

# Infection with human coronavirus NL63 enhances streptococcal adherence to epithelial cells

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Understanding the mechanisms of augmented bacterial pathogenicity in post-viral infections is the first step in the development of an effective therapy. This study assessed the effect of human coronavirus NL63 (HCoV-NL63) on the adherence of bacterial pathogens associated with respiratory tract illnesses. It was shown that HCoV-NL63 infection resulted in an increased adherence of *Streptococcus pneumoniae* to virus-infected cell lines and fully differentiated primary human airway epithelium cultures. The enhanced binding of bacteria correlated with an increased expression level of the platelet-activating factor receptor (PAF-R), but detailed evaluation of the bacterium–PAF-R interaction revealed a limited relevance of this process.

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## INTRODUCTION

The concept of excessive morbidity and mortality of bacterial infection occurring during or shortly after viral infection was first formulated for influenza virus in the early 19th century. Analysis of influenza pandemics showed that the incidence of bacterial pneumonia was increased and contributed substantially to mortality rates (Abrahams *et al.*, 1919; Muir & Wilson, 1919; Stone & Swift, 1919; Wilson & Steer, 1919). Comparison of bacteriological and virological data from children hospitalized for respiratory disease shows a high degree of occurrence of viral and bacterial infections positively correlating with the severity of illness (Duttweiler *et al.*, 2004; Kneyber *et al.*, 2005; Randolph *et al.*, 2004; Thorburn *et al.*, 2006). Although the role of a preceding viral infection in development and severity of bacterial respiratory diseases is a clinically well-documented phenomenon, the exact mechanism has not been elucidated fully.

Initially, it was proposed that respiratory viruses facilitate bacterial colonization through physical damage of the respiratory tract epithelium, with exposed basement membrane components being responsible for increased bacterial adherence (Louria *et al.*, 1959; Muir & Wilson, 1919; Wilson & Steer, 1919; Wolbach, 1919). Such a mechanism undoubtedly occurs for highly pathogenic viral species, but it does not explain the occurrence of increased severity of bacterial infection during and shortly after relatively mild viral infections. Analysis of published data

suggests that interplay between viruses and bacteria is a complex process, where the final outcome depends heavily on multiple factors, including modulation of innate immune responses resulting in delayed clearance of bacteria, hypersensitization of infected cells leading to enhanced immune-mediated lung damage and modulation of bacterial adherence (Okamoto *et al.*, 2004). The increase in bacterial adherence occurs due to exposure of novel binding sites for bacteria on the epithelial surface, either by expression of highly glycosylated viral proteins (McCullers & Bartmess, 2003; Peltola & McCullers, 2004) or by the alteration of a bacterial receptor expression pattern (McCullers & Rehg, 2002; Patel *et al.*, 1995; Terajima *et al.*, 1997).

Bacterial pathogens predominantly involved in secondary infection of the respiratory tract are recruited from respiratory tract pathogens, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In addition, they may include pathogens involved in the development of aspiration pneumonia. Aspiration pneumonia is commonly caused by anaerobic bacteria derived from the supra- or subgingival dental plaque (Brook & Frazier, 1993), with *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and *Fusobacterium nucleatum* as the most prevalent pathogens (Brook, 2002; Carlisle *et al.*, 2009; Finegold, 1991; Potempa & Pike, 2009; Scannapieco, 1999; Schreiner, 1979).

Mixed viral/bacterial infections have been studied *in vitro* and *in vivo*. Influenza virus infections exacerbate the severity of secondary streptococcal pneumonia, and increased pneumococcal adherence is proposed to be mediated at least partially by expression of the influenza virus neuraminidase protein. This protein promotes adherence and invasion of *Streptococcus pneumoniae* by cleavage of sialic acid from the surface of host cells, resulting in exposure of cryptic receptors for pneumococci (McCullers & Bartmess, 2003; Peltola *et al.*, 2005; Tong *et al.*, 2001). Other studies show a positive interaction between respiratory syncytial virus (RSV) infection and *Streptococcus pneumoniae*, *H. influenzae* and *Pseudomonas aeruginosa*. RSV is known to upregulate the surface expression of cellular receptors for these bacteria, namely intercellular adhesion molecule 1 (ICAM-1), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) and platelet-activating factor receptor (PAF-R), and it was reported previously that the virus is able to act as a coupling agent between bacteria (*Streptococcus pneumoniae* and *Pseudomonas aeruginosa*) and epithelial cells (Avadhanula *et al.*, 2006, 2007; Hament *et al.*, 2004, 2005; Van Ewijk *et al.*, 2007). Furthermore, a pathogenic synergism between rhinovirus and *Staphylococcus aureus* and *Streptococcus pneumoniae* has been identified. It was demonstrated that human rhinovirus infection promotes internalization of *Staphylococcus aureus* into epithelial cells by the secretion of inflammatory cytokines [interleukin (IL)-6 and IL-8] and overexpression of ICAM-1 on infected cells. Infection with rhinovirus stimulates adherence of *Streptococcus pneumoniae* to human tracheal epithelial cells via binding to overexpressed PAF-R molecules (Ishizuka *et al.*, 2003; Passariello *et al.*, 2006). In contrast, it was shown that increased adhesion of pneumococcus to influenza virus-infected cells is not PAF-R dependent (McCullers & Rehg, 2002; McCullers *et al.*, 2008). It was also confirmed that adenovirus infections facilitate *Streptococcus pneumoniae* colonization by increasing bacterial binding to epithelial cells (Håkansson *et al.*, 1994).

