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Investigating the synergism of influenza *and Streptococcus pneumoniae* in an *in vitro* model.

Master project:

Molecular Virology & Molecular Bacteriology

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Abstract

Most lethal cases during an influenza pandemic are caused by a secondary bacterial infection. A *S. pneumoniae* infection is frequently seen after an influenza infection. An influenza infection seems to predispose the airways to a secondary bacterial infection, but the underlying mechanisms to this viral-bacterial synergism are unknown. With this research we investigated the direct interaction of the influenza virus, *S. pneumoniae* and lung epithelial cells *in vitro*. Respiratory epithelial cells were infected with influenza for different periods of time, before adhesion and invasion of *S. pneumoniae* was determined. Also, we investigated possible causes responsible for changes in susceptibility to *S. pneumoniae*. Our results indicate that nasopharyngeal epithelial cells become more susceptible to an *S. pneumoniae* infection after an influenza infection. Also, the duration of the influenza infection seems to play a role in this increased susceptibility. It was previously assumed that viral neuraminidase (NA) contributed to an increased adhesion of *S. pneumoniae* after 30 minutes of influenza infection. Here we showed that 6 hours of influenza infection caused increased adhesion of *S. pneumoniae*, which is the time point where viral replication has started. This indicates that the activity of NA alone may not be the cause of increased adhesion of *S. pneumoniae*. Also, we showed that nasopharyngeal cells became more susceptible for bacterial invasion after an influenza infection. These results imply that there may be a role for the platelet activating factor (PAFr) and the poly immunoglobulin receptor (pIgR), but future research will be needed to specify their involvement. Interestingly, supernatants containing possible soluble factors did now show to have an effect on adhesion and invasion of *S. pneumoniae*, while TNF-a treatment caused increased invasion into nasopharyngeal cells. Also, *S. pneumoniae* does not seem to favour influenza infected cells over uninfected cells. These findings suggest that soluble factors, secreted by lung epithelial cells after an influenza infection, may contribute to the synergism of influenza and *S. pneumoniae*. This study indicates that there may be a role for direct interaction of influenza, *S. pneumoniae* and lung epithelial cells in the synergism of influenza and *S. pneumoniae*.

Introduction

Seasonal influenza outbreaks cause 3-5 million illnesses worldwide leading to an estimated 250.000-500.000 annual deaths [1]. Children under the age of 2, elderly and immunocompromised seem more susceptible for an influenza infection. Due to the high mutation rate of the influenza virus, new pandemics can develop. While the mortality rate of the recent H1N1 influenza pandemic was low, 43-89 million cases of severe illness occurred worldwide [2]. Future new pandemic strains may have more severe consequences.

Most lethal cases during influenza pandemics, however, are caused by a secondary bacterial infection [3]. Already during the Spanish flu pandemic in 1918, which caused between 40 and 50 million deaths worldwide [4], it was seen that a secondary bacterial infection frequently occurred after an influenza infection. An influenza infection seems to make the airways more susceptible for a bacterial infection, but the underlying specific mechanisms explaining this co-infection remain unknown. The classic dogma that epithelial cell damage of the lungs, caused by the influenza virus, leads to increased adhesion sites for bacteria seems to explain the synergism only partly [5].

Influenza A viruses are members of the *Orthomyxoviridae*. They are enveloped viruses with a negative sense, single-stranded RNA genome consisting of 8 segments. Influenza A viruses are subtyped based on differences in the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). HA has 16 different subtypes and NA has 9 different subtypes. The function of HA is to bind the virus to sialic acid on the epithelial cell surface receptors [6]. Furthermore, HA is also the main target for neutralizing antibodies to prevent influenza infection [7]. Due to antigenic drift, frequent mutations in the viral RNA leading to changes in the HA, annual seasonal outbreaks can occur. Another possibility for seasonal outbreaks to occur is through antigenic shift, when different strains of influenza combine and new subtypes of HA are expressed on the cell surface. NA is involved in the release of new virus particles from infected cells. Its function is cleaving sialic residues from the cell membrane [8]. Also, NA is the main target for antiviral drugs like Oseltamivir.

A secondary bacterial infection that is frequently seen after an influenza infection is a *S. pneumoniae* infection [5]. Influenza and *S. pneumoniae* are two of the most common infectious pathogens known threatening human health. Combined they rank as the sixth leading cause of death worldwide and as the leading infectious cause of death [8]. Secondary bacterial pneumoniae, after an influenza infection, is an important cause of mortality, especially in the elderly. Annually, in the United States alone, more than half a million cases of pneumococcal pneumoniae are reported [9]. Besides bacterial pneumoniae, the bacteria can cause a number of severe invasive diseases, depending on the site of invasion [10]. When entering the blood stream, *S. pneumoniae* can cause bacteremia. Also, when the bacteria enter brain tissue they can cause meningitis. These invasive diseases cause more than 5 million deaths among children worldwide [11]. Also, *S. pneumoniae* is able to cause otitis media that may lead to loss of hearing.

S. pneumoniae are lancet shaped gram positive bacteria, which commonly occur in pairs or groups. *S. pneumoniae* is a human commensal, and the bacteria are present in the nasopharynx of about 54% of healthy children by 1 year of age [12]. One single strain of

S. pneumoniae may persist in the nasopharynx for weeks or months [13]. Some asymptomatic individuals may be colonized with up to 4 different serotypes simultaneously [14].
While worldwide a lot of research is performed, aiming to clarify the underlying mechanisms in the synergism of influenza and *S. pneumoniae*, results show to be contradictory. Previously it was assumed that damage to the protective layer of epithelial cells in the lungs was the cause of increased susceptibility to a bacterial infection. The cell damage was thought to expose extracellular matrix molecules and basement membrane elements to which bacteria can adhere. McCullers et al. showed in mice studies, using a highly pathogenic, mouse-adapted PR8 influenza virus, that severe damage to the epithelial cell layer of the lungs indeed occurred [8, 15]. The cytotoxicity of the virus seemed to be the cause of the damage to lung epithelial cells, and also seemed to cause weight loss or even death in mice. When these mice successively were exposed to *S. pneumoniae,* an increased bacterial load was seen in the lungs and bacteria were detected bound to damaged parts of the respiratory tract. However, when a low pathogenic influenza virus was used to infect mice, there was no pathological damage observed in epithelial cells of the respiratory tract. Exposing these mice afterwards to *S. pneumoniae* indicated that the bacteria were able to persist in the lungs for prolonged periods of time [16]. These findings indicate that cell damage may only partly explain the increase in susceptibility to a secondary bacterial infection after an influenza infection. However, these *in vivo* studies indicate that adhesion and invasion of *S. pneumoniae* are an important factor in pneumococcal infection. Therefore, the effect of influenza infection on the adhesion and invasion of *S. pneumoniae* seems important in the synergism of both pathogens.
Another possible mechanism, underlying the lethal synergism between influenza and *S. pneumoniae*, might be that an influenza infection causes changes in the airway function besides cytotoxicity. The influenza virus may cause changes in the lungs that are beneficial for bacteria. Disruption of surfactant can cause obstructions in the small airways, increased mucinous secretions combined with fibrin and edema fluid, and an influx of inflammatory cells create dead spaces and a culture medium for bacteria [17]. These dead spaces not only reduce pulmonary functional capacity, but also diffusion capacity. These functional limitations then cause decreased mechanical clearance of bacteria, increased airway hyperreactivity and improved conditions for bacterial growth [5]. With an increased amount of bacteria present in the lungs, bacterial infections may occur more easily.
Besides physical changes in the lungs, the platelet activating factor receptor (PAFr) and the poly immunoglobulin receptor (pIgR) may be involved in the synergism of influenza and *S. pneumoniae*.
The platelet activating factor receptor (PAFr) is thought to be involved in the crossing of *S. pneumoniae* from the blood into the cerebrospinal fluid where it can induce meningitis [18]. The PAFr is a G-protein coupled receptor, which binds platelet-activating factor. The receptor is present on lung epithelial cell, endothelial cells and platelets. The PAFr is thought to be able to bind phosphocholine of the cell wall of *S. pneumoniae* [18]. Van der Sluijs et al. showed in an *in vivo* study that the PAFr expression was upregulated in the lungs after mice were infected with influenza [19]. In this experiment PAFr knockout mice were infected with influenza and successively exposed to *S. pneumoniae*. The mice

did not only display a reduced bacterial outgrowth in their lungs, but also a reduced dispersion of the infection in the lungs and prolonged survival, compared to mice expressing PAFr. Contradictory, McCullers et al. showed that PAFr was not upregulated in the lungs after an influenza infection [20]. This experiment showed no difference in secondary bacterial pneumonia in the presence or absence of competitive inhibitors of PAFr. Also, knockout mice lacking PAFr showed that although the PAFr seems to be involved in the transition of *S. pneumoniae* from the lung compartment into the blood, that the receptor is not necessary for the induction of bacterial pneumonia. These findings indicate that the PAFr may be involved in the synergism of influenza and *S. pneumoniae*, but the underlying mechanism of the possible involvement of the PAFr is still not fully understood.

Beside the PAfr, also the poly immunoglobulin receptor (pIgR) may be involved in the synergism of influenza and *S. pneumoniae*. The pIgR is a type 1 membrane spanning protein produced by mucosal epithelial cells, including respiratory and intestinal epithelial cells, in humans [21]. *S. pneumoniae* posses a variety of virulence factors which are thought to contribute to its pathogenesis. More than 73% of *S. pneumoniae* isolates express choline-binding protein A (CbpA) [22]. This protein possesses a C-terminal choline-binding domain and a unique N-terminal domain that is thought to mediate host cell attachment [23]. *In vivo* experiments have shown that a *S. pneumoniae* strain lacking CbpA was 100-fold less efficient at colonizing the nasopharynx in rats than an *S. pneumoniae* strain expressing CbpA [22]. Also, Zhang et al. have shown that *S. pneumoniae* is able to invade nasopharyngeal cells by binding CbpA to the human pIgR [24]. This experiment showed that interaction of CbpA with human pIgR enhances adhesion and invasion of *S. pneumoniae* in nasopharyngeal cells. Interestingly, rabbit pIgR did not seem to bind CbpA, and adhesion and invasion of *S. pneumoniae* was not increased in canine kidney cells. These findings suggest that *S. pneumoniae* are a new example of a pathogen using the transcytosis machinery of the pIgR to cross the mucosal layer of the lungs.

