et al.* [71] showed that TLR4 is expressed intracellularly in A549 and in BEAS-2B cells. In these cells, extracellular LPS stimulated TLR4 *via* unknown mechanisms. Therefore, the present authors conclude that TLR localisation is a dynamic process regulated by pathogen exposure, which may generally (but not always) lead to recruitment of TLRs at the cell membrane.

Multiple studies have reported that stimulation of airway epithelial cells by LPS induces the secretion of IL-8 *via* a cellular cascade involving a TLR4/myeloid differentiation primary response gene (MyD)88/nuclear factor-κB-dependent pathway [66, 72]. NAKANAGA *et al.* [73] reported that LPS-mediated IL-8 production is dependent on the Duox1-TACE-TGF-α-EGFR signalling pathway located on the cell surface. These data suggest that, after recognition of LPS by TLR4, a signalling cascade may lead to EGFR activation. In phagocytes, after TLR4 activation, MyD88 is recruited to the Toll/IL-1 receptor domain, resulting in phosphorylation of p47^{phox}, a critical component of NADPH oxidase, leading to ROS

production [74]. The current authors suggest that similar mechanisms occur in airway epithelial cells. Interestingly, MᴄNᴀᴍᴀʀᴀ and Bᴀsʙᴀᴜᴍ [75] reported that a dominant negative mutant MyD88 inhibited LPS-induced mucin production in NCI-H292 cells, supporting the speculation that MyD88 may activate Duox1 and EGFR.

A second group of receptors that signal epithelial responses are GPCRs [76]. These form the largest family of cell surface receptors and mediate signals from an enormous diversity of extracellular molecules, including acetylcholine, endothelins, prostaglandins, thrombin, ATP and UTP, PAF and lysophosphatidic acid. Interestingly, GPCRs also participate in the recognition of some inhaled noxious stimuli. For example, PAF receptors contribute to the recognition of Gram-positive bacteria (*e.g. Streptococcus pneumoniae* and *Staphylococcus aureus*) [53, 77], and acetylcholine receptors are activated by nicotine (*e.g.* from cigarette smoke) [78]. Activation of PAF receptors by *S. aureus* lipoteichoic acid induces mucin production *via* EGFR activation in airway epithelial cells [53]. In addition to the ability of GPCRs to participate in the responses to inhaled microbes, these receptors may respond to autocrine signals provided by locally produced molecules, such as ATP/UTP, for example as a result of a local inflammatory process. This is an area for future investigation.

Other non-TLRs and non-GPCRs are important for recognition of some pathogens by the airway epithelium. For example, *S. aureus* protein A is reported to interact directly with EGFR in airway epithelial cells, leading to EGFR activation [79]. Intercellular adhesion molecule (ICAM)-1, which is the major

human rhinovirus receptor in phagocytes [80], is also involved in the recognition of some rhinoviruses in airway epithelial cells [81]. The relationship between ICAM-1 signalling and EGFR activation is an interesting area for future studies.

Summary of the EGFR signalling pathway
When inhaled atmospheric irritants deposit on the airway epithelium and bind to a receptor such as a TLR or GPCR, activation of the receptor leads to a series of downstream signals and multiple outcomes. Among these signalling events is EGFR activation, which may result in cell migration and proliferation, wound repair and/or the production of proteins involved in innate immune responses (*e.g.* upregulation of IL-8, mucins and antimicrobial peptides). The sequence of events from stimulus to EGFR activation is best described as a cascade. There are probably multiple unknown steps in this cascade. Furthermore, the future investigation of positive and negative feedback systems involving this EGFR cascade could lead to important discoveries related to epithelial cell regulation in health and disease. A diagrammatic representation depicting surface mechanisms that lead to EGFR activation in response to inhaled pathogens is presented in figure 1.