Although many studies on viral and bacterial co-infections have been performed to date, no data are available on human coronaviruses. Human coronavirus NL63 (HCoV-NL63) is a relatively recently discovered human respiratory pathogen with a high worldwide prevalence (Fouchier *et al.*, 2004; Golda & Pyrc, 2008; Hofmann *et al.*, 2005; Pyrc *et al.*, 2004, 2006, 2007a, b, 2008; Schildgen *et al.*, 2006; van der Hoek *et al.*, 2004, 2006). Arguably, HCoV-NL63 is among the most clinically important human coronaviruses and is associated with upper and lower respiratory tract infections, occurring most frequently in the winter season and presenting more severe symptoms in children, the elderly and immunocompromised patients (Bastien *et al.*, 2005; Chiu *et al.*, 2005; Dijkman *et al.*, 2008; Kaiser *et al.*, 2005; Suzuki *et al.*, 2005; Vabret *et al.*, 2005; Wu *et al.*, 2008). HCoV-NL63 is currently considered to be the major pathogen involved in the development of croup in young children (van der Hoek *et al.*, 2005).

In this study, we evaluated the effect of HCoV-NL63 on adherence of a number of bacterial species to epithelial cells and showed that coronavirus infection results in increased adherence of *Streptococcus pneumoniae* to epithelial cells.

## RESULTS

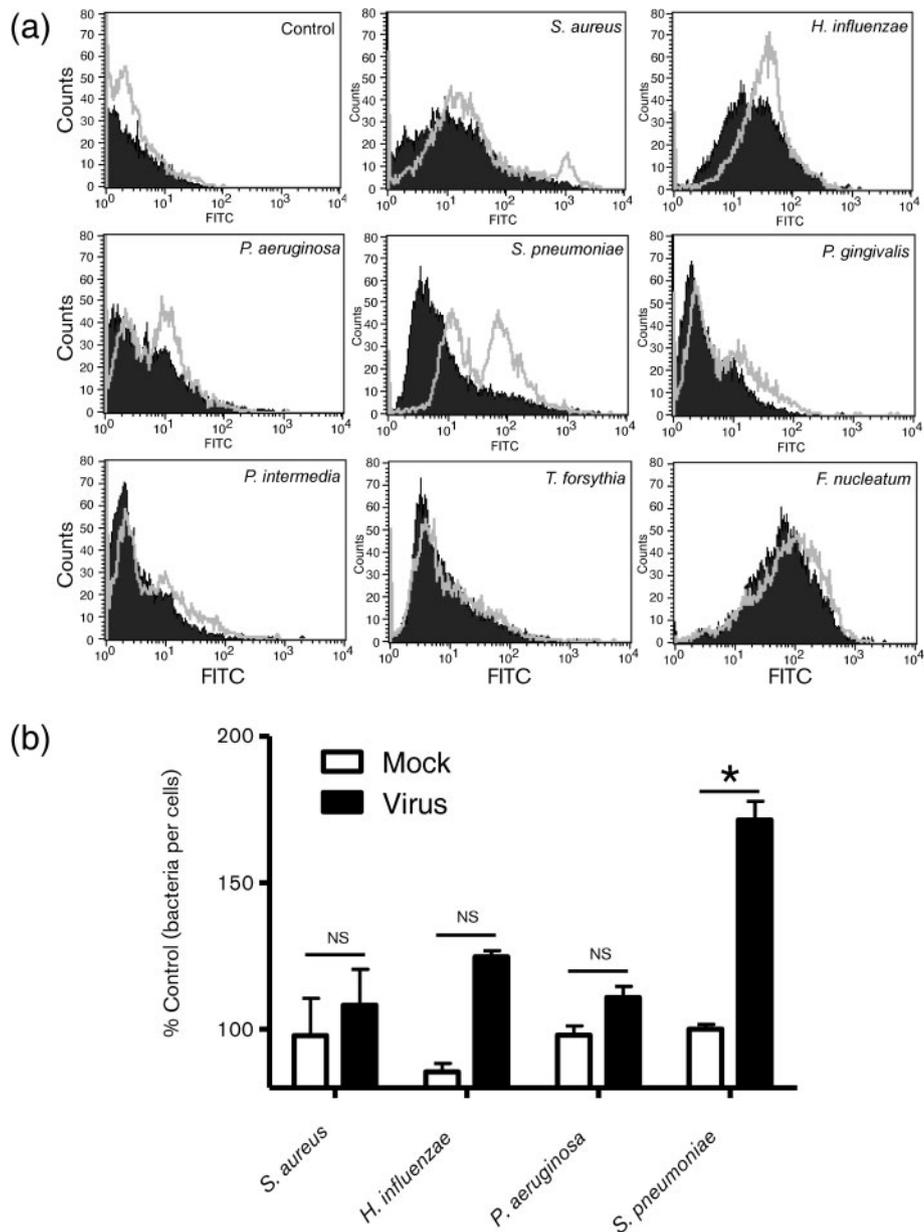
### Effect of HCoV-NL63 infection on adherence of bacteria to LLC-MK2 cells

The *in vitro* adherence of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *H. influenzae*, *Streptococcus pneumoniae*, *Porphyromonas gingivalis*, *T. forsythia*, *F. nucleatum* and *Prevotella intermedia* to LLC-MK2 cells (a monkey kidney cell line) infected with HCoV-NL63 or mock-infected cells was evaluated. For the primary screening assay, flow cytometry analysis was used, as it allows high-throughput testing of a large number of samples. Briefly, cells were incubated with *Streptococcus pneumoniae* at an m.o.i. of 500. Such a high dose of bacteria was required to visualize the binding of bacteria to mock-treated cells. Analysis revealed that at day 6 post-inoculation (p.i.), HCoV-NL63 infection significantly enhanced *Streptococcus pneumoniae* adherence to LLC-MK2 cell monolayers (Fig. 1a). In contrast, adhesion of the other bacteria tested to virus-infected cells was not markedly altered compared with mock-treated cells (Fig. 1a). To ensure that the observed effect was not related to binding of dead bacteria lacking membrane integrity, we evaluated bacterial viability with propidium iodide staining and subsequent fluorescence-activated cell sorting (FACS) analysis in the experimental setting described above. A significant change in bacterial binding was observed for the population of living cells, and the residual population of damaged cells did not influence the result (data not shown).