When cells of the respiratory tract are exposed to influenza, and the virus infects epithelial cells, monocytes, macrophages, and also the lung epithelial cells start to produce several pro-inflammatory cytokines [25]. Recently, le Goffic et al. showed in mice infected with influenza A virus up-regulation of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), Interleukin (IL)-1β, IL-6, and IL-33 in lung cells [26]. This suggests that there may be an indirect effect of the influenza infection on the adhesion and invasion of *S. pneumoniae*. In this study TNF-α is of particular interest. This pro-inflammatory cytokine can induce expression of several proteins, which are associated with pneumococcal adhesion [27]. Furthermore, TNF-α seems to be able to up-regulate the expression of PAFr. Dagenais et al. showed that after 1 hour of TNF-α stimulation the PAFr was up-regulated [28], and the up-regulation persisted up to 24 hours. Because of these findings the effect of TNF-α on the adhesion and invasion of *S. pneumoniae* seems of interest.

This study is aimed to investigate the direct interaction between influenza, *S. pneumoniae* and lung epithelial cells in an *in vitro* mode. The main goal is to investigate the effects of an influenza infection on the adhesive and invasive behavior of *S. pneumoniae*. We will expose cells, representing different parts of the respiratory tract, to influenza for 30 minutes, 6 hours or 17 hours to see if duration of influenza infection has an effect on the

susceptibility to *S. pneumoniae* infection. We hypothesize that lung epithelial cells become more susceptible for an *S. pneumoniae* infection after an influenza infection. Furthermore, we will investigate what gives cause to the possible increased susceptibility to *S. pneumonaie*. This will be studied by investigating the receptor expression of PAFr and pIgR. We hypothesize that these receptors are up-regulated after an influenza infection. Thereafter we will look into more detail at the adhesion and invasion of *S. pneumoniae* to investigate whether *S. pneumoniae* favors influenza infected cells over uninfected cells. Since we expect PAfr and pIgR to be up-regulated, due to the influenza infection, that *S. pneumoniae* favors influenza infected cells over uninfected cells. Furthermore, we will examine the effects of possible secreted cytokines on the adhesion and invasion of *S. pneumoniae* to investigate if there is a direct effect due to the influenza virus, or an indirect effect due to produced cytokines. We expect that the adhesion and invasion of *S. pneumoniae* will be up-regulated due to secreted soluble factors. Finally we will investigate the effects of TNF-α in more detail. We expect that adhesion and invasion of *S. pneumoniae* will be up-regulated after TNF-α treatment. We will perform the proposed experiments in nasopharyngeal cells, bronchial lung epithelial cells and alveolar basal epithelial cells which represent different sections of the respiratory tract.

Materials and Methods

Cell cultures
The human carcinoma nasopharyngeal cell line Detroit 562 (Peterson, 1971) was cultured in RMPI 1640 (+ 25mM HEPES, - L-glutamin) and supplemented with 10% Fetal Calf Serum (FCS), 1% L-glutamin, 1% MEM non-essential amino acids and 1% sodium pyruvate. The human bronchial lung epithelial cell line BEAS 2B (Reddel, 1989) was cultured in RMPI 1640 (+ 25mM HEPES, - L-glutamin) and supplemented with 10% FCS. The carcinoma alveolar basal epithelial cell line A549 (Lieber, 1976) was cultured in RMPI 1640 (+ 25mM HEPES, - L-glutamin) and supplemented with 10% FCS, 1% L-glutamin and 1% MEM non essential amino acids. Cells were grown in either 25 cm^2 or 75 cm^2 flasks (Costar, Cambridge, MA) at 37 ˚C with a 5% CO_2 humidified atmosphere.

Infectious agents
The Mount Sinai strain of mouse-adapted influenza virus A/Puerto Rico/8/34 (H1N1, virus titer 3,3 x 10^9), hereafter referred to as PR8, was grown in the Madin-Darby Canine Kidney (MDCK) cell line (Gaush, 1966). The *S. pneumoniae* TIGR4 (serotype 4 encapsulated, Tettelin, 2001) strain was grown in 0,5% glucose supplemented M17 (GM17) broth at 37 ˚C. Stocks were prepared with 11% glycerol and stored at -80 ˚C. Subsequently aliquots were made by growing TIGR4 in GM17 at 37 ˚C. At mid-logarithmic phase (optical density at 600 nm= 0,35) aliquots were supplemented with 11% glycerol and stored at -80 ˚C. Bacterial counts were derived from colony forming units (CFU). Therefore 10-fold serial dilutions of bacteria were made in M17 broth and plated on blood agar plates, supplemented with 3% (vol/vol) sheep erythrocytes.

Determination of susceptibility of epithelial cells to influenza infection
Cells were grown on glass cover-slips until a confluent monolayer was formed. To remove unbound and dead cells the cover-slips were washed twice with Dulbecco's phosphated buffer saline (PBS) before different multiplicities of infection (MOI) were added. Cells were exposed to influenza virus with MOIs of 0, 10, 20, 50, 100, or 200 and incubated for 1 hour at 37 ˚C with a 5% CO_2 humidified atmosphere. Controls were exposed to fresh medium without virus (MOI=0). After influenza infection, cells were washed once with PBS to remove unbound virus particles and a new layer of fresh prewarmed media was added. Then the cells were incubated for 17 hours at 37 ˚C with a 5% CO_2 humidified atmosphere. All infections were performed as duplicates.
To determine the susceptibility of the epithelial cells to influenza infection a nucleoprotein staining (NP) was performed after the 17 hours of incubation. First the cells were washed three times with PBS to remove possible detached cells, and fixed with 100% ethanol for 30 seconds. Then the cells were washed again twice with PBS to remove excessive fixative. Successively, the cells were incubated for 1 hour with mouse influenza A nucleoprotein specific primary antibody (Serotec, product code: MCA400, 1mg/mL), 1:100 diluted in PBS, supplemented with 1% bovine albumin. Subsequently, the cells were washed with PBS three times, and incubated for 1 hour with the secondary antibody anti-mouse IgG Alexa fluor antibody (Invitrogen, product code: A10037, 2mg/mL), 1:50 diluted in PBS supplemented with 1% bovine albumin. Then the cells

were washed two times with PBS, and once with distilled water. As a control to prove the presence of a monolayer of cells, cell nuclei were stained with DAPI (0,5 μg/mL) 1:100 diluted in milliQ for 3 minutes. Subsequently, the cells were washed with distilled water once and dried at room temperature. Finally the cover-slips were stuck to a microscope slide with Kaisers glycerol gelatine. The slides were observed using a fluorescence microscope (Leica, Orthoplan, 064436). The nucleoprotein staining was observed with a filter with wavelength of 543-560 nm, and the DAPI staining was observed with a filter with a wavelength of 340-410 nm. The percentage of influenza infected cells was determined by visual observation.

Streptococcal adhesion and invasion assessment

Detroit, BEAS-2B and A549 cells were grown in 12 well plates till a confluent monolayer was formed (after about 24 hours). Detroit cells and A549 cells were infected with a MOI of 50 of PR8, BEAS-2B cells were infected with a MOI of 200 of PR8. After 1 hour of influenza infection cells were washed once with sterile PBS and the cells were then additionally incubated for 30 minutes, 6 hours or 17 hours. After the different time periods of influenza infection cells were washed with sterile PBS twice. A volume containing 5 x 10^6 CFU/mL of TIGR4 was taken from a previously grown aliquot. The bacteria were centrifuged at 10.000 rpm during 3 minutes. Then the pellets were resuspended in the corresponding cell growth media. The monolayers were then covered with 100 μL/well of the *S. pneumoniae* suspension and 900 μL of fresh medium. Cells were incubated for 2 hours at 37 ˚C in a 5% CO_2 humidified atmosphere for bacteria to adhere to the cells. After incubation cells were washed with sterile PBS three times. Subsequently, cells were detached and lysed using a 1:1 mixture of 0,05% trypsin-EDTA (Life Technologies) and saponin.

For determination of invasion cells were incubated with media containing a mixture of penicillin-G (0,67 μg/mL) and gentamycin (13,33 μg/mL) to eliminate extracellular bacteria. Pilot studies indicated that not all cell lines are equally susceptible for *S. pneumoniae* invasion. Allowing bacteria to invade the cells for 1 hour showed a hardly detectible amount of invaded bacteria in Detroit cells and BEAS-2B cells. A549 cells did show detectable amount of invaded bacteria after 1 hour. Allowing bacteria to invade Detroit and BEAS-2B for 2 hours showed detectable amounts of invaded bacteria. Therefore we chose to incubate A549 cells for 1 hour, while Detroit cells and BEAS-2B cells were incubated for 2 hours. After incubation cells were detached and lysed using a 1:1 mixture of 0,05% trypsin-EDTA (Life Technologies) and saponin.

Bacterial counts were derived from colony forming units (CFU). Therefore the lysates, containing the free bacteria, were plated on blood agar plates supplemented with 3% (vol/vol) sheep erythrocytes after 10-fold serial dilutions were made in M17 broth.

Adhesion and invasion assessment of *S. pneumoniae* in controls was determined as previously described, only the cells were treated with media without PR8. All experiments were performed in duplicate wells and the average of a duplicate is considered as one experiment. Data for adhesion is presented as ratio of adhered bacteria; the number of adhered bacteria were divided by the total number of bacteria present in the well. Data for invasion is presented as ratio of invaded bacteria; the number of bacteria counted after invasion was divided by the number of bacteria counted after adhesion.