IL-8 synthesis *via* EGFR signalling
Neutrophils have been recognised to play important roles in chronic airway diseases such as CF [82], COPD and severe asthma. IL-8 is a chemokine that is expressed in many cell types, including airway epithelial cells [83]. IL-8 is a potent and selective chemoattractant for neutrophils *in vitro* [84] and *in vivo* [85]. IL-8 has also been shown to be an important
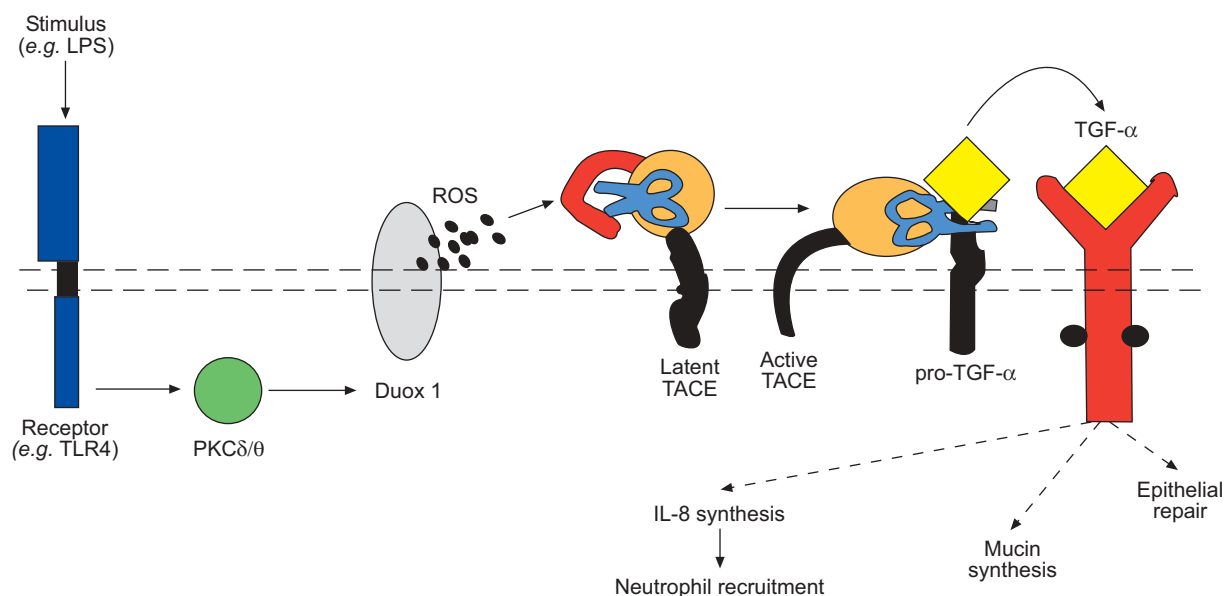

FIGURE 1. Schematic diagram depicting multiple innate immune responses induced by inhaled irritants. Inhaled irritants (exemplified here by bacteria) deposit on the airway epithelial surface. In the diagram, the stimulating bacterial lipopolysaccharide (LPS) binds to its receptor, Toll-like receptor (TLR)4, resulting in activation of protein kinase C (PKC) isoforms PKCδ and PKCθ. This recruits cytosolic components (*e.g.* p47[phox] and p67[phox]) to the plasma membrane to join Dual oxidase (Duox)1 to form an active enzyme system for generating reactive oxygen species (ROS). The ROS activate the latent form of tumour necrosis factor-α converting enzyme (TACE), removing the inhibitory prodomain and exposing the active domain to cleave epidermal growth factor receptor (EGFR) proligand (*e.g.* pro-transforming growth factor (TGF)-α). Soluble TGF-α is released, which binds to and activates EGFR, initiating multiple responses involved in innate immune responses. This cascade of events is implicated in mechanisms contributing to both pathogen elimination (*e.g.* production of mucins and recruitment of activated neutrophils *via* interleukin (IL)-8 synthesis) and maintenance of host integrity (*e.g.* epithelial repair).

neutrophil chemoattractant in the sputum of patients with chronic inflammatory diseases, including CF, bronchiectasis and chronic bronchitis [86]. IL-8 is produced by the airway epithelium in response to various stimuli, such as Pseudomonas bacteria [87], bacterial LPS [88], *S. aureus* [89] and ragweed antigen [90].

An important recent finding is that the synthesis and secretion of IL-8, a major neutrophil chemoattractant in airway epithelial cells, is also dependent, at least in part, on EGFR activation. SUBAUSTE and PROUD [91] first showed that treatment of airway epithelial cells with increasing concentrations of EGF or TGF-α, two EGFR ligands, resulted in increased secretion of IL-8. Subsequently, RICHTER *et al.* [92] showed that cigarette smoke, which has been shown to activate EGFR resulting in mucin production [93], also induces the gene transcription and protein production of IL-8, effects that are prevented by the selective EGFR tyrosine kinase inhibitor AG1478 [92]. They also showed that the EGFR-dependent IL-8 secretion in response to cigarette smoke was the result of TGF-α shedding from airway epithelial cells [92]. NAKANAGA *et al.* [73] showed that TACE is responsible for the synthesis and release of IL-8 from airway epithelial cells in response to bacterial LPS *via* a Duox1-TACE-TGF-α-EGFR signalling pathway on the surface of airway epithelial cells. Neutrophil elastase induces IL-8 production in airway epithelial cells *via* similar surface signalling [94].