To confirm this phenomenon and to rule out the possibility that FITC staining modulates the binding capacities of certain bacteria, a c.f.u.-based adherence assay was carried out for selected pathogens. We evaluated the adherence of *Staphylococcus aureus*, *H. influenzae*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* to LLC-MK2 cells infected with HCoV-NL63 or mock-treated cells at day 6 p.i. LLC-MK2 cells were incubated with bacteria under the conditions described above and, after an extensive washing step, the bacteria were harvested by trypsinization and plated on agar plates (as described in Methods). Analysis of c.f.u. numbers revealed a significant increase in the number of *Streptococcus pneumoniae* cells adhering to HCoV-NL63-infected cells (Fig. 1b). Adhesion of *Staphylococcus aureus*, *H. influenzae* and *Pseudomonas aeruginosa* to virus-infected cells did not increase significantly compared with mock-treated cells (Fig. 1b).

### Adherence of *Streptococcus pneumoniae* to HCoV-NL63-infected epithelium

To determine whether there is a link between HCoV-NL63 replication and pneumococcal adherence to LLC-MK2 cells,



**Fig. 1.** HCoV-NL63-mediated modulation of bacterial adherence to LLC-MK2 cells. (a) Bacterial adhesion was quantified by FACS. The histograms illustrate the fluorescence intensities and corresponding numbers of FITC-labelled bacteria attached to mock-treated or virus-infected cells (filled areas and grey lines, respectively). Data are representative of three independent experiments. (b) The number of adherent bacteria was quantified by plating and c.f.u. counting. Bars represent the number of bacteria that attached to mock- or HCoV-NL63-infected LLC-MK2 cells as a percentage of the control. Adhesion is expressed as a percentage of the control. The results are shown as means  $\pm$  SD of three independent experiments. The statistical significance of observed differences was estimated using Student's *t*-test; \*,  $P < 0.01$ ; NS, not significant.

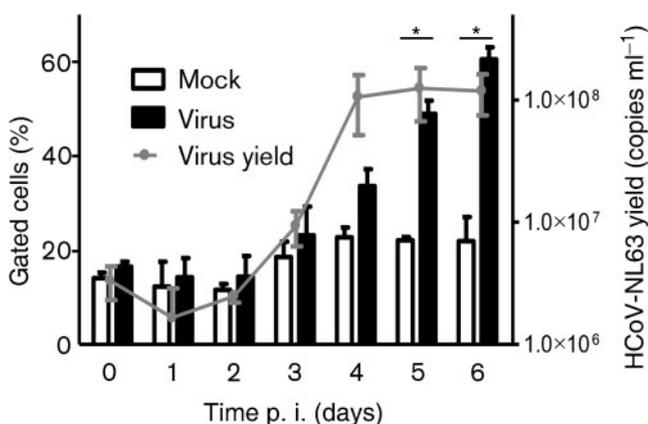
modulation of bacterial adhesion was studied on consecutive days following virus inoculation. Concurrently, the HCoV-NL63 yield was assessed by employing real-time PCR. Flow cytometric analysis detected no increase in the number of adhered *Streptococcus pneumoniae* in control and infected cells up to day 3 p.i. Compared with mock-treated LLC-MK2 cells, a significant enhancement in

*Streptococcus pneumoniae* adhesion to virus-infected LLC-MK2 cells was observed at day 4 p.i. The number of cells binding *Streptococcus pneumoniae* progressively increased over the 2 days following inoculation, coinciding with virus replication (Fig. 2). After an initial lag period, HCoV-NL63 replicated efficiently in LLC-MK2 cells, with a steep rise in virus yield at day 3 p.i. Importantly, *Streptococcus*

*pneumoniae* did not adhere to cells inoculated with UV-inactivated virus, suggesting that increased adherence is dependent on virus replication (data not shown).

Fluorescent microscopy was also used to visualize differences in the adherence of *Streptococcus pneumoniae* to mock- and HCoV-NL63-infected cells and to further substantiate and confirm this phenomenon. The analysis confirmed the significant increase in *Streptococcus pneumoniae* adherence to virus-infected LLC-MK2 cells. The bacteria attached to HCoV-NL63-infected cells in a non-random pattern and were found in clusters associated with a subset of cells (Fig. 3a).

To add physiological meaning to the finding, binding of *Streptococcus pneumoniae* to fully differentiated human airway epithelium (HAE) cells during consecutive days following HCoV-NL63 infection was examined. Virus replication in HAE cultures was assessed in the same manner as described above. A c.f.u.-based adherence assay revealed a major increase in the number of *Streptococcus pneumoniae* bacteria adhered to virus-infected cells at day 4 p.i., although no significant difference was observed on days 0 and 2 p.i. (Fig. 4). The observed increase in bacterial adherence coincided with virus replication. Furthermore, to visualize the increased adherence of *Streptococcus pneumoniae* to HCoV-NL63-infected HAE cultures, we also assessed the increase in bacterial adherence to infected epithelium by fluorescent microscopy. The results clearly showed a marked increase in *Streptococcus pneumoniae* adherence to HCoV-NL63-infected HAE cultures (Fig. 3b).



