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Signaling networks controlling mucin production in response to Gram-positive and Gram-negative bacteria

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Human lung cells exposed to pathogenic bacteria upregulate the production of mucin, the major macromolecular component of mucus. Generally this upregulation is beneficial for the host, however, in the lungs of cystic fibrosis patients, overproduction of mucin can lead to the plugging of pulmonary airways. Mucus plugging impedes airflow and creates an environment that is highly compartmentalized: those bacteria within the mucus layer are shielded from high doses of antibiotics whereas those outside the mucus are exposed. These conditions augment mutation rate and the development of drug resistance in bacteria that colonize the lungs of cystic fibrosis patients. While therapeutic inhibition of mucin induction would improve airflow and reduce antibiotic resistance in these patients, the challenge is to develop drugs that block excessive mucin production while leaving beneficial aspects of the response intact. To do this, we must understand the molecular mechanisms underlying mucin production. Here we review the signal transduction pathways that control mucin production in response to Gram-positive and Gram-negative bacteria.

Keywords: **bacterial pathogenesis, host defense, purinergic receptors, platelet activating factor receptor (PAFR), *P. aeruginosa*, *S. aureus*, flagellin, LPS**

Introduction

Lung mucus production is an adaptive mechanism whose function is to provide a barrier between lung cells and noxious stimuli in the inspired air. These include viruses, bacteria, dust particles and air pollution. As with other protective mechanisms such as pain and inflammation, mucus induction often exceeds useful limits, creating pathology in and of itself. Persistent mucus hypersecretion is associated with three common lung diseases: asthma, chronic bronchitis and cystic fibrosis. Multiple mediators can impinge on lung epithelial cells and contribute to the pathogenesis of mucin overproduction. Relevant stimuli in asthma include products of eosinophils and T lymphocytes [1–3]; relevant stimuli in chronic bronchitis include tobacco smoke [4] as well as products of neutrophils, macrophages and lymphocytes [5–11]; relevant stimuli in cystic fibrosis, a genetically determined disease, include both bacterial pathogens [12] and many of the mediators from inflammatory cells mentioned above. In this review, we focus on stimulatory mechanisms triggered by bacterial pathogens that contribute to mucin overproduction in cystic fibrosis. These mechanisms may be generalizable to any situation in which bacteria come into close and prolonged contact with mucosal epithelial cells.

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Cystic fibrosis

Cystic fibrosis (CF), an autosomal recessive disorder, is the most common lethal genetic disease in Caucasians [13]. Lung disease is the principal cause of morbidity and mortality, with 95% of CF patients dying of respiratory failure. This can be attributed to the deleterious effects of bacteria infecting the lung, which, in CF patients, correspond largely to the Gram-negative organism, *Pseudomonas P. aeruginosa* and the Gram-positive organism, *Staphylococcus (S.) aureus*. Although *S. aureus* infections can sometimes be cleared early in disease progression, *P. aeruginosa* infections are not typically eradicable and eventually lead to lung destruction and death.

The downward spiral precipitated by bacterial infection is probably set in motion by deficiencies in the ability of CF patients to clear bacteria through the mechanism of mucociliary transport. This is secondary to the genetic defect in the cystic fibrosis transmembrane conductance regulator (CFTR), which results in insufficient Cl^- ion and water movement into the

tracheal lumen. The failure of this system prolongs the dwell time of bacteria in proximity to lung epithelial cells. We now know that a variety of molecules shed during the life cycle of bacteria have the capacity to bind to epithelial cells and thereby trigger signaling cascades resulting in altered gene expression [14–16]. The signaling cascades activated by bacteria result not only in mucin induction, but also in the induction of bactericidal host proteins such as defensins and pro-inflammatory cytokines such as IL-8 [17,18].