Epithelial wound repair *via* EGFR signalling

Epithelia constitute an important barrier against invading microorganisms, and the epithelial innate immune mechanisms provide important pathways for repairing wounded epithelium. It has long been suspected that EGFR signalling induces accelerated wound repair [95]. Wound repair of human surface respiratory epithelium has been studied using cultured human epithelial respiratory cells in which wounds were created mechanically [96]. Investigators have shown that when EGFR is activated by its ligands (delivered exogenously), wound repair is accelerated in alveolar [97], airway [98], intestinal [99, 100], mammary gland [101] and skin epithelia [102, 103].

Damage to airway epithelial cells could cause accelerated wound repair *via* mechanical stimulation. Thus, after mechanical stress, endogenous nucleotides, such as ATP and UTP, are released into the extracellular space from mechanically stressed epithelium, stimulating epithelial cell proliferation [104, 105]. These nucleotides bind to specific GPCRs and to non-GPCRs. They stimulate IL-8 production in airway epithelial cells in a Pertussis toxin-sensitive manner, suggesting that GPCR activation plays a role in IL-8 production [105]. Other studies have shown that EGFR activation induces IL-8 production, and GPCR stimulation is known to cause EGFR activation [45]. Further investigation of the roles of mechanically damaged epithelium and other mechanical stresses on wound repair are needed.

Inhaled stimuli, such as H_2O_2 [106] and cigarette smoke [107], have been reported to inhibit wound healing in airway epithelium. Based on the knowledge that inhaled particulate invaders can inflict damage to the epithelium of the host and that the epithelium is capable of mounting a coordinated innate immune defence, KOFF *et al.* [108] hypothesised that the host epithelium intercepts the invader's signalling and responds by mounting responses that destroy the invader and repair associated epithelial damage. Gram-negative bacteria are important in the pathophysiology of airway diseases, including CF [109], bronchiectasis [110] and COPD exacerbations [111]. *P. aeruginosa* and its virulence factors damage the airway epithelium. KOFF *et al.* [108] showed that nontoxic levels of *P. aeruginosa* LPS accelerate wound repair *via* a complex signalling pathway that includes TLR4, PKC, Duox1, ROS, TACE, TGF-α and EGFR phosphorylation. However, at concentrations that were toxic to the epithelial cells, LPS inhibited wound repair. The response to low concentrations of the LPS stimulus suggests that airway epithelium provides an important function by activating host defences. Higher concentrations of LPS overcame this repair response. This cytotoxicity could initiate epithelial damage and facilitate invasion. Cigarette smoke extract is also reported to inhibit repair responses in human airway epithelial cells [107]. High concentrations of cigarette smoke extract were toxic to airway epithelial cells, whereas low concentrations enhanced cell survival [92]. Nicotine, a product contained in cigarette smoke, activates epithelial cell migration and wound repair *via* activation of the GPCR α3α5β2-nicotinic acetylcholine receptors [78]. It is suggested that nicotine at low concentrations promotes acetylcholine receptor-mediated EGFR activation that may result in cell migration, whereas higher concentrations of nicotine are toxic to the cells. Thus, the concentrations of the various molecules involved in surface signalling can determine the outcome, at least in part.

The epithelium may also cooperate with recruited cells in wound repair. Thus, macrophages isolated from wounds produce TGF-α [39]. Since EGFR ligands have been shown to stimulate wound repair, one of the roles that macrophages may play in the repair process is to provide EGFR ligands that can activate EGFR in the epithelium.

EGFR AND EGFR LIGAND EXPRESSION IN HUMAN AIRWAYS

In the airway epithelium of healthy subjects, EGFR expression is weak or absent [98, 112–114], but it is increased in the airway epithelium of asthmatics [98, 113, 114], smokers with normal lung function [11], patients with COPD [11, 115] and CF patients [116, 117]. This suggests that EGFR activation may play more obvious roles in inflammatory states than in healthy individuals.