Supernatant collection and infection
Detroit cells were infected with a MOI of 50 of PR8 for 1 hour and subsequently cells were washed with sterile PBS once. Cells were then overlaid with a layer of fresh prewarmed media and incubated at 37 ˚C with a 5% CO_2 humidified atmosphere. After 17 hours of exposing Detroit cells to influenza supernatants were transferred to a 1,5 ml microfuge tube and stored at -80 ˚C. Before use, the supernatants were tested for the presence of remaining influenza particles. Therefore supernatants were spun down at 1400 rpm during 5 minutes and added to a new confluent monolayer of Detroit cells (50% new media-50% supernatant). After 17 hours of infection a DAPI staining and anti NP staining was performed like previously described.
For investigating the effects of supernatants of influenza infected cells on adhesion and invasion of S. *pneumoniae* Detroit cells were incubated with previously collected supernatants (50% new media-50% supernatant). The cells were incubated with the supernatants for 17 hours before adhesion and invasion assessment were performed like previously described.

TNF-a stimulation
Detroit cells were grown in a 12 well plate until a confluent monolayer was formed. Cells were washed once with sterile PBS, and successively stimulated with TNF-a (10 ng/ml) (kindly provided by the surgery section of the University Medical Centre Groningen, The Netherlands) for 3 hours. After TNF-a stimulation an adhesion and invasion assessment was performed as previously described.

Determining receptor expression
For cell lysate preparation cells were grown in 12 well plates until confluence. Media were aspirated and cells were washed with sterile PBS once. Lysis buffer (containing 50 mM Tris-HCl (pH 7,4), 150 mM NaCl, 1% Triton 100x, 1% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and Protease Inhibitors) was added and cells were scraped to detach them from the bottom of the 12 well plate. Then the cells were transferred to an microfuge tube and kept on ice. DNAse was added and the cells again were kept on ice for 30-45 minutes. Samples were then frozen at -20 ˚C. At the time of the experiment lysates were thawed in a waterbath at 37 ˚C and 14 µl per sample was taken. Together with 2,5 lithium dodecyl sulfate (LDS) sample buffer (Invitrogen), 2,5 µl of milliQ and 1 µl of reducing agent (Invitrogen) per sample, the samples were boiled at 95 ˚C for 10 minutes. The samples were then added onto a 1,0 mm NU-PAGE Novex 10% Bis-Tris midi Gel (Invitrogen) in a 1x MOPS running buffer.
As a positive control for the PAFr a Raji Cell Lysate was used (recommended by the manufacturer of the PAFr antibody, Cayman). For the pIgR a cell lysate of Human promyelocytic leukemia cells (HL60) was used as a positive control (recommended by the manufacturer of the used pIgR antibody, Abcam). The lysate was prepared as previously described (cells kindly donated by the Oncology section of the University Medical Centre Groningen, The Netherlands).
For the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) samples ran 55 minutes at 200 Volt. For blotting the samples onto a nitrocellulose membrane, samples were blotted at 125 mA for 1 hour and 15 minutes. For staining the PAFr and

pIgR with antibodies the manufacturers' guide was followed. Briefly, the blot was blocked overnight at 4 ˚C with 1x PBS supplemented with 5% skim milk (OXOID. LTD, Basingstoke, Hampshire, England). After blocking, the blot was washed once with PBS-Tween-20 (PBS-T) and the first antibody was added to incubate for 2 hours on a shaker at room temperature. For PAFr human polyclonal PAFr antibodies were used (Cayman Chemicals, Uithoorn, The Netherlands, catalog number 160602), 1:200 diluted in 1x PBS-T, and for pIgR rabbit anti human polyclonal pIgR antibodies were used (Abcam, Cambridge, catalog number ab91269), 1:200 diluted in 1x PBS-T. Subsequently the blot was washed 4 times for 5 minutes with 1x PBS-T on a shaker. As a secondary antibody for both primary antibodies goat anti rabbit was used (488 nm, Invitrogen, catalog number C00830-01) diluted 1:5000 in sterile PBS. The secondary antibody was incubated 45 minutes in the dark on a shaker at room temperature. Then the blot was washed 4 times for 5 minutes with 1x PBS-T and 2 times for 5 minutes with PBS to remove the Tween-20. Then the blots were analyzed using an Odyssey scan (The Odyssey® Infrared Imaging System**,** LI-COR).

Staining procedures

For staining both influenza infected cells and *S. pneumoniae,* Detroit cells were grown in a 12 well plate on glass discs until a confluent monolayer was formed. An adhesion and invasion assessment was performed as previously described without lysing the cells. Cells were just washed three times with PBS and then fixed with 96% ethanol for 30 seconds. After fixation cells were washed twice with PBS and the primary antibodies for influenza infected cells and *S. pneumoniae* were added simultaneously. For staining influenza virus infected cells the nucleoprotein specific primary antibody mouse anti influenza PR8 nucleo protein was used (Serotec, product code: MCA400, 1mg/mL), and for staining *S. pneumoniae* anti capsule antibody type serum 4 (Statens Serum Institute, Denmark) was used. The primary antibodies were simultaneously incubated for 2 hours at room temperature. After incubation cells were washed once with PBS and the secondary antibodies were also added simultaneously. Goat anti mouse 594 nm was used as a secondary antibody for influenza (dilution 1:100 in PBS), and goat anti rabbit 488 nm was used as a secondary antibody for *S. pneumoniae* (dilution 1:200 in PBS) (2 mg/ml, both Invitrogen).

Statistical analysis

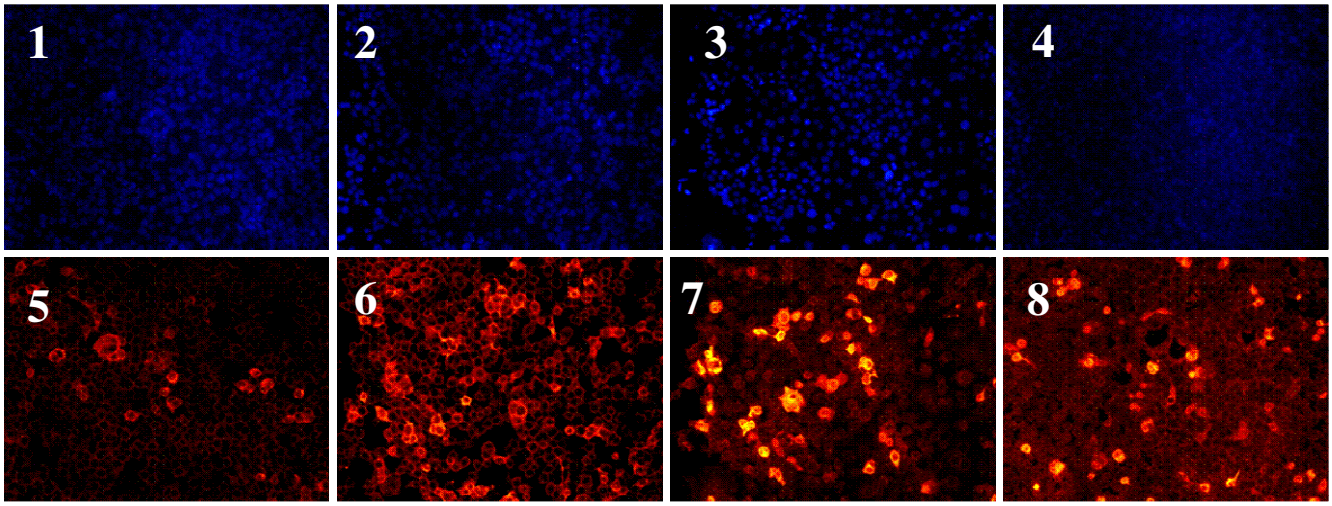
Statistical analysis was performed using the unpaired Student's *t*-test. Significant differences between groups are indicated with a *. A value of $p<0,05$ was accepted as indication of statistical significance. All experiments were performed as n=3, possible exceptions are indicated along the figures.

Results

Upper respiratory tract epithelial cells, as well as lower respiratory tract epithelial cells, are susceptible for influenza infection, but not all are equally susceptible.

First Detroit, BEAS-2B and A549 cells were tested for their susceptibility to influenza infection. This experiment was performed to test if the used cell lines can be infected with PR8 influenza virus, and to see if there were differences in susceptibility to influenza infection between the different cell lines. Cells were treated with media with different MOIs of PR8 influenza. For Detroit cells and A549 cells MOIs of 1, 10, 20, and 50 were tested (Figures 1A and 1C), while for BEAS-2B MOIs of 1, 10, 100, and 200 were tested (Figure 1B). Cells were infected with influenza for 17 hours before fixation with 100% ethanol. After fixation cells were stained either with DAPI to stain cell nuclei, or with anti NP to stain influenza infected cells.
Detroit cells showed to be susceptible for PR8 influenza virus infection (Figure 1A). A MOI of 1 showed 1% of the cells to be infected with influenza. At a MOI of 10, 20% of the cells were infected with influenza, and at a MOI of 20 this was 30%. With a MOI of 50 the highest amount of influenza infection was reached with 30% of the cells infected. Higher MOIs of virus did not show increased proportions of infected cells (data not shown). BEAS-2B cells were also susceptible for influenza infection (Figure 1B). However, the cells seemed less susceptible to influenza infection than Detroit cells since higher MOIs were needed to reach the same amount of influenza infected cells. At a MOI of 1, 1% of the cells were infected with influenza. At a MOI of 10, 5% of the cells were infected with influenza virus, and at a MOI of 100, 10% of the cells were infected. At a MOI of 200 the maximum amount of 30% of infected cells was shown. When higher MOIs were tested no increased proportion of infected cells were shown, instead cells seemed to become necrotic (data not shown). A549 cells were the most susceptible for influenza infection since the highest proportions of influenza infected cells could be reached in this cell line (Figure 1C). At a MOI of 1, 10% of the cells were infected with influenza virus. When a MOI of 10 was added, already 40% of the cells were infected with influenza. A MOI of 20 showed 60% of the cells to be infected with influenza virus. The maximum amount of influenza virus infected cells was reached with a MOI of 50 where 90% of the cells were infected with influenza.
Detroit cells, BEAS-2B cells and A549 cells were all susceptible for influenza infection. However, the alveolar epithelial cell line A549 seemed to be more susceptible to influenza infection than the nasopharyngeal cell line Detroit. The bronchial epithelial cell line BEAS-2B seemed to be the least susceptible to influenza infection. Even when high amounts of influenza virus were added only a small fraction of the cells showed to be infected with influenza virus.

