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The *Moraxella catarrhalis*-induced pro-inflammatory immune response is enhanced by the activation of the epidermal growth factor receptor in human pulmonary epithelial cells

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ABSTRACT

Background: Chronic lower airway inflammation is considered to be a major cause of pathogenesis and disease progression in chronic obstructive pulmonary disease (COPD). *Moraxella catarrhalis* is a COPD-associated pathogen causing exacerbations and bacterial colonization in the lower airways of patients, which may contribute to chronic inflammation. Increasing evidence suggests that the epidermal growth factor receptor (EGFR) modulates inflammatory processes in the human airways. The goal of this study was to investigate the role of EGFR in the *M. catarrhalis*-induced pro-inflammatory immune response in airway epithelial cells.

Methods: The effects of inhibition and gene silencing of EGFR on *M. catarrhalis*-dependent pro-inflammatory cytokine expression in human primary bronchial epithelial cells (NHBEs), as well as the pulmonary epithelial cell lines BEAS-2B and A549 were analyzed. We also assessed the involvement of EGFR-dependent ERK and NF-κB signaling pathways.

Results: The *M. catarrhalis*-induced pro-inflammatory immune response depends, at least in part, on the phosphorylation and activation of the EGF receptor. Interaction of *M. catarrhalis* with EGFR increases the secretion of pro-inflammatory cytokines, which is mediated via ERK and NF-κB activation.

Conclusion: The interaction between *M. catarrhalis* and EGFR increases airway inflammation caused by this pathogen. Our data suggest that the inhibition of EGFR signaling in COPD could be an interesting target for reducing *M. catarrhalis*-induced airway inflammation.

1. Introduction

1.1. EGFR expression in the airways in COPD

Chronic obstructive pulmonary disease (COPD) is associated with chronic neutrophilic inflammation of the lower airways [1]. The expression of epidermal growth factor receptor (EGFR) is increased in the airway epithelium of COPD patients [2]. EGFR activation is involved in repair and regeneration processes in airway epithelial cells, but has also been shown to be linked to the expression of pro-inflammatory cytokines and chemokines (e.g., via activation of MAPK pathways [3,4]). In particular, increased secretion of the chemokine interleukin (IL) 8 has a considerable impact on neutrophil recruitment in the airways and pulmonary tissues [4–8]. Chronic inflammation with the involvement of neutrophils leads to airway remodeling and progressing airflow limitation and so is believed to play a major role in the pathogenesis and progression of COPD [1,9]. However, current pharmacological therapies only have limited influence on neutrophil recruitment and chronic inflammation.

1.2. The role of bacterial infection in COPD

As the first line of pathogen defense in the human lung, the epithelium of the lower airways is equipped with multiple mecha-

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nisms of the innate immune system. In healthy subjects, the lower respiratory tract is considered to be sterile and bacteria can only be detected in around 4% of these individuals [10]. Due to chronic inflammation, mucus retention, and hypersecretion, defense mechanisms in the lower airways of COPD patients deteriorate. In particular, the function of the mucociliary clearance as a centrally important defense mechanism for removing invading pathogens from the lower airways is restricted by the chronic inflammatory process, leading to bacterial colonization of the lower airways [11]. The three most frequently detected bacterial pathogens in both stable and exacerbated COPD are *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* [10]. *M. catarrhalis* is responsible for 10–15% of exacerbations [12], colonizes in up to 32% of COPD patients [13,14], and is believed to play an important role in maintaining chronic inflammation and promoting disease progression [12].

1.3. Interaction of EGFR and pathogens

M. catarrhalis induces a pro-inflammatory immune response, which is dependent on mitogen activated protein kinase (MAPK) extracellular-signal regulated kinase (ERK), and activation of the transcription factor nuclear factor κB (NF-κB) [15]. Interestingly, EGFR is an important receptor that is known to activate the cells via ERK signaling pathways [3]. Several authors have reported the activation of EGFR by bacterial or viral pathogens [16–18]. *In vitro* studies have also indicated regulatory functions of EGFR on pro-inflammatory IL8 secretion via ERK signaling in infected airway epithelial cells [18,19]. We hypothesized that *M. catarrhalis* might induce a pro-inflammatory immune response by activation of EGFR in airway epithelium.