In humans, the airway epithelium expresses EGFR ligands constitutively, including EGF, TGF-α, HB-EGF, amphiregulin, heregulin and betacellulin [10, 115]. Expression of several EGFR ligands has also been investigated in disease (*e.g.* asthma, COPD and CF) [112, 115–120]. These studies either assessed only the expression of an individual EGFR ligand or relied on the specificity of antibodies to examine the expression of multiple EGFR ligands, which are structurally related and may cross-react with antibodies. The studies showed that EGFR ligands are expressed in normal and diseased epithelia, but their expression pattern and distribution are variable. The respective roles of individual EGFR ligands in eliciting various EGFR-dependent physiological responses are largely unknown. It is suggested that activation of EGFR by different ligands results in changes in the activation of different intracellular pathways, which causes

different responses [121]. Since cleavage of epithelial EGFR proligands leading to autocrine EGFR signalling appears to mediate the response to multiple noxious stimuli, it will be important to examine the roles of individual EGFR ligands in these responses to different stimuli.

INNATE IMMUNE RESPONSES IN HEALTH AND DISEASE

Airway mucus

Normal mucus

Normally, the mucociliary escalator provides a potent protective response to inhaled invaders. On the epithelial surface of conducting airways is a periciliary sol phase consisting of liquid that allows cilia to move, propelling the mucous gel floating above the cilia towards the mouth. In large conducting airways, coughing shears secreted materials and assists in their clearance. Mucus is a complex mixture of lipids and proteins, together with a liquid phase composed of water and electrolytes. Secreted gel-forming mucins are high molecular weight mucous glycoproteins that contribute to the formation of the mucous gel. Among these key constituents of mucus, MUC5AC and MUC5B mucins have been found most convincingly in human airway mucus [12, 122–124]. Mucins are normally produced by cells in the mucous tubules of submucosal glands and by goblet cells in the airway surface epithelium. In healthy individuals, submucosal glands are small [119] and the airway surface epithelium contains few goblet cells [113, 117, 124]. The secreted thin mucous gel spreads on the epithelial surface. Inhaled invaders (e.g. bacteria and other particulates) are trapped in this mucous gel and are removed from the airway via mucociliary clearance and coughing [7, 8]. These clearance mechanisms defend the host from attack by microbes and other inhaled irritants that deposit in the airways.

Mucous hypersecretion

In many respiratory diseases (e.g. asthma, COPD and CF), pathological studies report an increase in submucosal gland size in large airways [119, 125, 126] and/or an increase in epithelial goblet cells in both large [125, 127, 128] and small airways [117, 125, 129]. This increase in mucin-producing cells is associated with increased mucin expression [11, 117, 119, 124, 127, 130, 131]. Anatomical considerations predict that the consequences of mucin hypersecretion should be different in large versus small airways.

Mucins in large conducting airways are produced by both airway epithelium [11, 127] and submucosal glands [132]. Mucins secreted by glands are transported into gland ducts, which terminate in the airway lumen. Gland ducts are concentrated at airway bifurcations, which are also the sites of cough receptors [8]. This localisation facilitates cough clearance of mucus secreted by glands. Secretion of mucins by proximal airway glands is predicted to result in cough and sputum production, the main symptoms of chronic bronchitis.

Small (peripheral) airways do not contain glands, and mucins in these airways are produced only by surface epithelial cells. Mucous hypersecretion in peripheral airways may result in airway plugging (mucous obstruction). Local overproduction of mucins by the surface epithelium is presumed to produce peripheral plugging. However, retrograde movement of mucins produced in large airways by aspiration is another, possibly major, cause of plugging.

Several anatomical features make the peripheral airways more likely to become obstructed by accumulated mucus than the large conducting airways, for the following reasons. First, when mucin-containing cells degranulate, the mucin volume can increase >1,000-fold due to hydration [133]. Thus, degranulated goblet cell mucins are more likely to obstruct airways of small diameter. Secondly, epithelial cells of patients with goblet cell hyperplasia have a larger volume and, therefore, occupy a larger percentage of the airway lumen than the epithelium of healthy control subjects, exaggerating the effects of mucous secretion. Thirdly, associated contraction of airway smooth muscle in the affected airway narrows the airway lumen further, again exaggerating the effects of mucous secretion (fig. 2).