**Fig. 2.** Adhesion of *Streptococcus pneumoniae* to HCoV-NL63-infected LLC-MK2 cells increases in a time-dependent manner that correlates with virus yield. Pneumococcal adhesion was quantified by FACS and is expressed as the percentage of cells showing increased fluorescence. The results are shown as means  $\pm$  SD of three independent experiments. The statistical significance of observed differences was estimated using Student's *t*-test; \*,  $P < 0.05$ .

### Effect of HCoV-NL63 infection on eukaryotic receptors for *Streptococcus pneumoniae* expression

In order to reveal the mechanism of the enhanced bacterial binding to coronavirus-infected cells, the level of surface expression of known epithelial receptors for *Streptococcus pneumoniae*, including PAF-R, epithelial cadherin (E-cadherin), Toll-like receptor (TLR)-2 and TLR-4, was determined on HCoV-NL63-infected and mock-infected cells. The surface expression of receptors was assayed by flow cytometry. At day 6 p.i., PAF-R surface levels increased significantly compared with mock-treated cells, whilst E-cadherin levels did not change (Fig. 5). TLR-2 and TLR-4 expression on both virus-infected and mock-infected cells was marginal.

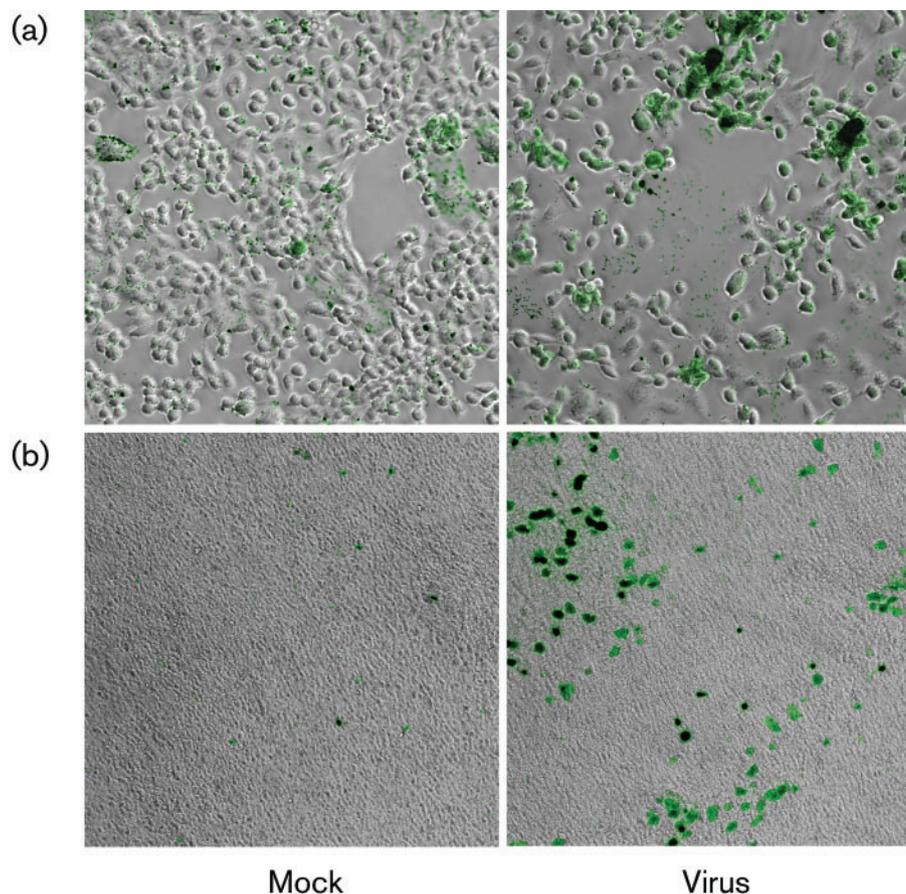
### Inhibition of PAF-R does not alter pneumococcal adherence to LLC-MK2 cells

It has been reported previously that PAF-R is a common receptor for *Streptococcus pneumoniae* that may be essential for the observed increase in bacterial adherence during viral infection (Ishizuka *et al.*, 2003). Indeed, we observed a marked increase in PAF-R surface levels during HCoV-NL63 infection. To test whether this phenomenon might serve as a mechanistic explanation for the observed increase in *Streptococcus pneumoniae* adherence, we evaluated platelet-activating factor (PAF), a PAF-R antagonist (ABT-491) and PAF-R-specific polyclonal antibody, previously reported to hamper the bacterium-PAF-R interaction (Radin *et al.*, 2005). Pre-incubation of HCoV-NL63-infected cells with PAF, PAF-R antagonist or PAF-R-specific antibody prior to the flow cytometry-based adherence assay did not affect the adhesion of the pneumococci to LLC-MK2 cells (Fig. 6). We were also not able to detect any decrease in *Streptococcus pneumoniae* adherence to human respiratory epithelial cultures following incubation with PAF.

## DISCUSSION

Previous reports have clearly shown that pneumococcal infections occurring during or shortly after respiratory viral illness are characterized by increased severity and more frequent hospitalization (Stille *et al.*, 1961). To illustrate the magnitude of the problem, it is worth mentioning that preceding viral infection could be detected in up to 90% of children with acute otitis media, which is the most common bacterial infectious sequela of initial viral infection. Moreover, clinical and epidemiological data showed that *Streptococcus pneumoniae* is a leading pathogen, involved in primary or secondary community-acquired pneumonia in hospitalized children, and in more than half of all cases a secondary viral agent could be detected (McCullers, 2006).