We therefore investigated the role of EGFR in the *M. catarrhalis*-induced pro-inflammatory immune response in airway epithelial cells. In addition, the possible involvement of dependent ERK and NF-κB signaling pathways was evaluated.

2. Materials and methods

2.1. Cell lines

Normal human bronchial epithelial cells (NHBEs) from healthy volunteers were obtained from Lonza (Cologne, Germany). Human bronchial epithelial cell line BEAS-2B was kindly donated by C. Harris (National Institutes of Health, Bethesda, MD, USA). A549 epithelial cells (type II alveolar lung epithelium cells; ATCC CCL85) were obtained from ATCC.

The NF-κB-dependent reporter cell line, A549 6Btkluc, was kindly given to us by R. Newton (University of Warwick, Coventry, UK). These cells contain a stably integrated plasmid with three tandem repeats of the sequence 5′-AGCTTACAAGGGATTTCCGCTGGGG ACTTTCCAGGGA-3′, which contains two copies of the decameric NF-κB binding site upstream of a minimal thymidine kinase promoter (-105–51) driving a luciferase gene 27.

All cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cells were grown to confluence (over 100%) in 75 cm^2 flasks and then were cultured in multi-well plates (both Falcon and Corning Star). Twelve hours before the experiment, cells were grown in a medium without antibiotic supplements.

2.2. M. catarrhalis strains

M. catarrhalis wild type strain O35E (serotype A) was kindly provided by Eric Hansen (University of Texas Southwestern Medical Center, Dallas, TX, USA). *M. catarrhalis* strain 25238 was purchased from ATCC. Strains were grown overnight at 37 °C in a humidified atmosphere with 5% CO_2 on Columbia agar with 5% sheep blood (BD Diagnostics, Germany). For infection experiments, single colonies of bacterial overnight cultures were expanded by re-suspension in BHI broth and incubation at 37 °C for 2 h, harvested by centrifugation, re-suspended in cell culture medium without antibiotics, and adjusted to an optical density (OD) at 405 nm of 0.3 [$\approx 1 \times 10^8$ colony-forming units (cfu)/ml] and used for infecting the cells at the indicated cfu/ml or multiplicity of infection (MOI).

2.3. IL8 and IL6 ELISA

Confluent BEAS-2B cells were stimulated as indicated. IL8 or IL6 secreted by the cells were evaluated with a commercially available ELISA kit according to the manufacturer's protocol (IL8 ELISA, BD Biosciences; IL6 ELISA, Ebioscience).

2.4. RT-PCR analysis

RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. All primers were purchased from TIB MOLBIOL (Berlin, Germany). PCR products were analyzed on 1.5% agarose gels, stained with ethidium bromide, and subsequently visualized. To confirm equal loading, PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed in parallel.

2.5. Western Blot analysis

Cells were lysed in buffer-containing Triton X-100, separated by SDS–PAGE, and blotted on Hybond-ECL membranes (Amersham Biosciences). Specific antibodies, as well as corresponding secondary antibodies labeled with IRDye (Li-COR Biosciences), were used for immunodetection of target proteins. Actin or Akt were measured simultaneously on the same membrane to confirm equal protein loading. Membranes were scanned and quantified with two different channels (channel 800, green; channel 700, red; Odyssey Infrared Imaging System; Li-COR Biosciences). The intensities of the green and the red channels were quantified by integrated image analysis.