Since small airways in normal lungs make only a small contribution to total airway resistance [135], abnormalities in these airways cause few clinical manifestations until most of the peripheral airways are occluded. However, extensive obstruction of small airways may result in significant airflow limitation in chronic airway inflammatory disease. It is predicted that plugging of 75% of all small airways is required before changes can be detected by routine pulmonary function tests (e.g. forced expiratory volume in one second) [136]. The relative absence of clinical manifestations, the limitations of physiological measures of small airway function in COPD and the difficulties of sampling these airways in human subjects are responsible for the limited recognition of the important pathophysiological roles of mucous plugging in small airways. In subjects with COPD, HOGG et al. [137] studied surgically removed tissues and found that the extent of small airway plugging increased in association with increasing airflow limitation, suggesting that mucous plugging is associated with the progression of COPD. In a subsequent study, HOGG et al. [138] showed that the extent of mucous plugging in peripheral airways predicts 3-yr mortality in subjects with severe COPD. SAETTA et al. [129] described goblet cell hyperplasia in peripheral airways of COPD subjects with chronic bronchitis. This hypertrophic epithelium increased the volume of tissue in the wall of small airways, another important determinant of airflow limitation in COPD [137]. Thus, it is suggested that both goblet cell hyperplasia and small airway plugging contribute to airflow limitation in COPD. These structural abnormalities are also found in subjects with advanced CF, contributing to the clinical deterioration of respiratory function in this condition [117].

Mucous plugging in small airways has long been known to occur in acute fatal asthma [130, 134, 139]. KUYPER et al. [140] studied airways in 93 patients who died with acute asthma and concluded that extensive mucous plugging was a major feature in most, if not all, patients dying of asthma. It is likely that mucous plugging in peripheral airways contributed to death, presumably by reducing ventilation and gas exchange. Whether plugging in small airways also occurs in chronic asthma and contributes to the development of poorly reversible airflow obstruction in severe asthmatics is unknown, because peripheral airway tissue from asthmatics is not widely available.
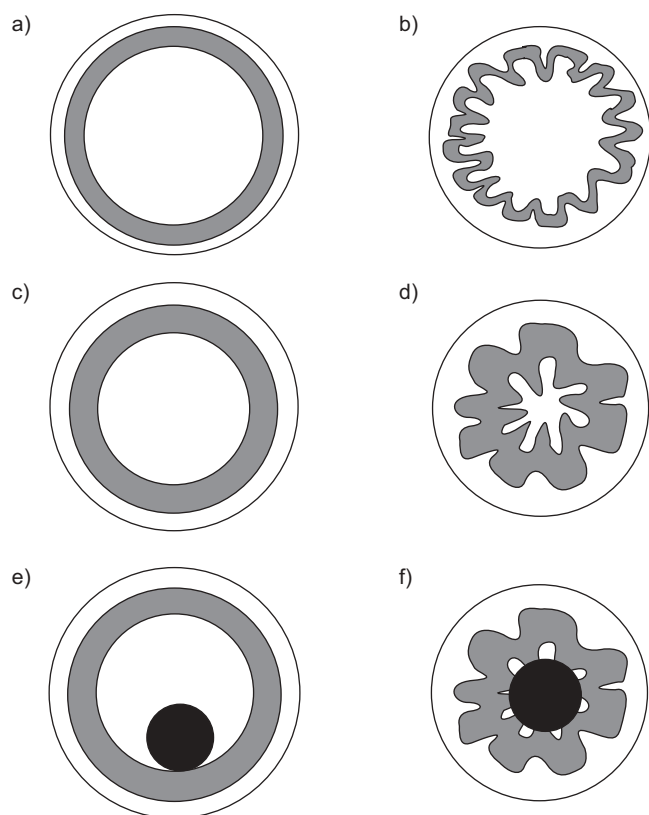
FIGURE 2. Schematic representation of the potential impact of goblet cell hyperplasia and mucous plugging on small airway obstruction. In normal individuals, the surface epithelium covering small airways contains few goblet cells and is composed of a thin layer of epithelial cells. Normal, thin epithelium (a and b) contributes little to luminal narrowing both when airway smooth muscle is relaxed (a) and when smooth muscle shortening occurs (b). In various airway inflammatory diseases (*e.g.* acute asthma, chronic obstructive pulmonary disease and cystic fibrosis), epithelial cell hyperplasia is present in small airway epithelium, resulting in epithelial thickening (c and d). When airway smooth muscle is relaxed (c), thickened epithelium contributes little to airflow limitation. However, because the thickness of the airway wall (composed of epithelium, lamina propria, muscle and adventitial compartments) is an important determinant of airflow limitation, when smooth muscle shortening occurs (d), hyperplastic epithelium is predicted to contribute to airflow obstruction [134]. When hyperplastic goblet cells release mucins in the airway lumen, swelling of secreted mucins during hydration may result in mucous obstruction (e and f). The contribution of mucous secretions to airflow limitation is likely to be increased when smooth muscle shortening occurs (f). Thus, both goblet cell hyperplasia and mucous obstruction in small airways are predicted to contribute significantly to the airflow limitation that occurs in chronic inflammatory airway diseases.