Although the clinical link between bacterial infection and lung mucus hypersecretion was obvious, it was unclear whether there was actually a causal relationship, and, if so, whether the stimulus directly activating the epithelial cell was bacterial in origin or instead derived from bacteria-induced inflammation. Our first step was to determine whether or not bacterial exoproducts directly up-regulate epithelial *MUC2* mucin transcription in human bronchial organ cultures as well as in cultured epithelial cells [14]. In these early studies, we used as stimulus the broth taken from late log phase bacterial cultures. We diluted this into cell or organ culture medium and performed *in situ* hybridization, Northern blot or RT-PCR to monitor effects on mucin production. Results clearly showed that mucin is upregulated in both the lung epithelial cell line, NCI H292 [19] and the intestinal epithelial cell line HM3 [20] upon exposure to bacteria and in the absence of any inflammatory cells. More recently, we performed experiments to examine the underlying mechanisms. Experiments described below indicate that *P. aeruginosa*, a common CF pathogen, activates *MUC2* mucin gene transcription via activation of a Src-dependent Ras-MEK1/2-ERK1/2-pp90rsk-NF-κB pathway [21].

Activation of NF-κB is required for *MUC2* mucin induction by *P. aeruginosa*

We began our investigation of the *P. aeruginosa* signaling pathway by defining response elements in the 5′-flanking region of the *MUC2* mucin gene and related transcription factors. Using a panel of MUC2-luciferase reporter gene deletion mutants, we identified a response element between −1627/−1308 bp. This region was narrowed to −1528/−1430 bp using heterologous constructs of the human *MUC2* promoter subcloned upstream of the thymidine kinase (TK) promoter. The region between −1458/−1430 bp was finally identified as the mucin response element using electrophoretic mobility shift assays with ^{32}P-labeled oligonucleotides. Sequence analysis showed that this region contained overlapping C/EBP and NF-κB sites. Selective mutagenesis of these sites and supershift assays using both anti- NF-κB (p65, p50 and c-Rel) and anti-C/EBPβ antibodies indicated that an NF-κB p65-p50 heterodimer was responsible for the observed protein binding. Mutation of the NF-κB site and chemical inhibition of NF-κB-DNA binding using caffeic acid phenethyl ester (CAPE) [22] abolished responsiveness of the wild-type *MUC2* luciferase construct. This confirmed that NF-κB binding to the κB site was critical for the upregulation of *MUC2* transcriptional activity induced by *P. aeruginosa*.

Activation of NF-κB by *P. aeruginosa* occurs via a Src-dependent Ras-MEK1/2-ERK1/2-pp90rsk pathway

Despite extensive analysis of NF-κB by previous investigators, the signaling events upstream of its activation were poorly understood. Using a dominant-negative mutant expression plasmid for the IκBα kinase pp90rsk (pp90rskΔC), we reduced the mucin response to *P. aeruginosa* by approximately 70%. pp90rsk has been recognized as a mitogen-activated, serine-threonine ribosomal S6 kinase involved in the transduction of signals induced by stimuli that activate the Ras-Raf1-MEK1/2-ERK1/2 cascade [23,24]. To test whether one or more MAP kinase pathways may be involved in the mucin response, we used dominant negative and chemical inhibitors of MEK1/2 (the kinase immediately upstream of ERK1/2) and SEK/JNKK (the kinase immediately upstream of c-Jun N-terminal kinase (JNK)) in our *MUC2* luciferase assay. These studies revealed activation of the classical MEK1/2-ERK1/2 pathway in *MUC2*-expressing epithelial cells which was abolished with a specific chemical inhibitor of MEK1/2, PD98059, and greatly enhanced by overexpression of wild-type MEK1.

The key issue of how *P. aeruginosa* activates MEK1/2 was addressed by looking for the involvement of a Src-dependent Ras pathway that had previously been described in bacterial-triggered macrophage signaling [24–28]. Co-transfection with a dominant-negative mutant form of Ras (RasN17) [29] reduced the response by 80% and co-transfection with a dominant-negative mutant form of c-Src (SrcRF) inhibited the response by 45% [30]. Consistent with this, the Src-specific chemical inhibitor PP1 reduced the response by 80% [31] and co-transfection of HM3 and NCIH292 cells with a constitutively active form of pp60c-Src (v-Src) greatly increased the response. This led us to conclude that mucin-induction by *P. aeruginosa* is both Ras- and Src-dependent.