A



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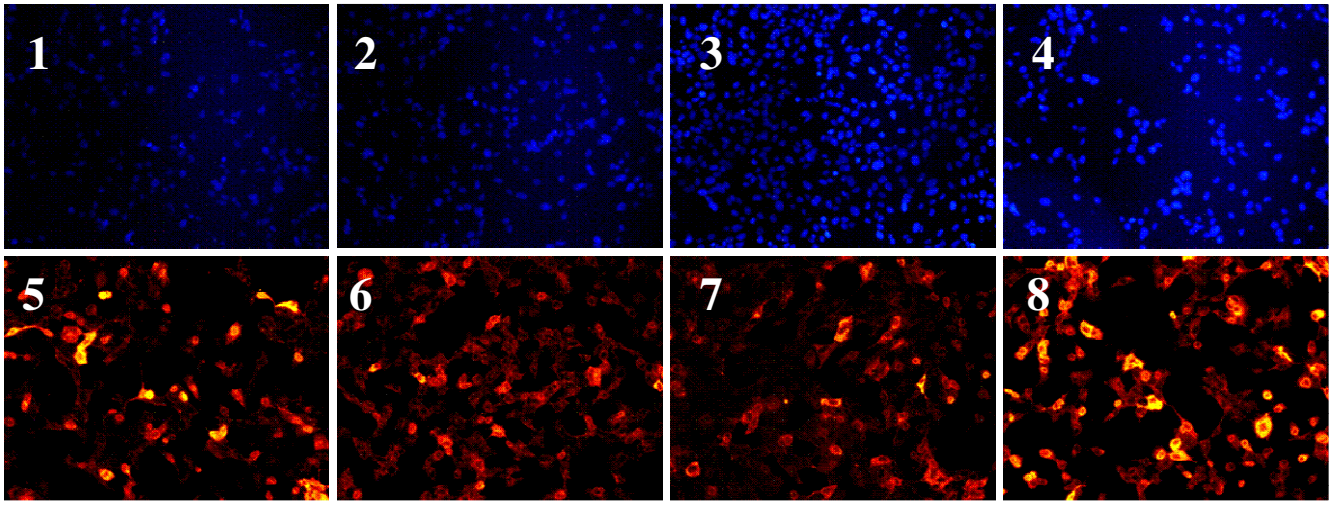

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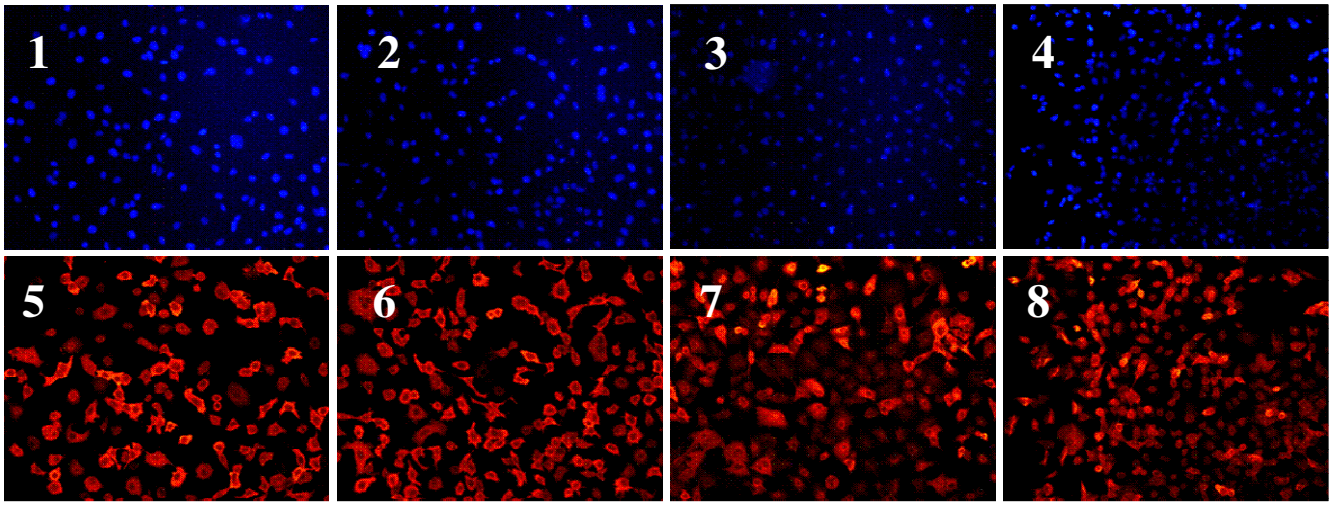


Figure 1: Susceptibility of lung epithelial cells to PR8 influenza virus.
Cells were incubated with different MOIs of PR8 influenza virus. After 17 hours of influenza infection the cells were fixed with 100% ethanol and immunofluorescent stainings were performed with DAPI to stain cell nuclei (blue) and anti NP to stain influenza infected cells (red). DAPI stainings (1-4) were compared to NP stainings (5-8).
A: Detroit cells were incubated with media containing MOIs of PR8 influenza virus of 1, 10, 20, or 50.
B: BEAS-2B cells were incubated with media containing MOIs of PR8 influenza virus of 1, 10, 100, or 200.
C: A549 cells were incubated with media containing MOIs of PR8 influenza virus of 1, 10, 20, or 50.

Thirty minutes of exposure to influenza virus has little effect on adhesion, but seems to increase susceptibility to S. pneumoniae invasion in nasopharyngeal cells.

Next we investigated the effects of 30 minutes of exposures to influenza virus on the adhesive and invasive behaviour of S. *pneumoniae*. Detroit, BEAS-2B and A549 cells were incubated with media containing PR8 influenza virus for 30 minutes, before adhesion (Figure 2A) and invasion (Figure 2B) were determined. For all experiments a starting concentration of 5 x 10^6 CFU/ml of *S. pneumoniae* was added. As a control, cells were incubated with medium without PR8 influenza virus for the same time as the cells that were exposed to PR8 influenza virus.
The nasopharyngeal cell line Detroit showed the smallest proportion of adhered bacteria in the control group with 0,73% adhered, whereas the BEAS-2B control group showed 0,84% adhered bacteria (Figure 2A). A549 cells indicated the highest proportion of adhered bacteria with 1,39% adhered in the control group. BEAS-2B cells showed a statistically significant decrease in adhesion of *S. pneumoniae* after 30 minutes of influenza infection with 58% less adhesion, compared to the control group (p=0,020). Both A549 and Detroit cells showed no difference in adhesion compared to the control groups (A549 p=0,112, Detroit p=0,554).
Only a small fraction of the adhered bacteria invaded the cells. Respectively in the Detroit control group 0,02% of the adhered bacteria invaded, for the BEAS 2B control group this was 0,16% and for the A549 control group this was 0,22% (Figure 2B). After 30 minutes of exposure to influenza virus, the invasion was trendwise increased in Detroit cells (p=0,061). For both A549 and BEAS-2B cells no differences in invasion were seen between the 30 minutes influenza infected cells and the control cells (A549 p=0,484, BEAS-2B p=0,078).
These findings indicate that 30 minutes of influenza infection has little effect on the adhesive behaviour of *S. pneumoniae* in epithelial cells of the respiratory tract. Nasopharyngeal cells may become more susceptible for bacterial invasion after 30 minutes of influenza infection. Interestingly, BEAS-2B cells seem to become less susceptible for bacterial adhesion after 30 minutes of influenza infection.