This study was carried out to evaluate whether infection with HCoV-NL63, a recently discovered viral respiratory



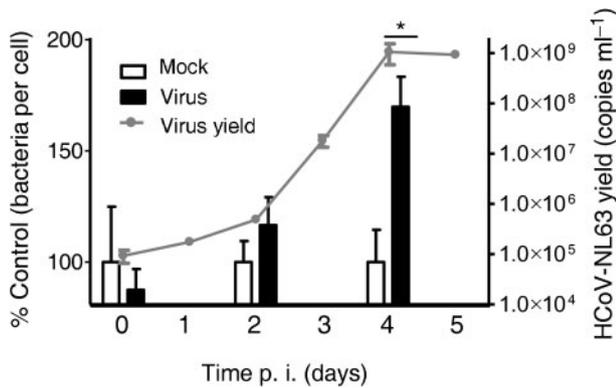
**Fig. 3.** Adhesion of *Streptococcus pneumoniae* to LLC-MK2 cells (a) and HAE cultures (b) is increased following infection with HCoV-NL63. Fluorescence images from FITC-labelled bacteria and bright-field images were obtained using a fluorescence microscope. Magnification:  $\times 200$  (a);  $\times 100$  (b). Data are representative of two independent experiments.

pathogen, resulted in increased vulnerability to secondary bacterial infection. The bacterial pathogens included in the study were chosen because of their prevalence and their association with respiratory tract infections. Within the studied group, *Staphylococcus aureus*, *H. influenzae*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* were taken as representatives of bacteria frequently associated with respiratory illness. *Porphyromonas gingivalis*, *T. forsythia*, *F. nucleatum* and *Prevotella intermedia* were chosen because they are aetiological agents of aspiration pneumonia, most prevalent in young children and elderly patients (Brook, 2002; Okuda *et al.*, 2005; Scannapieco, 1999).

Detailed analysis showed that adhesion of *Streptococcus pneumoniae* to epithelial cells was vastly enhanced by a preceding HCoV-NL63 infection. This finding is in accordance with the concept that viral infections augment pneumococcal adherence to the local epithelium, thereby facilitating bacterial infection (Avadhanula *et al.*, 2006, 2007; Hament *et al.*, 2004, 2005; McCullers & Bartmess, 2003; Peltola *et al.*, 2005; Tong *et al.*, 2001). As the viral infection had no effect on adherence of the other bacteria

tested, we assumed that there is a specific synergistic interaction between *Streptococcus pneumoniae* and HCoV-NL63.

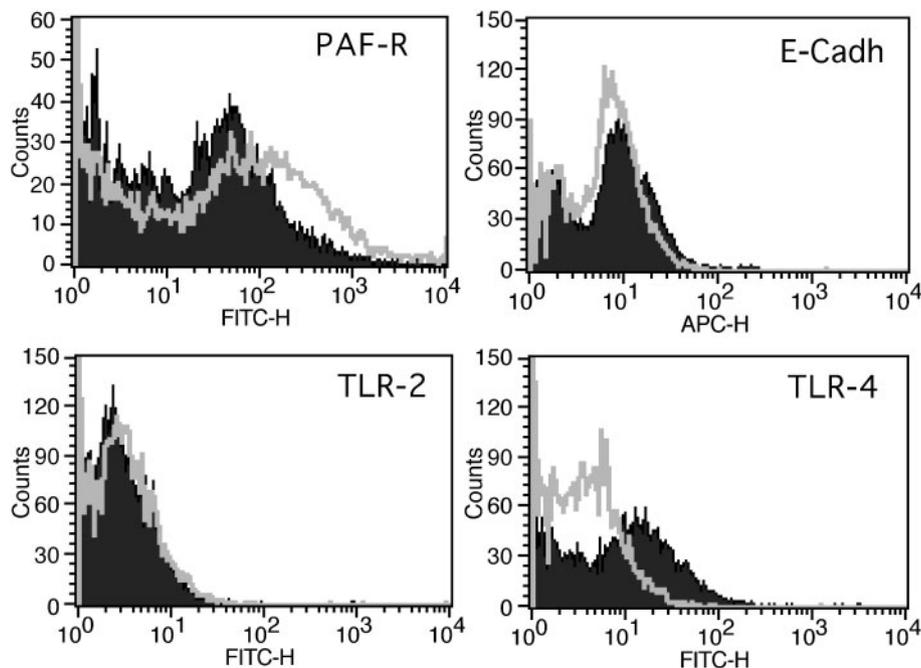
To evaluate our findings in a more natural environment for human respiratory pathogens, we employed the fully differentiated HAE cell line generated by primary human airway epithelial cells cultivated in an air-liquid interface. This system results in tissue culture that morphologically, structurally and functionally mimicks human cartilaginous airway epithelium. Mature, fully differentiated HAE cells contain multiple cell species forming multi-layered, ciliated, three-dimensional tissue culture that has been shown previously to support a wide range of respiratory viruses, including human coronavirus HKU1 (Pyrce *et al.*, 2010), severe acute respiratory syndrome coronavirus (Sims *et al.*, 2006), HCoV-NL63 (Orenstein *et al.*, 2008), influenza virus (Thompson *et al.*, 2006), parainfluenza virus (Zhang *et al.*, 2005) and respiratory syncytial virus (Zhang *et al.*, 2002). HCoV-NL63 infection of HAE culture resulted in a significant increase in the number of adherent *Streptococcus pneumoniae* cells in comparison with control, mock-infected culture. As it has been reported previously