P. aeruginosa culture supernatant and lipopolysaccharide up-regulate *MUC2* transcription via activation of the same Src-dependent MEK1/2-ERK1/2-NF-κB pathway

After confirming by RNAase protection assay that the endogenous *MUC2* gene and the *MUC2* promoter-driven luciferase reporter gene were regulated similarly, we next sought to identify the active bacterial factor(s) in *P. aeruginosa* supernatant. The major outer membrane component of Gram-negative bacteria is lipopolysaccharide (LPS). LPS is a potent activator of host defense [14,32] and both commercially available LPS and LPS purified from the bacterial culture supernatant caused MUC2 upregulation in our system. Further experiments showed that lipid A could essentially mimic the LPS effect, indicating that lipid A was a key factor in *MUC2* stimulation.

Despite evidence for involvement of LPS in the transcriptional activation of mucin by *P. aeruginosa*, it became clear that the response induced by LPS did not account for that induced by the whole supernatant (McNamara and Basbaum, unpublished data). This suggested that there were additional bacterial factors

that could stimulate mucin production and led to the discovery of a distinct, yet overlapping signaling pathway activated by the *P. aeruginosa* outer membrane protein, flagellin.

Flagellin ligation of asialoGM1 activates mucin transcription

The protein flagellin is a major structural component of bacterial flagella; an organelle required for chemotaxis, motility, and nutrition in both Gram positive and Gram negative bacteria. It has been shown that *P. aeruginosa* flagellin can elicit host cell responses through binding to a glycolipid receptor, asialoGM1 (ASGM1) [33]. It is unclear how this occurs, however, since ASGM1 lacks transmembrane and intracellular domains and is therefore incapable of direct contact with cytoplasmic signaling molecules. To study this, we used an *in vitro* system to investigate cellular signaling mechanisms by which ASGM1 ligation stimulates transcription of the mucin *MUC2*. As described below, results indicated that this involves the release of ATP extracellularly followed by activation of cell surface ATP receptors, Ca^{2+} mobilization and Erk 1/2 phosphorylation [15].

ASGM1 ligation activates mucin transcription in a Ca^{2+}-dependent manner

To conveniently monitor mucin responses to purified bacterial flagellin, we stably transfected HM3 cells with a construct consisting of the *MUC2* mucin 5′ upstream flank [34] ligated to the luciferase gene as a reporter (HM3MUC2 cells). Flagellin purified from *P. aeruginosa* and anti-ASGM1 antibody (α-ASGM1, referred to below as 'agonist antibody') stimulated *MUC2*-luciferase activity in a dose-dependent manner up to ~15 and ~40 fold, respectively. The endogenous *MUC2* gene responded similarly [15].

To dissect the signaling pathway downstream of ASGM1 ligation, we first monitored the activity of signaling molecules implicated in host defensive responses studied previously. Elevations in intracellular Ca^{2+} have been shown to mediate both cytokine production [35] and phagocytosis [36] in cells exposed to bacteria or their products. In our system, HM3 cells loaded with the fluorescent Ca^{2+} indicator Fura-2 showed a transient increase in intracellular Ca^{2+} approximately 20 seconds after administration of the agonist antibody or flagellin. To confirm that this increase in intracellular calcium was necessary for mucin induction, we showed that activation of MUC2 gene transcription following ASGM1 ligation was blocked with (a) BAPTA-AM, a Ca^{2+} chelator and (b) thapsigargin, which depletes Ca^{2+} stores by blocking Ca^{2+} ATPases in the endoplasmic reticulum [15].