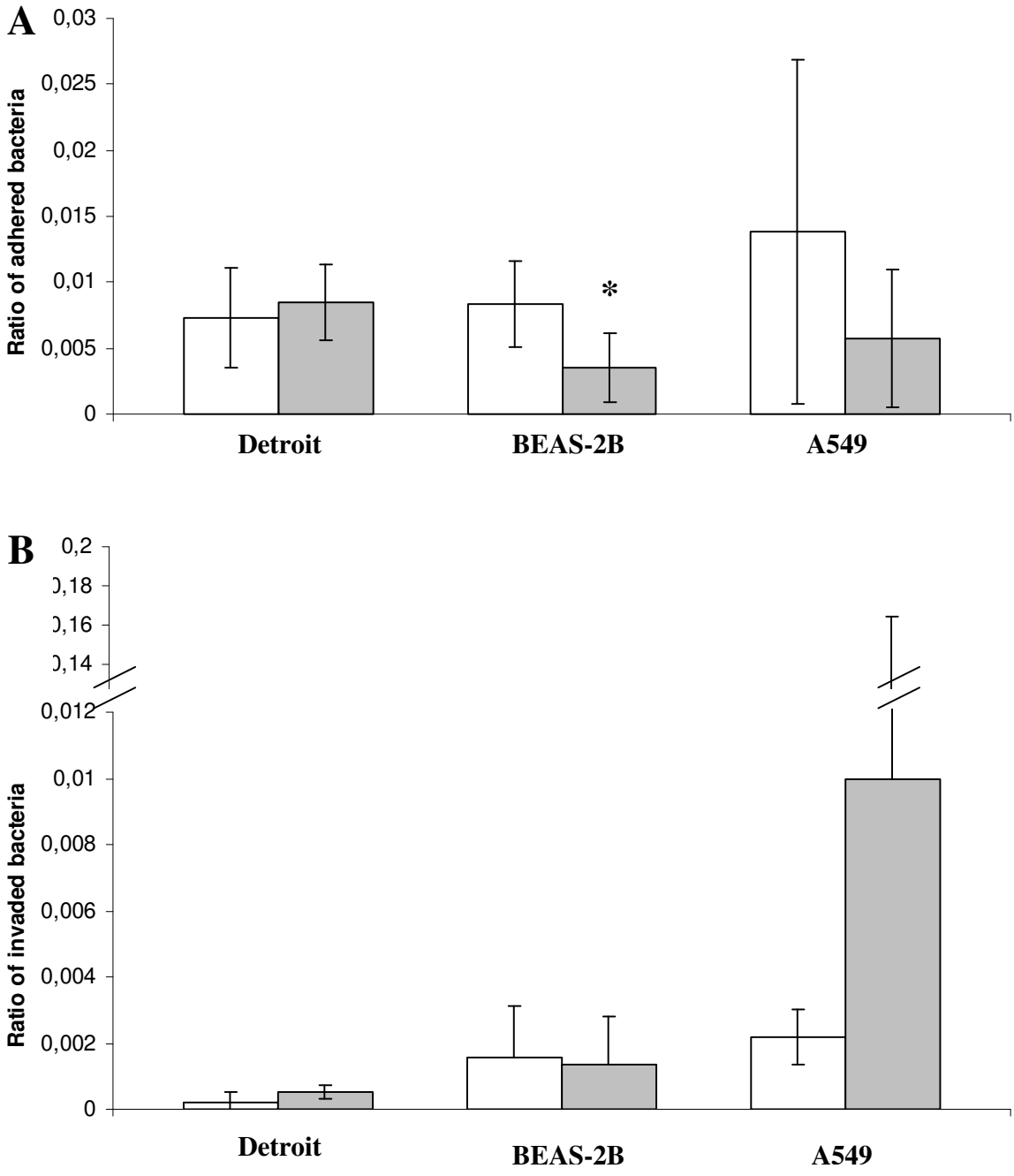


Figure 2: The effect of 30 minutes of influenza infection on the adhesion and invasion of *S. pneumoniae*.

A: Detroit and A549 cells were incubated with media with or without a MOI of 50 of PR8, BEAS-2B cell were incubated with media with or without a MOI of 200 of PR8. White bars indicate controls and grey bars indicate 30 minutes of influenza infection. After 30 minutes of influenza infection 5 x 10^6 CFU/ml of *S. pneumoniae* (TIGR4) was added, and adhesion was determined after 2 hours. Statistical significance compared to controls is indicated with * ($p<0,05$ using the unpaired T-test). Error bars indicate standard deviations (n=3).

B: Invasion into Detroit cells and BEAS-2B cells was determined after 2 hours. Invasion into A549 cells was determined after 1 hour. Statistical significance compared to controls is indicated with * ($p<0,05$ using the unpaired T-test). Error bars indicate standard deviations (n=3).

Six hours of exposure to influenza virus increases adhesion and invasion in Detroit cells.

Then we investigated the effects of 6 hours of exposure to influenza virus on the adhesion and invasion of S. *pneumoniae*. Detroit, BEAS-2B and A549 cells were incubated with media with PR8 influenza virus for 6 hours before adhesion (Figure 3A) and invasion (Figure 3B) were determined. As a control, cells were incubated for 6 hours with medium without PR8 influenza virus before adhesion and invasion were determined.
In the nasopharyngeal cell line Detroit a small proportion of adhered bacteria was seen in the control group with 0,46% adhered, whereas the BEAS-2B control group showed 0,75% adhered bacteria. A549 cells indicated the highest proportion of adhered bacteria with 0,90% adhered in the control group (Figure 3A). After 6 hours of influenza infection only Detroit cells showed a statistical significant increase in adhesion, with 40% more bacteria adhered than in the control group ($p=0,040$). Adhesion in BEAS-2B in A549 cells, after 6 hours of influenza infection, showed no differences compared to the controls (BEAS-2B $p=0,247$, A549 $p=0,673$).
Of the adhered bacteria, only a small fraction invaded the cells. Respectively, in the Detroit control group 0,02% of the adhered bacteria invaded, for the BEAS 2B control group this was 0,21% and for the A549 control group this was 0,06% (Figure 3B). After 6 hours of influenza infection, Detroit cells showed a significantly increased invasion compared to the control group ($p=0,007$). A549 cells showed no difference in invasion after 6 hours of influenza infection compared to the control group ($p=0.366$). BEAS-2B cells indicated a statistical significant decrease in invasion compared to the control ($p=0.029$).
These results indicate that Detroit cells seem to become more susceptible for both bacterial adhesion and invasion after 6 hours of influenza infection. While after 30 minutes of influenza infection only a trendwise increase in invasion was seen, a significant increased adhesion was shown after 6 hours of influenza infection. Interestingly, BEAS-2B cells seem to become less susceptible to bacterial adhesion.

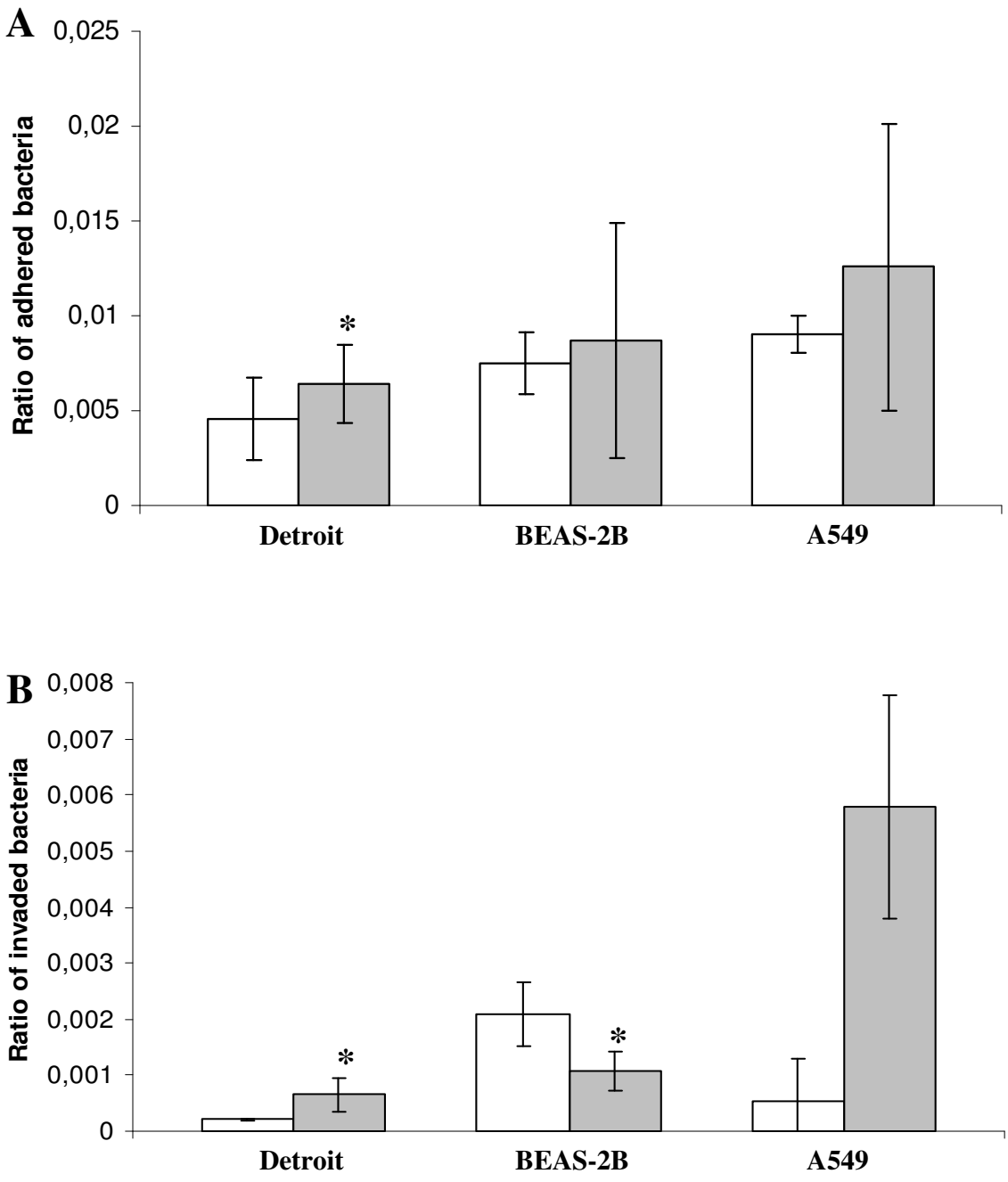


Figure 3: The effect of 6 hours of influenza infection on the adhesion and invasion of *S. pneumoniae*.
See legend figure 2A and 2B.

Seventeen hours of influenza infection has no effect on adhesion, but invasion is increased in Detroit cells

This experiment was performed to investigate the effects of 17 hours of exposure to influenza virus on the adhesion and invasion of S. *pneumoniae*. Detroit, BEAS-2B and A549 cells were incubated with media with or without PR8 virus for 17 hours before adhesion (Figure 4A) and invasion (Figure 4B) were determined. Controls were incubated for the same amount of time, only with medium without PR8 influenza virus.
Like previously observed, Detroit cells showed to remain least susceptible for bacterial adhesion with 0,65% of the added bacteria bound in the control group (Figure 4A). For BEAS-2B, 0,89 % of the bacteria adhered in the control group and for the A549 control group this was 1,01%. After 17 hours of influenza infection all epithelial cells showed no difference in adhesion, compared to their controls (Detroit p=0,494, BEAS-2B p=0,328 and A549 p=0,980).
After 17 hours of influenza infection the control groups indicate that only a small fraction of the adhered bacteria seem to be able to invade the cells (Figure 4B). For the Detroit control group 0,02% of the adhered bacteria invaded the cells. For the BEAS-2B control group this was 0,13% and for the A549 control group this was 0,14%. With an 1314% increase in invasion, the Detroit cells indicated a statistically significant increased invasion after 17 hours of influenza infection compared to the control group (p=0,003).. Both BEAS-2B and A549 showed no difference compared to the controls (BEAS-2B p=0,512, A549 p=0,888).
This experiment indicated that 17 hours of influenza infection seem to have little effect on the adhesion of *S. pneumoniae*. Detroit cells seem to become more susceptible for bacterial invasion after 17 hours of influenza infection. While we saw a trendwise increase in invasion after 30 minutes in the Detroit cells, and a significant increase in invasion after 6 hours, the largest increase in invasion was seen after 17 hours of influenza infection. Interestingly, after 17 hours of influenza infection the proportion of bacteria that seems to be able to invade the cells after adhesion is smaller than after 30 minutes and after 6 hours of influenza infection.