2.6. RNA-mediated interference

Control non-silencing siRNA (siRNA control, MWG Biotech AG, Germany) and siRNA targeting EGFR mRNA (siRNA EGFR silencer select® validated, Ambion) were used. A549 or BEAS-2B cells were transfected with the Amaxa Nucleofector kit (Amaxa) according to the manufacturer's protocol (Nucleofector Solution V; Nucleofector program G-16) with EGFR siRNA at a dose of 2 μg (25 nM) per 1×10^6 cells as described previously [20].

2.7. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was carried out as described previously [15]. Briefly, cells were lysed in ChIP radio-immunoprecipitation assay (RIPA) buffer and chromatin was sheared by sonication. All antibodies were purchased from Santa Cruz Biotechnology. Immunoprecipitations from soluble chromatin were carried out overnight at 4 °C. Immune complexes were collected with protein A/G agarose (Santa Cruz Biotechnology) for 60 min and washed thoroughly with RIPA buffer and high-salt buffer. We extracted immune complexes in elution buffer which were then digested with RNase for 30 min at 37 °C. After proteinase K digestion for 6 h at 37 °C and 6 h at 65 °C, DNA was extracted with a PCR purification kit (Qiagen, Hilden, Germany). IL8 promoter DNA was amplified by PCR using Hot Star Taq (Qiagen) DNA

polymerase. PCR products were separated by agarose gel electrophoresis and detected by ethidium bromide staining. Equal amounts of input DNA were controlled by gel electrophoresis.

2.8. Inhibitors

EGFR tyrosine kinase inhibitor AG1478, ERK inhibitor U0126, and IKK-NBD peptide (inhibiting NF-κB activation) were purchased from Calbiochem (Merck, Bad Soden, Germany).

2.9. Luciferase activity

Luciferase activity was measured using a luciferase reporter gene assay (Promega, Mannheim, Germany). All other chemicals used in the study were of analytical grade and obtained from commercial sources.

2.10. Statistical analysis

Data are shown as means ± s.e.m. of at least three independent experiments. A one-way ANOVA followed by a Newman–Keuls post-test was used to compare means of two samples. Statistical significance was considered to be reached for $p < 0.05$, as indicated by asterisks.

3. Results

3.1. M. catarrhalis activates EGFR in pulmonary epithelial cells

Firstly, we analyzed the EGFR expression in primary human bronchial epithelial cells (NHBE), as well as in the pulmonary epithelial cell lines BEAS-2B and A549 via Western Blot and RT-PCR. As shown in Fig. 1A and B in all epithelial cells EGFR expression was confirmed.

Upon activation of EGFR, phosphorylated receptor tyrosine residues serve as docking sites of a range of proteins, whose recruitment activates downstream signaling pathways [3]. In a Western Blot analysis we demonstrated that *M. catarrhalis* causes a considerable phosphorylation of EGFR tyrosine residues and that this effect increases with time (Fig. 1C). Inhibition of EGFR with selective EGFR tyrosine kinase inhibitor AG1478 led to a significant decrease in *M. catarrhalis*-induced EGFR phosphorylation (Fig. 1D).

3.2. Inhibition or knock-down of EGFR decreases M. catarrhalis-induced IL8

In a next step, we were able to demonstrate that inhibition of EGFR with AG1478 causes a significant decrease in *M. catarrhalis* induced IL8 and IL6 secretion in NHBE as assessed by an ELISA essay (Fig. 2A and B). These data suggested a considerable impact of EGFR on the pro-inflammatory immune response towards *M. catarrhalis* in the human airways.

To confirm the regulatory function of EGFR for the *M. catarrhalis*-induced immune response in pulmonary epithelial cells, we performed RNA-mediated interference experiments. The viability of NHBEs was much lower during the gene-knockout procedure, because of the transfection reagents (data not shown). Therefore, we used the pulmonary epithelial cell line BEAS-2B. Firstly, we confirmed the EGFR-dependent *M. catarrhalis*-induced IL8 secretion in BEAS-2B cells, which again was reduced after pretreatment of the cells with the selective EGFR inhibitor AG1478, as assessed by RT PCR (Fig. 2C) and ELISA (Fig. 2D). Knock-down of EGFR expression via RNA interference was confirmed by RT PCR (Fig. 2E) and revealed a significant decrease in IL8 secretion after infection with *M. catarrhalis* (Fig. 2F).