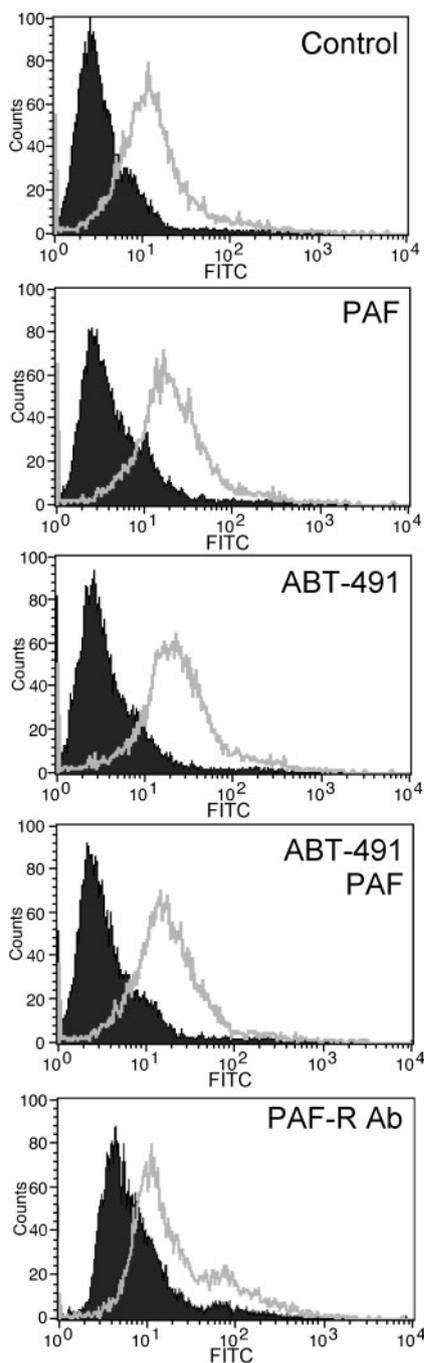
**Fig. 4.** HCoV-NL63 affects pneumococcal adherence to the apical surface of HAE cultures. Adherence of *Streptococcus pneumoniae* to virus-infected HAE cultures increased in a time-dependent manner that correlated with virus yield. The number of adherent bacteria was quantified by plating and c.f.u. counting. Bars represent the number of *Streptococcus pneumoniae* that attached to mock- or HCoV-NL63-infected HAE cultures as a percentage of the control. The results are shown as means  $\pm$  SD of two independent experiments. Statistical significance of the observed differences was estimated using Student's *t*-test; \*,  $P < 0.05$ .

that virus-mediated modulation of bacterial adherence is highly cell-line specific (Avadhanula *et al.*, 2006), these data validated the results obtained with LLC-MK2 cells as clinically relevant.

The mechanistic explanations for increased bacterial binding to cells infected with other viral pathogens are ambiguous and several factors have been considered (Hament *et al.*, 1999). One hypothesis suggests that an increase in the density of cellular receptors facilitates bacterial adherence. Here, we studied the levels of specific cell-surface proteins identified previously as receptors for *Streptococcus pneumoniae*. Although we observed no effect of viral infection on TLR-2, TLR-4 and E-cadherin expression, there was a marked increase in the surface level of the PAF-R protein. PAF-R is a cell-surface G protein-coupled receptor, facilitating adherence of *Streptococcus pneumoniae* to epithelial cells by binding to phosphorylcholine, a component of the bacterial cell wall (Barletta *et al.*, 2002; Cundell *et al.*, 1995). It has been suggested previously that PAF-R is an essential factor for the development of secondary bacterial pneumonia after viral infection (McCullers & Rehg, 2002). During rhinovirus and RSV infection, upregulation of PAF-R surface levels has been documented (Avadhanula *et al.*, 2006; Ishizuka *et al.*, 2003). In an *in vivo* experiment, it was also shown that influenza virus infection in mice caused enhanced PAF-R expression in the lungs, further supporting the contention of PAF-R importance for *Streptococcus*



**Fig. 5.** Infection with HCoV-NL63 affects receptor expression on the cell surface. Levels of bacterial receptors on the cell surface were determined by FACS on HCoV-NL63- or mock-infected LLC-MK2 cells (grey lines and filled areas, respectively). Analysis was performed for PAF-R, E-cadherin (E-Cadh), TLR-2 and TLR4. The data are representative of three independent experiments.



**Fig. 6.** Inhibition of PAF-R with PAF, ABT-491 and PAF-R-specific polyclonal antibodies does not decrease the adherence of pneumococci to LLC-MK2 cells. Adherence of *Streptococcus pneumoniae* to the cell surface was determined by FACS on HCoV-NL63- or mock-infected cells (grey lines and filled areas, respectively). Data are representative of three independent experiments.

*pneumoniae* adhesion. Moreover, it was demonstrated that *Streptococcus pneumoniae* employs PAF-R to invade the host's epithelium previously infected with influenza virus (van der Sluijs *et al.*, 2006).

In this study, we found that HCoV-NL63 infection was associated with a marked increase in PAF-R expression on infected cells, suggesting that enhanced adherence of *Streptococcus pneumoniae* through binding to PAF-R may be one of the mechanisms involved in the development of secondary bacterial infections after HCoV-NL63-associated respiratory illness. However, the presence of PAF, PAF-R antagonist (ABT-491) or PAF-R-specific antibody did not affect the adherence of *Streptococcus pneumoniae* to cells pre-infected with HCoV-NL63. This strongly suggests that increased adherence of pneumococci following HCoV-NL63 infection is not solely mediated by PAF-R, and that some other cellular receptors are involved in this interaction. It is also possible that increased bacterial adherence is caused by virus-mediated modification of the cellular glycan layer.