MUC2 activation by flagellin is Erk 1/2-dependent

To determine whether the host signaling in response to flagellin overlaps with that induced by LPS, we initially focused on the mitogen-activated protein kinase (MAPK) Erk 1/2 [21]. Using a phospho-Erk-specific antibody, we observed strong phosphorylation of both Erk 1 and 2 in cells treated with either agonist antibody or flagellin, but not control cells. Moreover, the MEK 1/2 inhibitor PD98059 and the MEK 1/2 dominant negative mutant MEK K97R inhibited mucin induction by these agents [15]. Interestingly, we obtained nearly complete inhibition of the mucin response using inhibitors of calcium metabolism, yet only about 50% inhibition when the MAPK pathway was blocked. This suggests a bifurcation of the ASGM-1 signaling pathway after Ca^{2+} mobilization, giving rise to downstream events that are alternatively Erk-dependent or -independent.

ATP release and an ATP receptor link ASGM1 with Ca^{2+} signaling

Mobilization of Ca^{2+} from intracellular stores is triggered by phasic generation of IP_3 [37]. The production of IP_3 is dependent on the activity of phospholipase C (PLC) [38,39]. By directly measuring IP_3 levels in stimulated cells vs controls we obtained evidence that PLC is activated by ASGM1 ligation. That PLC figures importantly in mucin induction by flagellin was evident from the blocking effect of the PLC inhibitor ET-18-OCH_3 [15].

We next asked how ligation of ASGM1, a membrane glycolipid, leads to activation of PLC, a cytoplasmic enzyme. PLC isoforms often act downstream of G protein-coupled receptors (GPCRs). Among the most extensively studied GPCRs known to exist on epithelial cells are receptors of the P2Y family that are activated by extracellular nucleotides [40–42]. We observed that P2Y antagonists Reactive Blue 2 and Acid Blue 129 [43,44] strongly inhibited flagellin-induced mucin gene expression [15], which reinforced the view that GPCRs are required for the response and more specifically implicated P2Y nucleotide receptors.

One possible mode of interaction between ASGM1 and P2Y receptors would be through ASGM1 stimulating the extracellular release of ATP followed by ATP binding to P2Y receptors on the same or adjacent cells. Using a luciferin-luciferase ATP assay system, we showed that ATP is released extracellularly in response to agonist antibody. This suggested a novel mechanism of pathogenesis in which the ligation of a receptor lacking a transmembrane domain (ASGM1) stimulates autocrine nucleotide signaling. We obtained additional evidence for this using as reporter cells HEK 293 cells that had been transfected with the P2Y2 nucleotide receptor and GFP (HEK-P2Y2-GFP cells). While the HEK-P2Y2-GFP cells did not show Ca^{2+} mobilization in response to either flagellin or agonist antibody when grown by themselves, they displayed clear Ca^{2+} responses when grown with ATP-releasing cells [15].

In summary, stimulation of host gene expression by *P. aeruginosa* flagellin or an ASGM1 agonist antibody appears to be mediated by an autocrine mechanism involving ATP release and then binding of ATP to a nucleotide receptor. Our results are significant both in demonstrating a role for nucleotide receptors in bacterial pathogenesis and in explaining how the glycolipid

bacterial receptor ASGM1 is able to engage cytoplasmic signaling networks.

This phenomenon could play a major role in the initiation of defensive responses throughout a mucosal epithelium, even when only a small fraction of the surface is directly exposed to pathogen. Yet, the means by which ATP is released from HM3 and NCIH292 cells (and mucosal epithelial cells in general) is unknown. Some reports suggest that ATP is pumped out of cells by members of the ABC transporter family, one of which is the cystic fibrosis transmembrane conductance regulator (CFTR) [45,46]. Other reports [47] dispute this, however, leaving the question of how ATP exits cells in a non-exocytotic manner still unresolved.

The signaling pathway by which ASGM1 stimulates mucin transcription is similar to that stimulated by the Gram-negative endotoxin, LPS [21], in that it requires activation of Erk 1/2. Notably, the Gram-positive cell wall component, lipoteichoic acid [16]) also requires activation of Erk 1/2. Thus, both Gram-positive and Gram-negative bacteria activate *MUC2* transcription via the same mitogen-activated protein kinase (Erk 1/2), yet the cell surface receptors and early parts of the signaling pathways are distinct. Below, we describe the signal transduction pathway activated by a cell wall component of Gram-positive bacteria, lipoteichoic acid (LTA).