3.3. The EGFR-dependent IL8 response is mediated via ERK and NF-κB signaling

In a next step we analyzed specific pathways for their involvement in the *M. catarrhalis*-induced and EGFR-dependent cytokine release. As shown in Fig. 3A, in pulmonary epithelial cells infected with *M. catarrhalis,* inhibition of ERK by U0126 resulted in a significant decrease in IL8 secretion (Fig. 3A). Involvement of ERK phosphorylation was further confirmed by Western Blot. Our results revealed that the infection of the cells with *M. catarrhalis* causes phosphorylation of ERK and that this effect could be reduced by the inhibition of EGFR (Fig. 3B).

Moreover, we found that the activation of transcription factor NF-κB was also involved in the EGFR-dependent immune response

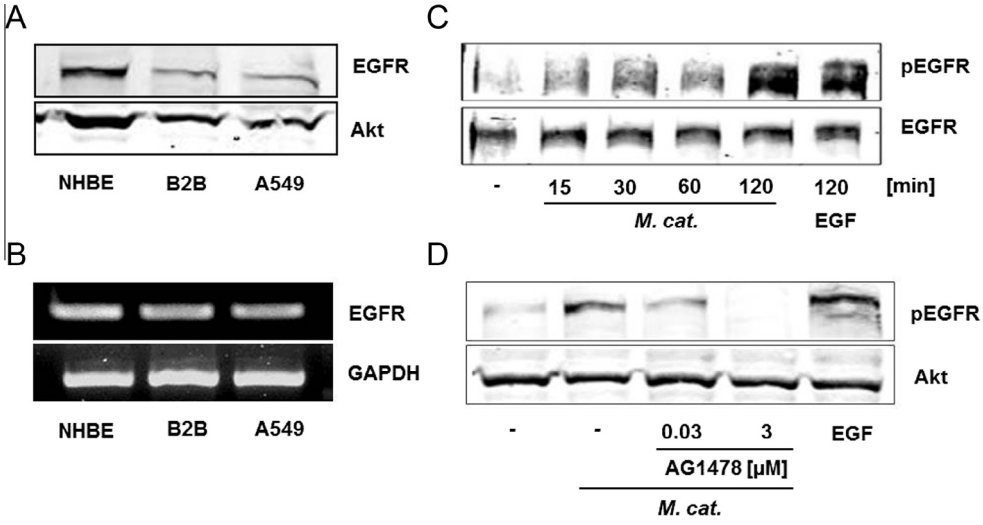

Fig. 1. (A) Immunoblot analysis of EGFR in cell lysates of NHBE, BEAS-2B, and A549 cells. Protein kinase Akt served as a loading control. (B) RT-PCR analysis of EGFR mRNA in cell lysates of NHBE, BEAS-2B, and A549 cells. (C) BEAS-2B cells were infected with *M. catarrhalis* (10^7 cfu/ml) for the periods of time indicated. EGF (25 ng/ml) was used as a stimulation control. Cell lysates were analyzed for phosphorylated EGFR (pEGFR) by Western Blot. Detection of EGFR served as a loading control. (D) BEAS-2B cells were pretreated as indicated with EGFR inhibitor AG1478 2 h before infection with *M. catarrhalis* (10^8 cfu/ml). After 2 h, cell lysates were analyzed for phosphorylated EGFR (pEGFR) by Western Blot. EGF (100 ng/ml) was used as a stimulation control. Detection of Akt served as a loading control.