Taken together, we have provided a possible reason for the severity of *Streptococcus pneumoniae*-related illness during or shortly after HCoV-NL63 infection. As this virus is a relatively recently described human pathogen, available clinical data are relatively limited and further epidemiological studies on the interactions between *Streptococcus pneumoniae* and HCoV-NL63 are required to elucidate fully the clinical relevance of the described process.

## METHODS

**Cell culture.** LLC-MK2 cells were maintained in minimal essential medium (MEM), containing two parts Hanks' MEM and one part Earle's MEM (PAA Laboratories) supplemented with 3% heat-inactivated FBS (PAA Laboratories), penicillin (100 U ml<sup>-1</sup>) and streptomycin (100 µg ml<sup>-1</sup>). Cells were cultured on six-well plates (Sarstedt) at 37 °C with 5% CO<sub>2</sub>.

Human tracheobronchial epithelial cells were obtained from airway specimens resected from patients undergoing surgery under protocols approved by the bioethical committee of the Silesian Center for Heart Diseases, Poland (approval no: KNW/0022/KB1/17/10 dated 16 February 2010). Primary cells were expanded on plastic to generate passage 1 cells and plated at a density of  $3 \times 10^5$  cells per well on permeable Transwell inserts (6.5 mm diameter) supports. HAE cultures were generated by provision of an air-liquid interface for 6–8 weeks to form well-differentiated, polarized cultures that resemble *in vivo* pseudo-stratified mucociliary epithelium.

**Virus preparation, titration and infection.** HCoV-NL63 stocks were generated by infecting LLC-MK2 cells. Cells were lysed at 6 days p.i. by two freeze-thaw cycles. The virus-containing fluid was aliquotted and stored at -80 °C. A control LLC-MK2 cell lysate from mock-infected cells was prepared in the same manner as the virus stocks.

Virus yield was assessed by virus titration on fully confluent LLC-MK2 cells in 96-well plates, according to the method of Reed & Muench (1938). Plates were incubated at 32 °C for 6 days and the occurrence of a cytopathic effect was scored using an inverted microscope.

In subsequent experiments, fully confluent cells were exposed to HCoV-NL63 at a concentration of 2000 TCID<sub>50</sub> ml<sup>-1</sup>. Cell-culture supernatant samples used for real-time PCR were collected every 24 h for 7 days.

HAE cultures were infected with HCoV-NL63, which was inoculated at the apical surface. Following 2 h incubation at 32 °C, unbound virus was removed by extensive washing with PBS and the HAE cultures were maintained at an air-liquid interface for the rest of the experiment. To generate replication curves at specific times, 100 µl PBS was applied to the apical surface of the HAE cells and collected after 10 min incubation at 32 °C. All samples were stored at -80 °C.

**Bacterial strains, storage and growth conditions.** *Pseudomonas aeruginosa*, *H. influenzae* and *Streptococcus pneumoniae* were kindly provided by J. Fiett (National Medicines Institute, Warsaw, Poland) and *Staphylococcus aureus* strain Newman by T. J. Foster (Moyné Institute of Preventive Medicine, Trinity College, Dublin, Ireland). *Porphyromonas gingivalis*, *Prevotella intermedia*, *T. forsythia* and *F. nucleatum* were obtained from S. Eick (University Hospital of Jena, Jena, Germany).

*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* were cultured in tryptic soy broth (TSB). *H. influenzae* was grown in brain-heart infusion broth (BHI) with haemin (10 µg l<sup>-1</sup>) and NAD (2 µg ml<sup>-1</sup>). *Porphyromonas gingivalis*, *Prevotella intermedia*, *T. forsythia* and *F. nucleatum* were grown anaerobically in Schaedler broth supplemented with L-cysteine (0.05 g ml<sup>-1</sup>), 1% DTT, menadione (0.5 mg ml<sup>-1</sup>) and haemin (1 mg ml<sup>-1</sup>). Bacteria were stored in TSB or BHI medium supplemented with glycerol (50%, v/v) at -80 °C. During experiments, 10 ml cultures were inoculated with bacterial stock. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were grown overnight under constant rotation (180 r.p.m.) to stationary growth phase at 37 °C. *H. influenzae* and *Streptococcus pneumoniae* were grown for 6 h to stationary phase at 37 °C (anaerobic conditions).

Prior to each inoculation, bacteria were washed twice in PBS. The concentration of bacteria was assessed based on optical density at 600 nm. Absolute numbers were determined based on a standard growth curve generated previously by counting c.f.u. on agar plates. Each time, in order to verify the absolute numbers of cells, bacteria were plated and colonies were counted after 16 h of incubation at 37 °C.

**Labelling of bacteria.** In order to label bacterial cells with a fluorescent dye for subsequent analysis, bacteria were collected by centrifugation at 4000 r.p.m. in a microfuge for 6 min and resuspended in 900 µl PBS. Samples were mixed with 100 µl FITC (1 mg ml<sup>-1</sup>) and incubated for 20 min at 37 °C in the dark. Subsequently, bacteria were washed three times with sterile PBS and resuspended in PBS to a final concentration of 1 × 10<sup>9</sup> c.f.u. ml<sup>-1</sup>.