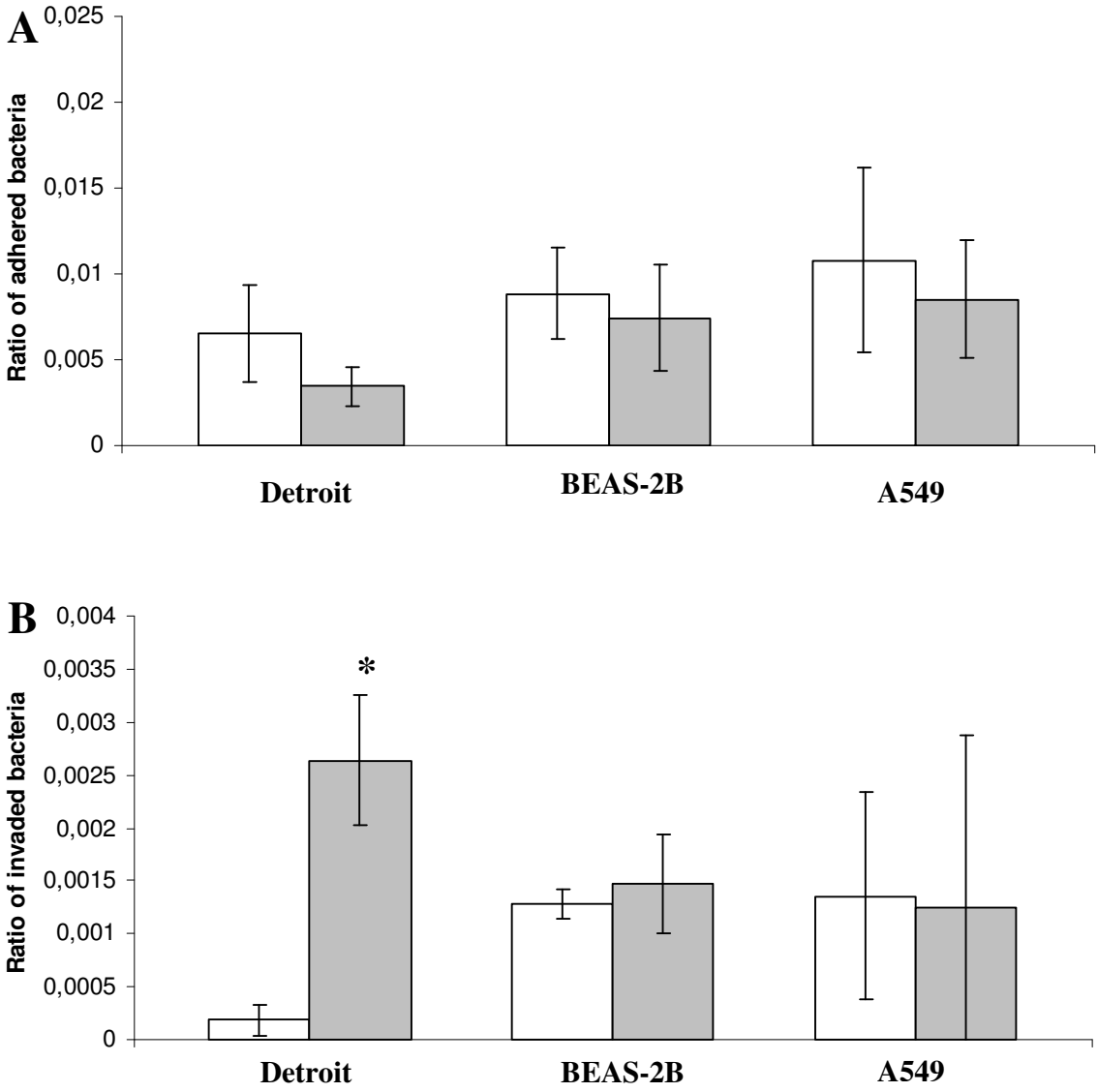


Figure 4: The effect of 17 hours of influenza infection on the adhesion and invasion of *S. pneumoniae*.
See legend figure 2A and 2B.

Detroit cells express the PAF receptor, and the receptor might be up-regulated after an influenza infection.

Previous results showed an increased susceptibility to bacterial adhesion and invasion in Detroit cells after an influenza infection, compared to uninfected cells treated with medium without PR8 influenza virus. Therefore, we studied this cell line in more detail. To investigate the involvement of the PAF receptor Western analysis was performed. After infecting Detroit cells with PR8 influenza virus for 30 minutes, 6 hours, or 17 hours, cell lysates were collected and blotted on a nitrocellulose membrane. With human polyclonal PAFr antibodies the PAF receptor was stained (Figure 5, lane A-E). To determine the molecular weight, a marker was loaded before adding the samples (marker not shown). As a positive control to mark the PAFr a Raji Cell Lysate was used (Figure 5, lane A).
According to the manufacturers guide, a band was expected at 48 kDA. A band was seen between 35 kDa and 50 kDa which is thought to be the PAFr. The positive control did not show a strong signal (Figure 5A), but untreated Detroit cells seemed to express PAFr (Figure 5B). After 30 minutes, 6 hours and 17 hours of influenza expression the receptor seemed to be slightly upregulated (Figure 5 C-D-E). This experiment indicates that Detroit cells seem to express PAFr.
PIGr expression was also investigated but a clear signal could not be detected due to possible malfunctioning of the used antibodies.

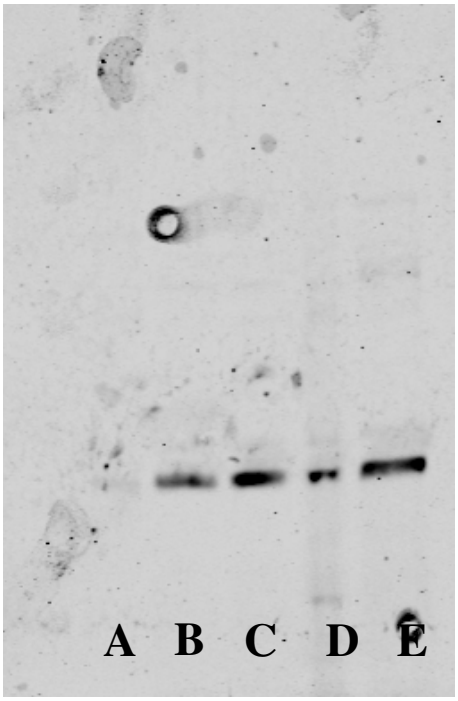
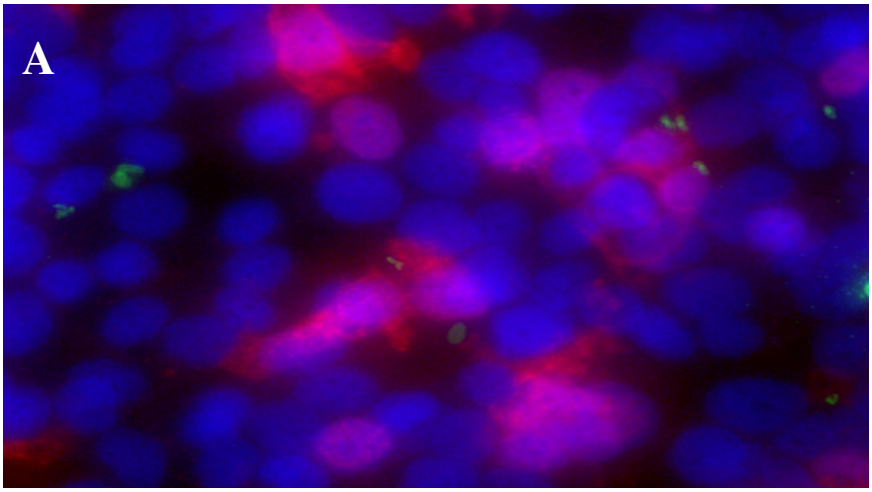


Figure 5: PAF receptor expression in Detroit cells
Cell lysates were collected and Western analysis was performed (n=3). As a positive control a Raji Cell Lysate was used (A). B indicates basic PAFr expression of Detroit cells treated with media without PR8 for 17 hours. C-D-E represent Detroit cells treated with media containing a MOI of 50 of PR8 for 30 minutes (C), 6 hours (D) or 17 hours (E).

S. pneumoniae does not seem to favour influenza infected cells over uninfected cells for both adhesion and invasion in Detroit cells.