The major MUC2-inducing activity in Gram-positive bacterial supernatant is LTA

To determine whether bacterial exoproducts of Gram-positive pathogens caused upregulation of *MUC2* transcription, we used an approach similar to that already described for the Gram-negative pathogen, *P. aeruginosa*. Culture supernatant prepared from the Gram-positive organisms *S. aureus* and *Streptococcus (S.) pyogenes* stimulated *MUC2*-luciferase activity ∼nine-fold in HM3MUC2 cells. Approximately 90% of the activity was resistant to protease treatment followed by boiling, indicating the activity was not protein in nature. This suggested a key role for lipids or polysaccharides. Teichoic acid and peptidoglycan (PGN) are major polysaccharides in the Gram-positive cell wall [48]. Teichoic acid is also present linked to a lipid moiety as LTA [48]. Direct administration of *S. aureus* or *S. pyogenes* LTA and PGN to epithelial cultures showed that LTA is a potent, and PGN a weak mucin stimulus. This was confirmed by the fact that immunodepletion of LTA from the supernatant yielded large reductions, whereas immunodepletion of PGN yielded small reductions, in the ability of bacterial supernatant to stimulate MUC2-luciferase. Thus, LTA stimulates mucin production and is responsible for most of the MUC-2-inducing activity of Gram-positive bacteria [16].

MUC2 induction by LTA is platelet-activating factor receptor (PAFR)-dependent

Previous reports showed that Gram-positive bacteria anchor themselves to human cells via PAFR [49,50]. This prompted us to ask whether PAFR mediates LTA signaling. LTA, like PAF, caused phosphorylation and internalization of PAFR and PAFR antagonists CV3988 and HAGPH blocked mucin induction by LTA. PAF, itself, also stimulated mucin transcription in a concentration-dependent manner with a maximum potency approximately equal to that of LTA [16]. These results supported the view that LTA stimulates PAFR and thereby stimulates *MUC2*.

PAFR induces mucin via transactivating the epidermal growth factor receptor (EGFR) in a metalloproteinase- and heparin-binding EGF (HBEGF)-dependent manner

PAFR is a G protein-coupled receptor, and thereby potentially capable of signaling through transactivation of EGFR [51–53]. Anti-phosphoprotein immunoblots showed that *S. aureus* supernatant, LTA or PAF, but not *P. aeruginosa* supernatant or lipopolysaccharide (LPS), stimulated phosphorylation of EGFR. Previous work showed that transactivation of EGFR by certain G protein-coupled receptors (GPCRs) requires a metalloproteinase that cleaves transmembrane HBEGF [52]. That both GM6001 (a metalloproteinase inhibitor) and CRM (an HBEGF inhibitor) blocked phosphorylation of EGFR by LTA, suggested that a similar mechanism was occurring in bacterial pathogenesis. Moreover, the same inhibitors strongly attenuated mucin induction by LTA, but not by LPS [16].

To further support these data and to define the specific metalloproteinase involved (at least in the setting of *S. aureus* infection), we tested the effects of morpholino anti-sense oligonucleotides [54] directed against various ADAM metalloproteinases. Results demonstrated a requirement for the membrane metalloproteinase kuzbanian (ADAM 10). While the ADAM 10 anti-sense oligo potently inhibited receptor phosphorylation induced by LTA, however, it had no effect on phosphorylation induced by EGF, indicating specificity. Moreover, neither ADAM 10 oligos with inverted sequence nor anti-sense constructs corresponding to other ADAMs (ADAMs 17, 9 and 15) blocked receptor phosphorylation by LTA. These results identified ADAM 10 as a major control point for epithelial cell responses to LTA. As expected, anti-sense ADAM 10 also blocked the induction of mucin transcription by LTA.