**Adherence of bacteria to LLC-MK2 cells (flow cytometry-based adherence assay).** To examine the effect of HCoV-NL63 infection on the adherence of bacteria to epithelial cells, LLC-MK2 monolayers were inoculated with the virus and mock-infected as described above. To assess the ability of bacteria to adhere to the cell surface, cells were incubated with FITC-labelled bacteria (1 × 10<sup>9</sup> c.f.u. ml<sup>-1</sup>, m.o.i. 500) for 1 h at 4 °C and subsequently rinsed three times with ice-cold PBS to remove non-adherent bacteria. The cells were then detached from the plate surface by scraping. Collected cells were resuspended in 1 ml PBS, spun at 260 g for 6 min and fixed in 3% paraformaldehyde (PFA). Samples were analysed on a FACSCalibur instrument (Becton Dickinson) using CellQuest software. A total of 10 000 events was acquired. During the procedure, the cell culture medium was not supplemented with antibiotics.

**Adherence of bacteria to LLC-MK2 cells (c.f.u.-based adherence assay).** Cell monolayers were incubated with bacteria (1 × 10<sup>9</sup> c.f.u. ml<sup>-1</sup>, m.o.i. 500) for 1 h at 4 °C and subsequently rinsed three times with ice-cold PBS to remove non-adherent bacteria. Cells were detached from the plate with 1 × trypsin/EDTA solution (PAA

Laboratories) and immediately placed on ice. Serial dilutions were prepared in ice-cold PBS and plated onto agar plates. Cell number was verified using a haemocytometer. After overnight incubation at 4 °C, colonies were counted.

To test whether the increase in PAF-R surface protein levels might alter *Streptococcus pneumoniae* adherence, virus-infected cells were incubated with 10 µM platelet activating factor-16 (PAF; Calbiochem), 10 µM PAF-R antagonist (ABT-491; Calbiochem) or PAF-R-specific polyclonal antibody (20 µg ml<sup>-1</sup>; Cayman Chemical) for 30 min. Subsequently, the cells were incubated with *Streptococcus pneumoniae* and tested in a flow cytometry-based adherence assay as described above.

**Adherence of *Streptococcus pneumoniae* to HAE cultures (c.f.u.-based adherence assay).** HAE cultures were incubated with bacteria overlaid on the apical surface of the culture (1 × 10<sup>8</sup> c.f.u. ml<sup>-1</sup>) for 1 h at 4 °C and subsequently rinsed three times with ice-cold PBS to remove non-adherent bacteria. Cells were detached from the plate with 1 × trypsin/EDTA solution and instantly placed on ice. Serial dilutions were prepared in ice-cold PBS and plated onto agar plates. After overnight incubation at 4 °C, colonies were counted.

**Virus detection by reverse transcription and quantitative PCR.** HCoV-NL63 total nucleic acids were isolated from cell-culture supernatant or apical washes from HAE cultures using a Total RNA Mini kit (A&A Biotechnology), according to the manufacturer's instructions. Reverse transcription was carried out with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer's instructions. HCoV-NL63 virus yield was determined using real-time PCR. Five microlitres of cDNA was amplified in a 20 µl reaction mixture containing 1 × TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 200 nM specific probe labelled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) and 900 nM each primer. Rox was used as a reference dye. The following primers were used for HCoV-NL63 amplification: sense, 5'-AAACCTCGTTGGAAGCGTGT-3'; antisense, 5'-CTGTGAAAAACCTTGGCATC-3'; and probe, 5'-FAM-ATGTTATTTCAGTCT-TGGTCTCGTGAT-TAMRA-3'. The reaction was monitored on a 7500 Fast Real-Time PCR machine (Applied Biosystems) with the following settings: 2 min at 50 °C and 10 min at 92 °C, followed by 45 cycles of 15 s at 92 °C and 1 min at 60 °C.

**Surface expression of molecules involved in bacterial adherence.** LLC-MK2 cells were scraped from plates, washed with PBS, incubated with 1% BSA (Sigma-Aldrich) in PBS and fixed with 3% PFA. To assess the surface expression of PAF-R, cells were incubated for 2 h at room temperature with a 1:50 dilution of rabbit anti-human PAF-R polyclonal antibody (Cayman Chemicals), followed by incubation with a 1:200 dilution of Alexa Fluor 488-labelled goat anti-rabbit antibody (Molecular Probes) for 1 h prior to analysis. To analyse surface expression of E-cadherin, cells were incubated with a 1:50 dilution of Alexa Fluor 647-conjugated anti-E-cadherin (CD324; eBioscience) antibody. To analyse surface expression of TLR-2 and TLR-4, cells were incubated with mouse anti-TLR-2 mAb (Abcam) and mouse anti-TLR-4 mAb (Abcam). For TLR staining, 1:200-diluted goat Alexa Fluor 488-conjugated anti-mouse secondary antibody (Molecular Probes) was used. In each case, after incubation with antibodies, cells were washed, suspended in PBS and analysed with Becton Dickinson FACSCalibur using CellQuest software.

**Fluorescence microscopy.** LLC-MK2 monolayers were inoculated with virus and mock infected as described above. FITC-labelled bacteria (1 × 10<sup>9</sup> c.f.u. ml<sup>-1</sup>) were added to cell cultures and incubated for 1 h at 4 °C. Cell monolayers were washed three times with ice-cold PBS and subsequently fixed with 3% PFA (Sigma-

Aldrich). Samples were examined under a fluorescent microscope (Nikon Eclipse Ti).

HCoV-NL63-infected or mock-infected HAE cultures were cooled, inoculated at the apical surface with bacteria ( $1 \times 10^8$  c.f.u. ml<sup>-1</sup>) and subsequently incubated for 1 h at 4 °C. The apical side of the cultures was then rinsed three times with ice-cold PBS to remove non-adherent bacteria. Samples were examined under a fluorescent microscope.

**Statistical analysis.** All experiments were performed in triplicate and results are expressed as means  $\pm$  SD. To determine the significance of the obtained results, comparisons between groups were made using Student's *t*-test. *P* values <0.05 were considered significant.

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