To determine whether *S. pneumoniae* favours adhering and invading to influenza infected cells over uninfected cells, immunofluorescent stainings were performed. Detroit cells were infected with a MOI of 50 of PR8 for 17 hours before 5 x 10^6 CFU/ml of *S. pneumoniae* (TIGR4) was added. Bacteria were allowed to adhere for 2 hours before immunofluorescent staining was performed (Figure 6A). For invasion bacteria were stained after allowing them to invade for 2 hours after adhesion (Figure 6B). An immunofluorescent staining was performed to visualize influenza infected cells (red), *S. pneumoniae* (green) and cell nuclei (blue). For each staining 10 bacteria were scored and their position was determined. Due to cell culturing problems at the time of the experiment it was not possible to count for more bacteria or to repeat the experiment.
The staining for influenza infected cells showed that about 30% of the Detroit cells were infected with influenza. Bacteria only seem to be present on the outside of the cells (Figure 6A). *S. pneumoniae* does not seem to favour adhering to influenza infected Detroit cells over uninfected cells, since the bacteria seem to bind randomly.
For the staining of invaded bacteria also 30% of the Detroit cells seemed to be infected with influenza (Figure 6B). Since the cells were incubated for 2 hours with medium containing antibiotics before staining, the visible bacteria are assumed to be inside the cells. *S. pneumoniae* does not seem to favour invading into cells that are infected with influenza over uninfected cells. Bacteria seem to invade into Detroit cells randomly.
These findings suggest that previous observations concerning changes in adhesion and invasion are not restricted to influenza infected cells. The influenza infection seems to cause changes in all cells leading to increased susceptibility to *S. pneumoniae* infection.



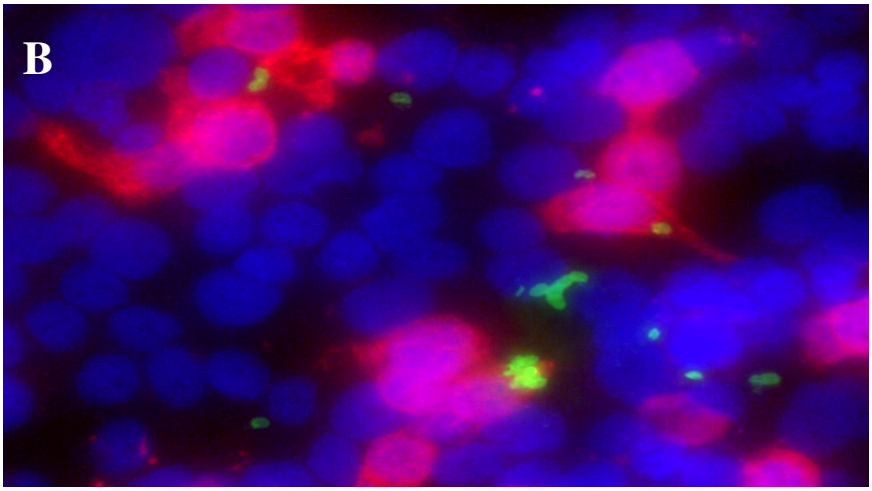


Figure 6: Staining for adhesion and invasion of *S. pneumoniae* in Detroit cells
A: Detroit cells were infected with a MOI of 50 of PR8 for 17 hours before 5 x 10^6 CFU/ml of *S. pneumoniae* (TIGR4) was added. After 2 hours of adhesion cells were fixed with 96% ethanol. Influenza infected cells were stained with NP staining (red), *S. pneumoniae* were stained with anti capsule antibodies (green) and cell nuclei were stained with DAPI staining (blue). Merge images were obtained with ImageJ (n=1).
B: After allowing the adhered bacteria to invade the cells for 2 hours, cells were fixed with 96% ethanol. Further, see legend 6A.

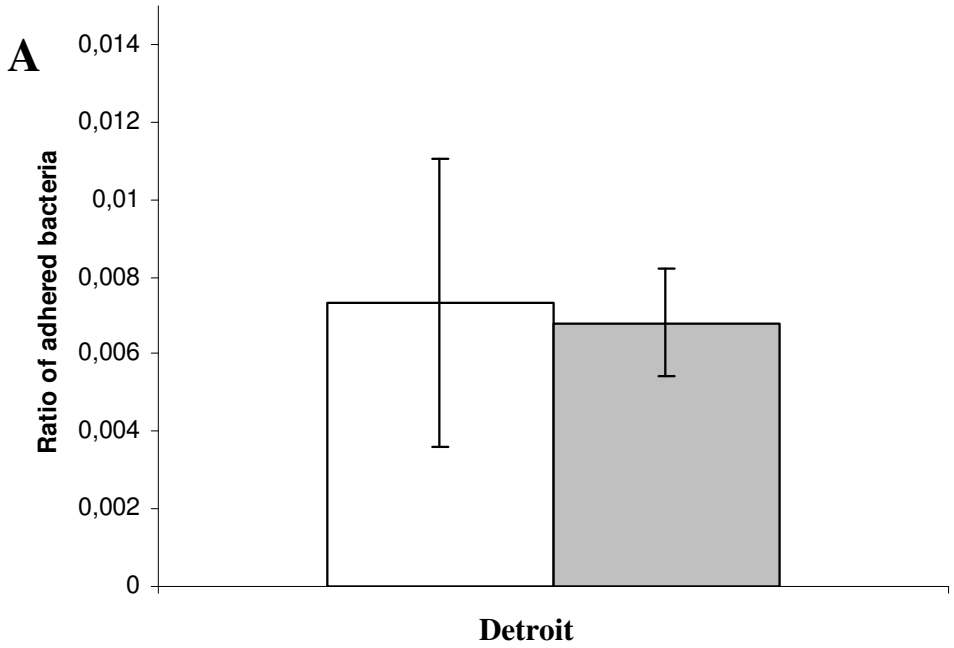
Observed changes in adhesion and invasion in Detroit cells seem to be caused by the influenza virus and not by factors secreted after an influenza infection.

This experiment was performed to investigate the effect of possible soluble factors, secreted after an influenza infection, on the adhesion and invasion of *S. pneumoniae*. Detroit cells were infected with a MOI of 50 of PR8 for 17 hours before supernatants were collected and frozen at -80 ˚C. To avoid the effects of possible nutrition depletion in the 17 hours old supernatants, new Detroit cells were infected with the supernatants and new medium (50% supernatant and 50% new media). Detroit cells were incubated with this mixture for 17 hours before adhesion (Figure 7A) and invasion (Figure 7B) were determined. Controls were incubated with medium for the same amount of time. As a control, to check for remaining influenza virus in the supernatants, supernatants were added to Detroit cells and incubated for 17 hours before DAPI staining and anti NP stainings were performed. About 10% of the cells turned out to be infected with influenza, indicating that influenza virus was still present in the supernatant (data not shown).
For the Detroit control group, treated with medium without supernatant, 0,65% of the added bacteria adhered to the cells. The cells treated with supernatant of previously 17 hours influenza infected cells showed no difference in adhesion (data not shown). For invasion a small part of the adhered bacteria invade the cells. For the Detroit control group 0,02% of the adhered bacteria invaded the cells. Also for invasion there was no

difference between the control group and the cells treated with supernatants of cells previously infected with influenza for 17 hours (data not shown).
These findings indicate that the effects on adhesion and invasion previously described seem to be due to the influenza virus itself, in stead of possible factors secreted by lung epithelial cells after an influenza infection.

Short TNF-α stimulation does not have effect on adhesion of S. pneumoniae, but invasion is increased in Detroit cells.

Since TNF-α is thought to be involved in the expression of several proteins which can be used in pneumococcal adhesion, we investigated the effects of TNF-α on adhesion and invasion of *S. pneumoniae*. Detroit cells were treated with medium with TNF-α (10 ng/ml) for 3 hours. After 3 hours of TNF-α exposure, adhesion (Figure 7A) and invasion (Figure 7B) of *S. pneumoniae* was determined. Controls were treated the same, only medium was added without TNF-α.
For the Detroit control cells 0,73% of the added bacteria adhered to the cells (Figure 7A). Adhesion of *S. pneumoniae* after TNF-α stimulation showed no difference compared to the control group.
In the control group 0,04% of the adhered bacteria invaded the cells (Figure 7B). After TNF-α stimulation the invasion increased with 198% compared to the control group.
This experiment indicates that Detroit cells seem to become more susceptible for bacterial invasion after 3 hours of TNF-α stimulation. Interestingly, this observed effect is not seen when Detroit cells were stimulated with supernatants of Detroit cells which were previously infected with influenza for 17 hours.



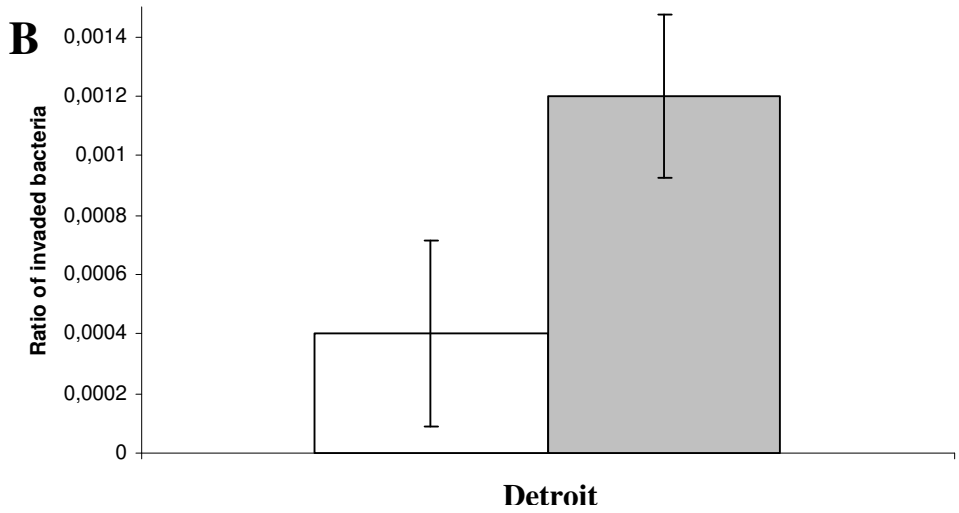


Figure 7: The effect of short TNF-α stimulation on the adhesion and invasion of *S. pneumoniae*.

A: Detroit cells were exposed to media with or without TNF-α. White bars indicate controls and grey bars indicate TNF-α stimulation. After 3 hours of TNF-α stimulation 5 x 10^6 CFU/ml of *S. pneumoniae* (TIGR4) was added, and adhesion was determined after 2 hours. Error bars indicate standard deviations (n=2).