Another intriguing question is the relationship between bacterial colonisation of the airways and plugging in small airways. Normal airways are sterile [141]. However, in diseases associated with chronic small airway plugging (*e.g.* COPD and CF), bacterial colonisation is a frequent finding [109, 141]. Bacteria (*e.g.* P. aeruginosa and *Haemophilus influenzae*) are known to adhere to mucins [5, 6, 142]. The adhesion of bacteria to mucins may result in bacterial phenotypic modification, *e.g.* production of alginate in a hypoxic environment within the mucous plugs [142], which contributes to the persistence of bacteria in the airways.

Relationship of EGFR expression to mucous hypersecretion

Since multiple stimuli induce mucin production *via* EGFR activation in cultured airway epithelial cells and in the airways in animals, studies have been performed to determine EGFR and mucin expression in human airway tissues. Associations have been found between EGFR and mucin expression. TAKEYAMA *et al.* [113] studied bronchial biopsies obtained from healthy volunteers and subjects with mild-to-moderate asthma and found a positive correlation between EGFR immunoreactivity and MUC5AC mucin staining in the epithelium of healthy and asthmatic subjects, suggesting a causal relationship. O'DONNELL *et al.* [11] also studied expression of several members of the EGFR family of receptors in relation to mucin expression, comparing nonsmokers with current smokers with or without COPD. They concluded that long-term cigarette smoking is associated with enhanced expression of EGFR and mucin MUC5AC in the epithelium of proximal airways. Additionally, BURGEL *et al.* [117] found a positive correlation between mucin and EGFR staining in the small airway epithelium of subjects with advanced CF. Although these studies are compatible with the hypothesis that EGFR activation is responsible for increased mucin production in airway diseases, proof for this concept requires clinical studies using selective drugs that inhibit the EGFR cascade in individuals with chronic respiratory diseases associated with mucous hypersecretion.

Neutrophil recruitment

Recruitment of neutrophils to airways in disease

Chronic airway diseases (*e.g.* CF and COPD) have long been known to be associated with neutrophil infiltration into the airways [143, 144], and acute [145, 146] and chronic severe asthma [147] have been added to this list. IL-8 is believed to be one of the major neutrophil chemoattractants in airway diseases and is a target for the treatment of neutrophilic airway diseases [148]. For example, sputum from patients with CF was found to contain high levels of IL-8, and an antibody to IL-8 inhibited neutrophil chemotactic activity in the sputum of patients with CF [86]. Neutrophilic inflammation is a common and important manifestation of chronic airway diseases, so it is surprising that effective therapeutic intervention has not been accomplished.

Relationship between EGFR and IL-8 expression and neutrophil recruitment

In CF airway tissues removed at transplantation, IL-8 staining was increased in the epithelium and was co-localised with mucins [117]. Furthermore, IL-8 and mucin expression were positively correlated with EGFR expression in the airway epithelium, and neutrophils were increased in the epithelium and in the lumen of CF airways [117]. HAMILTON *et al.* [149] also found a strong correlation between IL-8 and EGFR expression in the epithelium of subjects with severe asthma. Animal studies have definitively indicated that both IL-8 and mucin production in airway epithelial cells involve EGFR activation. However, determination of the importance of these findings in human diseases will require clinical studies using EGFR inhibitors.

Interactions between neutrophils and mucins

In response to noxious stimuli (*e.g.* bacteria), airway epithelial cells produce both mucins and IL-8, suggesting that neutrophils

and mucins could cooperate in the defence of the host. Thus, neutrophils and their products have been implicated in the production of mucins by epithelial cells, in the secretion of mucins in the airways and in the degradation of the secreted mucins.