Finally, since inhibition of EGFR transactivation by CRM tentatively identified the membrane protein being cleaved by ADAM 10 as HBEGF, we examined this by monitoring levels of immunoreactive EGFR ligands (EGF, HBEGF, amphiregulin, betacellulin, transforming growth factor alpha and epiregulin) in conditioned medium from LTA-exposed or control NCIH292 cells. Immunoblots showed an LTA-dependent band corresponding to HBEGF and function-perturbing antibodies directed against HBEGF blocked LTA-induced phosphorylation of EGFR [16].

Mucin induction by LTA does not involve Toll-like receptors

Toll-like receptors (TLRs) [55] are a family of conserved pattern recognition receptors that mediate defensive responses to

diverse pathogens. Although *TLR2* and/or *TLR4* have been implicated in LTA-induced responses of macrophages, dendritic cells and HEK 293 cells [56–59], and despite expression of both receptors in our epithelial cell lines, we could not obtain evidence for their involvement in LTA-induced mucin transcription. Transfection with dominant negative (DN) mutants of *TLR2*, *TLR4* or *Myd 88* (an adapter protein essential for TLR signaling [60] failed to inhibit LTA effects in epithelial cells, whereas monocyte responses to LTA were strongly suppressed by DN *TLR2* [56,59]. Thus, epithelial cells and monocytes appear to differ in their responses to LTA.

Convergence of LPS- and LTA-initiated pathways: Requirement for Ras, Mek 1/2, Erk 1/2, pp90 rsk and NFκB

Since mucin induction by LPS is *TLR*-dependent while mucin induction by LTA is not, we were interested to know whether the LPS and LTA pathways intersect. Looking for overlap between the pathways, we exposed cells to LTA in the presence of several of the same inhibitors previously found to attenuate LPS signaling. Inhibitors of Ras (DN-RAS), Mek 1/2 (PD98059 or DN-MEK 1/2), DN-pp90rsk and the NFκB inhibitor CAPE blocked mucin induction by LTA and *S. aureus* supernatant just as they had blocked induction by LPS and *P. aeruginosa* supernatant [21]. This argued strongly that the LPS- and LTA-stimulated pathways converge at Ras to induce Erk 1/2 phosphorylation, NFκB translocation, and ultimately, mucin transcription. To assess the validity of the LTA signaling pathway *in vivo*, we administered LTA to rats intratracheally and prepared tracheal lysates 10 min later to monitor Erk 1/2 phosphorylation. Consistent with results *in vitro*, intratracheal LTA evoked Erk 1/2 phosphorylation in the rat tracheas, and phosphorylation was blocked by the same inhibitors of PAFR, metalloproteinase and EGFR used to inhibit the response pathway *in vitro.*

Upregulation of *MUC5AC* by bacterial pathogens

In addition to the effects of bacterial pathogens on *MUC2* transcription, hypersecretion of mucin in the cystic fibrosis airway can also involve the mucin gene, *MUC5AC*. *MUC5AC* shows relatively high levels of expression even in healthy human airways [61] and shows an altered tissue distribution in CF airways [62]. *In situ* hybridization revealed elevated *MUC5AC* mRNA levels in CF nasal polyps and bronchi with respect to analogous samples from non-CF subjects [63]. This upregulation of *MUC5AC* was observed in both the surface epithelium and submucosal glands of bronchial explants and was more abundant on a per cell basis in CF subjects. Since *P. aeruginosa* is a common pathogen in the CF airway that has been shown to participate in the upregulation of *MUC2*, we exposed bronchial explants to *P. aeruginosa* supernatants for 6 hours *in vitro* and examined *MUC5AC* mRNA by *in situ* hybridization. Not only did we see upregulation of *MUC5AC* RNA, but we also saw elevated levels of mucin protein following exposure of NCIH292 cells to *P. aeruginosa* exoproducts [63].