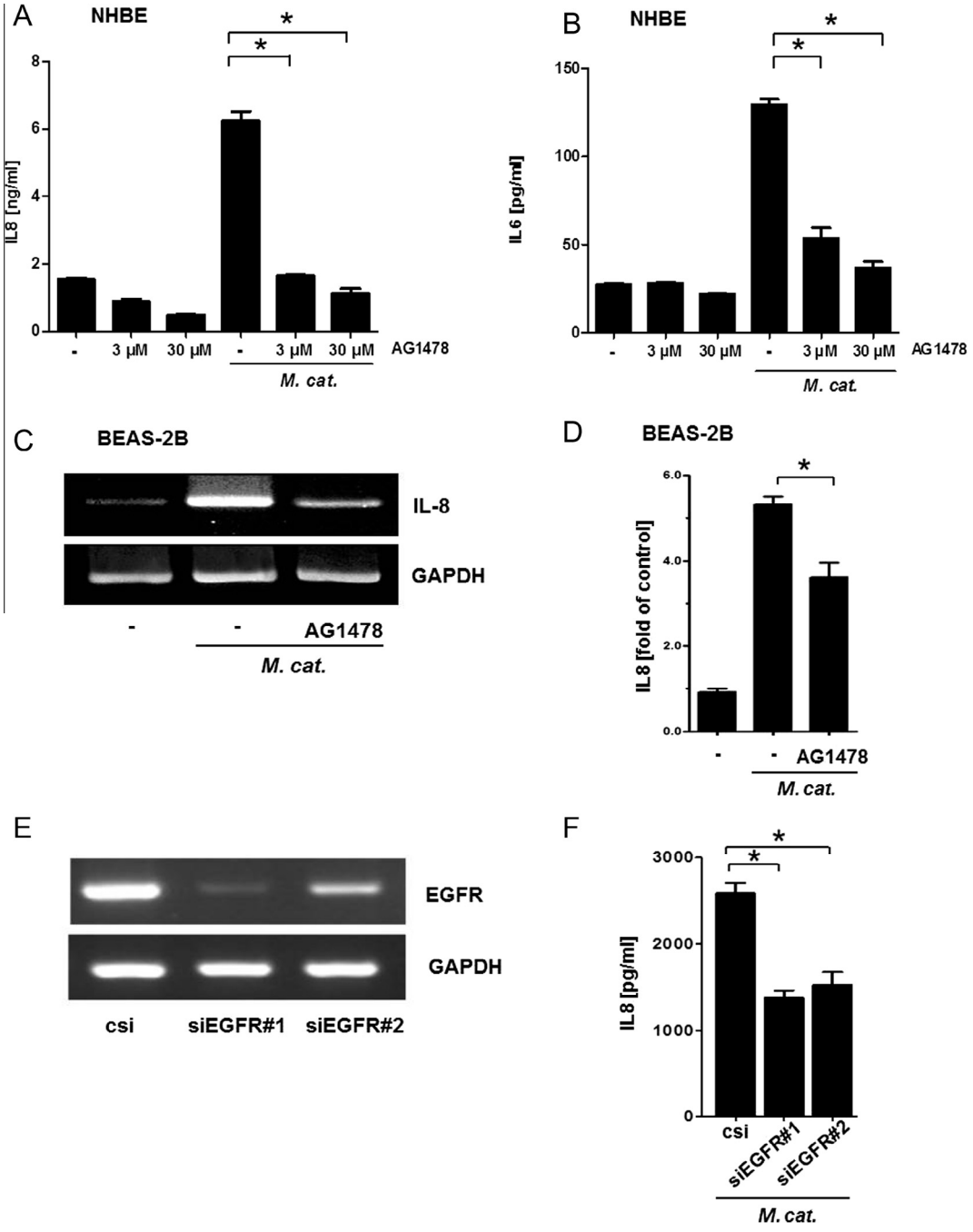

Fig. 2. (A/B) Confluent NHBE cells were pretreated with the doses of EGFR inhibitor AG1478 indicated 1 h before infection with *M. catarrhalis* (MOI of 0.1). After 24 h, concentrations of IL8 (A) or IL6 (B) were assessed in the supernatants by ELISA. (C) Confluent BEAS-2B cells were pretreated as indicated with AG1478 (30 µM) 2 h before infection with *M. catarrhalis* (10^7 cfu/ml). After 12 h IL8 mRNA was detected by RT-PCR. (D) Confluent BEAS-2B cells were pretreated as indicated with AG1478 (3 µM) 2 h before infection with *M. catarrhalis* (10^7 cfu/ml). After 16 h, IL8 was assessed in the supernatants by ELISA. (E) BEAS-2B cells were transfected with control siRNA (csi) or two different types of EGFR siRNA (siEGFR#1; siEGFR#2). After 48 h, EGFR silencing was confirmed by RT-PCR. (F) 48 h after transfection, cells were infected with *M. catarrhalis* (MOI 0.1). After 24 h, IL8 concentration in supernatants was assessed by ELISA. $^*p < 0.05$ in one-way ANOVA.

towards *M. catarrhalis*. As shown in Fig. 3A, inhibition of NF-κB by inhibitory-κB-kinase NF-κB-essential-modulator-binding-domain peptide (IKK-NBD) also led to a decrease in *M. catarrhalis*-induced cytokine secretion. In addition, NF-κB activation was confirmed by making use of a NF-κB-dependent reporter cell line (A549 6Btkluc). Infection of the cells resulted in a profound increase in NF-κB-dependent luciferase activity which decreased after incubation of the cells with the selective EGFR inhibitor AG 1478 (Fig. 3C). Inhibition of EGFR also resulted in reduced binding of the NF-κB sub-unit p65 and RNA polymerase II (Pol II) to the IL8 Promoter (Fig. 3D). Our data show that *M. catarrhalis* induces an EGFR-dependent, pro-inflammatory immune response which is mediated via activation of ERK and NF-κB.

3.4. EGFR induced IL8 is not strain specific or dependent on the viability of the pathogen

Next we analyzed whether the *M. catarrhalis*-induced and EGFR-dependent pro-inflammatory immune response was dependent on different *M. catarrhalis* strains.