Neutrophil products induce mucin gene and protein synthesis in airway epithelial cells [28]. Thus, both neutrophil-derived ROS [150] and neutrophil elastase [47, 60] have been shown to induce mucin gene and protein synthesis in airway epithelial cells via EGFR-dependent mechanisms.

Experimental studies indicate that when neutrophils are recruited to the airways, their close contact with airway epithelial goblet cells triggers mucin secretion from epithelial cells [151, 152]. This effect of recruited neutrophils on mucin secretion is mediated by neutrophil elastase proteolytic activity [152]. Thus, neutrophil proteases (e.g. elastase, cathepsin G and proteinase 3) are potent secretagogues for airway and submucosal gland epithelial cells [153, 154]. In CF tissues, BURGEL et al. [117] found that mucin secretions were increased in the lumens of small airways. The presence of mucins varied among different areas of epithelium, and neutrophil numbers in epithelium were inversely correlated with mucin contents in epithelium, suggesting that recruited neutrophils trigger mucin secretion [117]. Representative photomicrographs are shown in figure 3.

Neutrophil proteases also induce the proteolytic degradation of mucins in vitro [28, 155, 156] and in vivo [155, 156]. DAVIES et al. [157] suggested that neutrophil proteases present in CF sputum cleave MUC5AC and MUC5B mucins into small fragments that do not react with the usual antibodies. They also suggested that MUC5AC mucin is more susceptible to proteolytic degradation than MUC5B [157]. The roles of neutrophil-dependent mucin degradation in the clearance of mucous secretions and in the pathogenesis of airway mucous obstruction deserve future studies.

The complex interactions between neutrophils and mucins have important implications for studies of mucins in clinical diseases. HAYS and FAHY [119] found only a modest increase in mucin content in airway epithelium obtained from bronchial biopsies of CF subjects compared with the epithelium of healthy volunteers. They suggested that the relatively low amounts of mucins in goblet cells in CF epithelium could be related to ongoing degranulation signals (via recruited neutrophils) [119]. Since ongoing mucin secretion decreases mucin content in epithelium that actively produces and secretes mucins, the current authors suggest that in diseases associated with marked neutrophilic infiltration (e.g. CF and exacerbations of COPD), investigators should, when possible, examine both gene and protein expression of mucin.

Epithelial damage in airway diseases
Evidence for epithelial damage in airway diseases
Experimental airway epithelial damage in humans has proven difficult to examine because wounding causes significant side-effects (e.g. bleeding). Furthermore, sampling of the airway epithelium in humans often involves bronchial biopsy, which can itself mechanically damage the epithelium, making the assessment of epithelial damage subject to bias [158].

Inhaled bacteria that deposit on the airway epithelial surface can damage the protective barrier of the airway epithelium [159]. Inhaled allergens and other irritants may also induce epithelial damage. In experiments with guinea pigs, allergic sensitisation with ovalbumin caused localised areas of tracheal epithelial damage associated with an intense, rapid restitution process [160]. In atopic humans sensitised to house dust mite, inhalation of this allergen was associated with proliferation of the airway epithelium [161]. Obvious epithelial damage was not identified in biopsies sampled 48 h after the challenge [161].

LAITINEN et al. [162] studied bronchial biopsies and reported epithelial cell disruption in asthmatic airways. MARGUET et al. [163] reported increased numbers of epithelial cells in the bronchoalveolar lavage fluid of asthmatic children and suggested that epithelial shedding occurred. Autopsy studies of subjects dying of status asthmaticus also showed extensive epithelial shedding [139], suggesting that airway epithelial damage is a significant aspect of the pathophysiology of asthma [164].
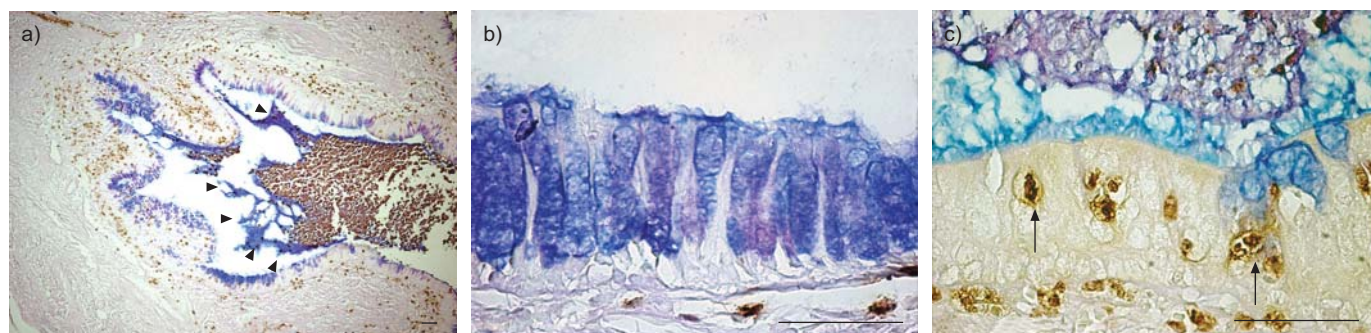