Since upregulation of *MUC5AC* likely contributes to morbidity and mortality associated with CF, it is important to understand the transcriptional control of this gene. By cloning approximately a 4 kb region of genomic DNA upstream of the transcription start site and subcloning it into the *MluI/SmaI* site of the pGL3 basic luciferase reporter, we constructed a stably transfected cell line containing the *MUC5AC* 5′-flanking region [64]. Using this cell culture model, we observed a 15–20 fold induction of *MUC5AC* transcriptional activity by *P. aeruginosa* or its exoproducts in cell-free supernatants. These results indicated the presence of elements responsive to *P. aeruginosa* in the 4-kb DNA fragment immediately upstream of the *MUC5AC* transcription start site and confirmed that bacterial-induced upregulation of *MUC5AC* is controlled at the level of transcription. This upregulation is mimicked by LPS and can be blocked by the tyrosine kinase inhibitor genistein [64]. Further analysis has shown that *MUC5AC* is inducible by supernatants from a wide variety of Gram-positive bacteria (e.g., *S. aureus* and *S. pyogenes*). Additional studies using the cloned *MUC5AC* 5′-flank will permit precise identification of transcriptional control mechanisms and elucidation of upstream signaling pathways.

Another bacterial agent associated with mucin overproduction in the lung is *Bordatella pertussis*, the causative agent of whooping cough [65]. We collaborated with Belcher and colleagues in microarray expression analysis to show that Bordetella pertussis induces MUC2 RNA in the human bronchial epithelial cell line, BEAS-2B [66]. We confirmed and extended these results in MUC2 and MUC5AC transcription assays, showing that *B. pertussis* stimulated these genes in both a dose-and time-dependent manner. Transcription reached maximum levels of 32-fold and 17-fold, respectively, following 6 hours of exposure to live pathogen. Although mucin production is generally considered protective for the organism, mucin overproduction favors the pathogen by improving its ability to adhere to cell surfaces and providing a nutritional source. More comprehensive examination of transcriptional responses of both the pathogen and its hosts will help define specific strategies used by both and identify potential therapeutic targets.

Nontypeable *Haemophilus influenza* is an important human pathogen that causes chronic otitis media in children and exacerbation of chronic obstructive pulmonary disease (COPD) in adults [67,68]. A common hallmark of both diseases is mucin overproduction, which can lead to conductive hearing loss in otitis media and airway obstruction in COPD. We collaborated with Wang and colleagues to uncover information regarding the transcriptional activation of *MUC5AC* by *H. influenza* [69]. Specifically, we showed that bacterial cytoplasmic proteins upregulate *MUC5AC* maximally, whereas surface membrane proteins induce *MUC5AC* only weakly. The bacterial cytoplasmic proteins stimulate *MUC5AC* via p38; in contrast, the phosphoinositide 3-kinase-Akt pathway acts as a negative regulator. The inductive role of bacterial cytoplasmic proteins