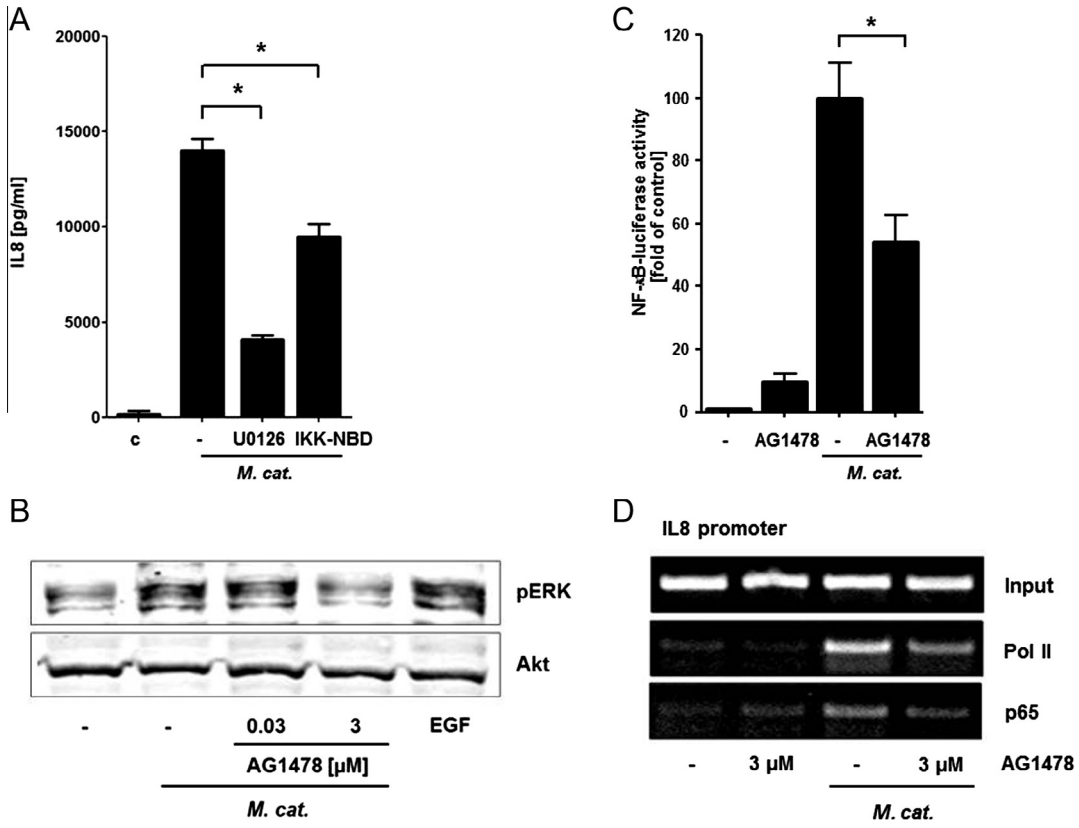

Fig. 3. (A) Confluent BEAS-2B cells were pretreated with the inhibitor U0126 and IKK-NBD peptide at a concentration of 10 μM 1 h before the infection with *M. catarrhalis* (10^6 cfu/ml). After 24 h, IL8 was assessed in the supernatants by ELISA. (B) BEAS-2B cells were pretreated as indicated with EGFR inhibitor AG1478 2 h before infection with *M. catarrhalis* (10^8 cfu/ml). After 2 h, cell lysates were analyzed for phosphorylated ERK (pERK) by Western Blot. EGF (100 ng/ml) was used as a stimulation control. Detection of Akt served as a loading control. Fig. 3B and Fig. 1D share data from the same experiment. (C) Confluent cells of the NF-κB-dependent reporter cell line A549 6Btkluc were pretreated as indicated with AG1478 (3 μM) 1 h before the infection with *M. catarrhalis* (10^7 cfu/ml). After 5 h, luciferase activity was measured. (D) BEAS-2B were pretreated as indicated with AG1478 (3 μM) 2 h before infection with *M. catarrhalis* (10^8 cfu/ml). After 4 h, DNA binding of RNA polymerase II (Pol II) or p65 to the IL8 promoter was analyzed using a ChIP assay. $^*p < 0.05$ in one-way ANOVA.

Using RNA interference, we knocked down the expression of EGFR in A549 cells. Subsequently, cells were infected with *M. catarrhalis* wild type strain O35E, ATCC strain 25238, and heat-killed O35E. Expression of IL8 was assessed via RT-PCR. Knock down of EGFR significantly reduced IL8 expression in response to all pathogens used (Supplementary Fig. 1).

Consequently, our data indicate that the interaction of *M. catarrhalis* with EGFR was not strain specific. We also found that signaling via EGFR activation is not only a feature of vivid bacteria, since heat killed *M. catarrhalis* also caused an EGFR-dependent IL8 response.

4. Discussion

In this study, we were able to demonstrate that *M. catarrhalis* causes a considerable immune response in pulmonary epithelial cells which is dependent on the expression of EGFR. Moreover, we found an activation of EGFR by *M. catarrhalis* via phosphorylation and subsequent activation of the cells by the ERK pathway, as well as by NF-κB. Our data suggest that the interaction of *M. catarrhalis* with EGFR might have an impact on the immune response and the extent of *M. catarrhalis*-induced airway inflammation in COPD patients.