FIGURE 3. Representative photomicrographs of the relationship between mucins and neutrophils in airway sections from a patient undergoing lung transplantation for cystic fibrosis. Sections were stained with a monoclonal antibody to human neutrophil elastase (brown) and counterstained with Alcian blue/Periodic acid-Schiff (AB/PAS) for mucous glycoconjugates. a) Large numbers of neutrophils were seen within the airway lumen and AB/PAS in the lumen was localised to the periphery of the plug (arrowheads). b) In the epithelium where large amounts of mucous glycoconjugates were present, no neutrophils were found in the epithelium. c) In areas where large numbers of neutrophils were found in the epithelium (arrows), only small amounts of mucous glycoconjugates were present within the epithelium, but large amounts of mucous glycoconjugates were present in the lumen adjacent to the epithelial cells, suggesting goblet cell degranulation. Reproduced from [117] with permission from the publisher. Scale bars=50 μm.

In asthma, the current authors conclude that, although the methods of sampling can create artefacts in epithelial tissue, there is considerable data implicating epithelial damage. Future studies of mechanisms underlying the causes of this epithelial damage may provide new avenues for improving asthma treatment.

Relationship of EGFR expression to epithelial damage
PUDDICOMBE *et al.* [98] studied the expression of EGFR in airway epithelium in relation to epithelial disruption in asthma. They reported that areas of epithelial damage exhibited a strong EGFR immunoreactivity and suggested that EGFR activation plays an important role in the epithelial damage/repair process in asthma [98]. However, it is unlikely that epithelial damage is the only reason for increased EGFR expression in asthmatic epithelium, since EGFR staining was also increased in morphologically intact epithelium [98]. Other stimuli, such as tumour necrosis factor-α secretion, which is increased in asthmatic airways [165] and induces EGFR expression in airway epithelium [32], could explain the increase in EGFR expression.

SUMMARY, IMPLICATIONS FOR THERAPY, AND FUTURE PERSPECTIVES
The airway epithelium is the first site of contact with inhaled and potentially damaging particulates. In healthy individuals, low concentrations of inhaled invaders are cleared *via* immune mechanisms, usually without symptoms or obvious pathological findings. Inhaled microbes and foreign particulates depositing on the airway epithelial surface activate epithelial receptors, which transmit signals *via* various pathways including EGFR signalling. EGFR activation results in the production of molecules such as mucins (which assist in the clearance of foreign particulates) and IL-8 (which potently recruits neutrophils), and also stimulates epithelial repair [98, 108]. However, high concentrations of the pathogens, often in combination with specific abnormalities in the host, may induce or exaggerate disease. Thus, mucous hypersecretion and exaggerated neutrophil recruitment are thought to play important roles in the pathogenesis of chronic airway diseases. Current therapies have only limited effects on mucin production and secretion [166] and on neutrophil recruitment [143, 144]. Therapies inhibiting various aspects of the EGFR signalling cascade could be beneficial in individuals with excessive mucous, neutrophilic and other EGFR-mediated responses. In considering potential therapies, it will be important to weigh potential therapeutic benefits with possible side-effects.

Much remains to be learned concerning this signalling cascade, especially concerning selective signalling that determines specific outcomes. It is predicted that a careful temporal analysis of the signalling molecules involved in this immune signalling and the effects of different modes of stimulation and signalling (*e.g.* roles of different EGFR ligands and phosphorylation sites) will provide new insights. Future studies are also needed in order to evaluate the use of therapeutic modalities involving the cascades described in the present review.

Modulation of airway epithelial cell activity and responses to external and internal stimuli is complex, involving many molecules and multiple pathways. One aim of the present review is to encourage future investigations of the interactions of the epidermal growth factor receptor signalling pathways with other important epithelial signalling pathways.

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