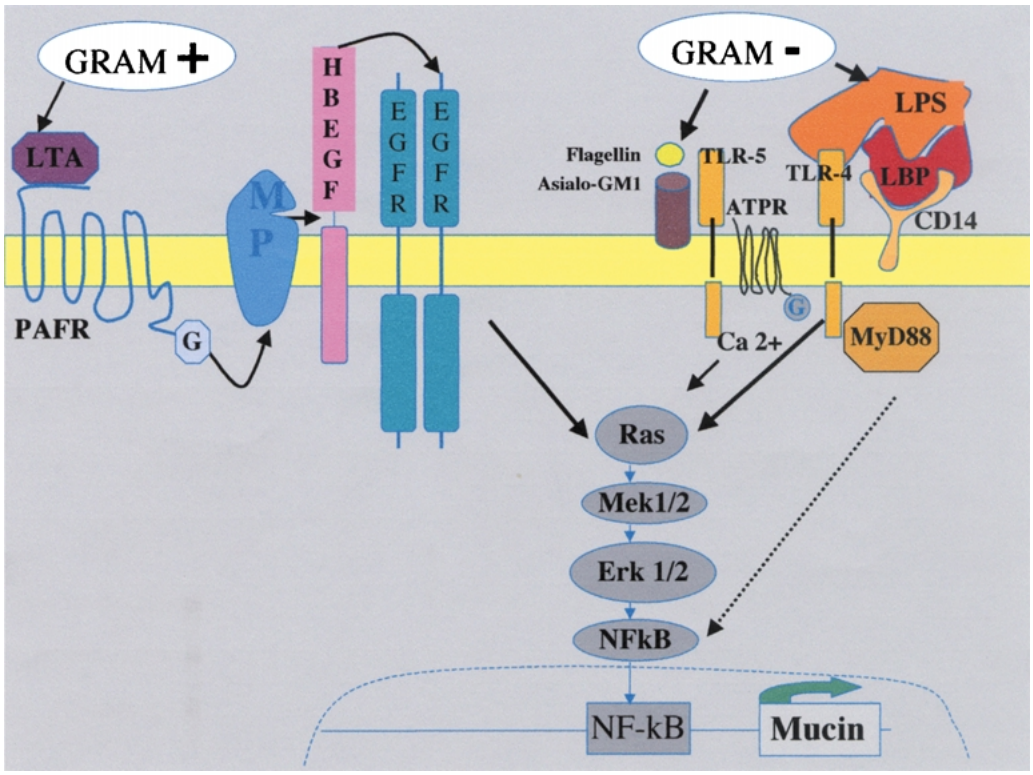

Figure 1. Convergence of signaling pathways initiated by LTA, flagellin and LPS. LTA contacts the host cell surface by binding to the PAF receptor (PAFR). Through a heterotrimeric G protein, this results in the activation of the metalloproteinase (MP) ADAM 10 (kuzbanian). Kuzbanian cleaves HBEGF, freeing it to ligate a cell surface EGF receptor, initiating signaling through Ras, Raf, Mek, Erk and NFκB. Flagellin contacts the cell surface via binding to a glycolipid, asialo-GM1. A consequence of this is autocrine activation of an ATP receptor (ATPR). Preliminary data suggest that Toll-like receptor 5 (TLR-5) is required for this interaction, which in turn results in the mobilization of intracellular Ca^{2+} and activation of Erk 1/2. LPS contacts the cell surface through binding to the serum protein LBP, which in turn binds to the lipoprotein CD14. CD14 uses TLR-4 as a co-receptor, thereby engaging cytoplasmic signaling molecules including Myd 88 that eventuate in the activation of NFκB.

means that antibiotic therapies leading to bacterial lysis should increase mucin production in the short term. New therapeutic approaches will have to take this into account.

Discussion

Our studies reveal previously unknown signaling pathways that mediate defensive responses of epithelial cells to Gram-positive and Gram-negative organisms (see Figure 1). The work introduces two novel concepts in bacterial pathogenesis. First, distinct bacterial agents such as LPS, flagellin and LTA bind to distinct receptors on a given cell and signal via convergent pathways to activate common defense mechanisms such as mucin production. Second, a single bacterial agent (e.g., LTA) stimulates defensive responses by two distinct pathways in macrophages versus epithelial cells (one requiring *TLR*s, the other not).

Within the specific context of mucin production in bacterial pathogenesis, it is possible to note significant common themes. For example, the signaling pathway by which flagellin stimulates mucin transcription is similar to that stimulated by both LPS [21] and LTA [16], in that it requires activation of Erk 1/2. Convergence of these bacterial recognition pathways on Erk 1/2 is consistent with the idea that mucin activation pathways evolved from a single phylogenetically ancient network [70]. Presumably, conserved mutations have allowed the host to develop responses to new pathogens. This can be appreciated by noting the similarities and differences between signaling pathways triggered by LPS, flagellin and LTA.

With respect to cystic fibrosis, there is no doubt that lung mucin plays a central role in morbidity and mortality. Mucin is now understood as a contributor to both airflow obstruction and antibiotic resistance [12]. Thus, therapeutic inhibition of mucin production has the potential to not only reduce airflow obstruction, but also to stem the emergence of antibiotic-resistant bacterial strains.

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