4.1. Activation of EGFR by M. catarrhalis

Several authors have reported the activation of EGFR by bacterial or viral pathogens [16–18]. So far, there is only a small amount of evidence that the pathogens themselves or components of them directly bind to EGFR [21,22]. However, the EGFR tyrosine kinase can be transactivated by cross-communicating signaling pathways [3], among which toll-like receptors (TLRs) signaling pathways have been identified [23]. TLRs are expressed in airway epithelium and recognize pathogen-associated molecular patterns of various bacteria, viruses, and fungi [24]. Koff et al. recently demonstrated that TLR1/2, TLR3, TLR5, and TLR2/6 communicate with EGFR via a complex, integrated signaling cascade [23]. Upon TLR stimulation, airway epithelial cells release the EGFR ligand transforming growth factor (TGF)-α by activating metalloprotease TNF-alpha-converting enzyme (TACE) [23]. Transactivation of EGFR by *M. catarrhalis* via this pathway seems likely, as the bacterium is known to be an activator of TLR1/2 and TLR2/6 in airway epithelial cells [20]. Beswick et al. demonstrated that, in gastric epithelium, macrophage migration inhibitory factor (MIF) and IL8 also induced phosphorylation of EGFR signaling during *Helicobacter pylori* exposure [25]. Therefore, it is possible that secreted IL8 may also contribute to EFGR activation in airway epithelium during *M. catarrhalis* infections.

4.2. Modulating effects of EGFR on pro-inflammatory signaling

EGFR activation is associated with an increase in IL8 expression of airway epithelial cells [6,7,18,19]. Accordingly, IL8 secretion has been found to be positively correlated with the extent of EGFR expression in bronchial biopsies of patients with severe obstructive lung disease [8].

In our study, we demonstrated that *M. catarrhalis* activates ERK in airway epithelial cells and induces pro-inflammatory cytokine responses [20]. We showed that the *M. catarrhalis*-induced EGFR activation seems to play a major role in activating ERK and ERK-dependent IL8 expression. EGFR is known as a main ERK activator [3] and the involvement of EGFR/ERK signaling pathways has been reported in various cellular innate immune responses [18,19,26–28]. Recent research suggests that constitutive or aberrant activation of ERK contributes to several COPD-associated phenotypes, including mucus overproduction and secretion, cytokine expression and inflammation [29].

Chronic inflammation in the lower airways is characteristic for COPD [1]. NF-κB has a key role in regulating various pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukins, and cyclooxygenases [30]. The expression of NF-κB sub-unit p65 is positively correlated with disease severity in COPD [31]. NF-κB is activated by the most prevalent bacterial pathogens in COPD *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* [15,32,33]. In our study, *M. catarrhalis* activated NF-κB over an EGFR-dependent pathway. An ERK dependent activation of IκB kinase may be a possible underlying mechanism for this observation [34].

4.3. Possible role of EGFR activation in COPD

Our data support our previous work that *M. catarrhalis* causes a pro-inflammatory immune response in airway epithelial cells *in vitro* [15]. Therefore, it can be presumed that the colonization of this pathogen in the lower airways of COPD patients contributes to chronic inflammation [12]. EGFR expression is increased in the airways of COPD patients [4]. We found that activation of EGFR by *M. catarrhalis* induces pro-inflammatory IL8, which is known as a chemo-attractant for neutrophil transmigration into the airway tissue, thereby causing inflammation and recurring destruction and repair processes, which lead to airway remodeling, continuing airway limitation, and consequently disease progression [1,9]. It is likely that the interaction with EGFR serves as an important trigger of chronic airway inflammation in the lower airways of COPD patients that are colonized or infected with *M. catarrhalis*. New therapies target EGFR-mediated signaling with the aim of reducing mucus hyper-secretion in COPD patients [35]. Therefore, modulation of EGFR-induced airway inflammation caused by *M. catarrhalis* and possibly other bacterial airway pathogens may also be an interesting strategy for treating COPD and so should be further explored.

We demonstrated that the infection of airway epithelial cells with *M. catarrhalis* activates EGFR, which leads to an ERK and NF-κB-mediated pro-inflammatory immune response. Our study suggests that the inhibition of EGFR signaling in COPD could be an interesting target for reducing airway inflammation caused by this bacterial pathogen.

Conflict of interest statement

All authors declare no conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.102.

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