B: Invasion was determined after 2 hours. Further, see legend 7A.

Discussion

With this research we tried to gain more insight in the mechanisms behind the lethal synergism of influenza and *S. pneumoniae*. We studied the direct interaction between influenza, lung epithelial cells and *S. pneumoniae*. We hypothesized that lung epithelial cells would become more susceptible to bacterial infection after an influenza infection.
Contradictory to McCullers et al., we showed that only 6 hours of influenza infection would lead to increased adhesion of *S. pneumoniae* to nasopharyngeal cells. This is, to our knowledge, the first time that 6 hours of influenza infection was proven to increase adhesion of *S. pneumoniae*. McCullers et al. showed that 30 minutes of exposure to influenza would lead to significant increased adhesion of *S. pneumoniae* in alveolar epithelial cells, compared to untreated cells [8]. After 30 minutes of influenza infection, viral NA activity was thought to have an effect on adhesion by cleaving sialic acid on the cells, and therefore priming epithelial cells for a *S. pneumoniae* infection. This was proven by McCullers et al. by using the NA inhibitor Oseltamivir. Adhesion of *S. pneumoniae* was decreased when viral NA function was inhibited. In their study viral particles were washed away after 30 minutes of exposure to lung epithelial cells. In our study however, viral particles were washed away after 1 hour of exposure to lung epithelial cells. Cells were then incubated for an additional 30 minutes. This difference in exposure time may have an effect on the viral NA activity, possibly diminishing its effects on the adhesion of *S. pneumoniae*. This difference in experimental set-up may be the reason why we didn't see increased adhesion after 30 minutes of influenza infection. After 6 hours of influenza infection the viral particles will have entered the lung epithelial cells, and viral replication may have started at the time of *S. pneumoniae* infection [29]. The viral NA activity may therefore not be the only factor contributing to the observed increased adhesion of *S. pneumoniae,* after 6 hours of influenza infection. After 17 hours of influenza infection we observed no changes in adhesion between the influenza infected cells and the cells treated with media for the same amount of time.
While performing our experiments in bronchial epithelial cells and alveolar epithelial cells, we experienced great variation within the repeats of the experiments. Since a number of (yeast) infections occurred in these cell lines during the time of the experiments, this may be the cause of the observed variations. Therefore we can not draw clear conclusions from these experiments.
Furthermore, our results show that nasopharyngeal epithelial cells become more susceptible for *S. pneumoniae* invasion after an influenza infection. As explained in the introduction, the PAFr may be used by *S. pneumoniae* to adhere, and invade epithelial cells [19]. It is thought that phosphorylcholine, expressed on the pneumococcal cell wall, can bind to the PAFr [18]. Multiple, *in vivo,* studies have been performed to investigate the expression of the PAFr, providing contradictory results about a possible up-regulation of the receptor after an influenza infection [18, 19, 20]. Nonetheless, this possible up-regulation was never proven to be caused by the influenza infection itself, since multiple factors can cause this effect *in vivo*. Our findings do not give a clear indication of increased PAFr expression after an influenza infection *in vitro*. *S. pneumoniae* binds to the PAfr after it has been phosphorylated [30]. An influenza infection may be able to activate the PAFr directly. After 30 minutes of influenza infection we saw a trendwise

increase in invasion of *S. pneumoniae* compared to the control group. After 6 hours there was a significant increased invasion, and after 17 hours we saw the largest increase in invasion. This might indicate that the PAfr gets activated over time. Therefore, it might be worthwhile to investigate the proportion of activated PAFr on lung epithelial cells, especially after different time periods of influenza infection.
Even though we could not generate a clear signal to prove the presence of pIgR due to practical setbacks, Brock et al. proved the presence of pIgR on nasopharyngeal cells [21]. That study also indicates that the pIgR receptor is used by *S. pneumoniae* to invade epithelial cells. Future research should look into the expression of the pIgR on nasopharyngeal cells in more detail. It would be interesting to know if the receptor was indeed upregulated after different time periods of influenza infection.
Interestingly, we observed in our control groups that the highest proportion of *S. pneumoniae* was able to invade after 30 minutes of exposing cells to the appropriate medium. The proportion of invaded bacteria in the control group was smaller after 6 hours of exposure to medium, and after 17 hours the smallest fraction of invaded bacteria was observed in the control group. Exposing cells for a long time to medium may lead to depletion of nutrients present in the medium. This was especially seen after 17 hours of influenza infection since the colour of the media changed, indicating pH changes. This may have an effect on the capability of *S. pneumoniae* to bind to the cells. While we performed a visual control to check for cell stress, cells did not seem to become stressed. However, stressed cells may have been washed away during our adhesion and invasion assessments, which may give a distorted view of the situation.
Le Goffic et al, showed that lung epithelial cells, after an influenza infection, start to produce a number of pro-inflammatory cytokines, among which TNF-α, Il-1β, and IL-6 [26]. Therefore we hypothesised that soluble factors secreted after an influenza infection would cause increased susceptibility to *S. pneumoniae*. When investigating the preference of *S. pneumoniae* to bind to influenza infected cells or to uninfected cells, preliminary results seemed to confirm this hypothesis. *S. pneumoniae* showed to bind randomly to cells. It has to be taken in account that this experiment was only performed once, scoring a low number of bacteria. When we investigated the effect of soluble factors on the adhesion and invasion of *S. pneumoniae,* we did not see any difference between the cells infected with previously collected supernatant and untreated cells. One reasonable explanation may be that the supernatants were frozen before the experiments were performed. Freezing might have an effect on the activity of the pro-inflammatory cytokines, possibly present in the supernatant. Repeating these experiments with freshly collected supernatants might indeed indicate an effect on adhesion and invasion of *S. pneumoniae,* which would be in line with our hypothesis.
Additionally, it might be interesting to investigate which pro-inflammatory cytokines were produced by the used cell lines. With our experiment we showed that TNF-α induced increased susceptibility to *S. pneumoniae* invasion in nasopharyngeal cells. However, it was not investigated if this pro-inflammatory cytokine was present in the collected supernatants. Furthermore it might be interesting to look at the interaction between the PAFr and pro-inflammatory cytokines. Besides an effect of pro-inflammatory cytokines on the expression of PAFr, there may also be an opposing effect. Recently, Hasegawa et al. have shown in a rodent model that the PAFr may be involved in the expression of the pro-inflammatory cytokines TNF-α and IL-1ß [31], indicating

that the involvement of the PAFr in the synergism of influenza and *S. pneumoniae* may be more complex than previously assumed.

With our cell based *in vitro* model we were able to perform an influenza infection in cells representing different parts of the respiratory tract. While the used nasopharyngeal epithelial cells, bronchial epithelial cells and alveolar epithelial cells poses many characteristics representing the human respiratory tract, our used cell model has its limitations. In a living organism many factors play a role in the adhesive and invasive behaviour of *S. pneumoniae*. While we investigated the direct interaction of influenza, lung epithelial cells, and *S. pneumoniae,* this is only a small part of the underlying mechanism leading to the co-infection of influenza and *S. pneumoniae*. The innate and adaptive immune system play an important role in the synergism of influenza and S. *pneumoniae*. When we investigated the effect of TNF-α on the adhesion and invasion of *S. pneumoniae*, we found that nasopharyngeal cells became more susceptible to bacterial invasion after TNF-α stimulation. While these findings might suggest that epithelial cells play a signalling role in the immune response, this role of is possibly only of minor relevance. Recently, Wu et al. have shown that successive influenza virus infection and *S. pneumoniae* stimulation alter the dendritic cell function [32]. During their study it was shown that the challenge of influenza virus and pneumococcus altered dendritic cell functions. While an influenza infection alone indicated a mild induction of TNF-α, IL-6 and IFN-γ from dendritic cells, a pneumococcal challenge induced production of Il-6, IFN-γ, TNF-α, IL-12 and IL10 from dendritic cells. However, successive challenge of dendritic cells with influenza virus and pneumococcus resulted in synergistic up-regulation of pro-inflammatory cytokines with simultaneous down-regulation of the anti-inflammatory cytokine Il-10. These findings indicate that signalling of dendritic cells may play an important role in the synergism of influenza and *S. pneumoniae*. Therefore, it might be interesting to expand our used cell model with dendritic cells to investigate the effect of direct interaction of the two pathogens with lung epithelial cells, and their ability to activate dendritic cells.

In conclusion, our results indicate that nasopharyngeal epithelial cells become more susceptible to invasion of *S. pneumoniae* after an influenza infection, and different time periods of influenza infection do play a role in this effect. While we proposed some possible causes for this increased susceptibility, several questions remain to be answered. One important issue that needs to be further investigated is the effect of secreted pro-inflammatory cytokines after an influenza infection, and their effect on PAFr and pIgR. Besides TNF-α, many other soluble factors secreted after an influenza infection may be interesting targets for future research. Also, looking at the activity of PAFr and pIgR in more detail, especially after different time periods of influenza infection might be of valuable importance. Furthermore, adding dendritic cells to our used cell model might give more insight in the interactions of the local effects of influenza, lung epithelial cells, and *S. pneumoniae* on evoking the immune response.

With recent outbreaks of 'New' influenza pandemics like H1N1 and the avian flu a renewed focus is brought to the co-infection of influenza and *S. pneumoniae*. More knowledge in the mechanisms underlying this synergism may help reduce lethality during influenza pandemics, and may create new therapeutic targets in prevention and treatment of bacterial pneumonia.

ACKNOWLEDGEMENTS

With recent outbreaks of H1N1 and avian flu influenza infections are a hot topic. With this project I learned more about the underlying mechanisms which are involved in influenza infections and the synergism of the virus with *S. pneumoniae*. I thank Anke Huckriede and Jetta Bijlsma for giving me the opportunity to work on this project and for giving me valuable input during my research project. The past 6 months have been very interesting and informative. I would also like to thank Jacqueline de Vries and Federico Iovino for teaching me basic laboratory skills which I will need during my future scientific career, and for their help with my project.

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