Budesonide Inhibits Intracellular Infection with Non-Typeable *Haemophilus influenzae* despite Its Anti-Inflammatory Effects in Respiratory Cells and Human Lung Tissue: A Role for p38 MAP Kinase

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Key Words

Budesonide · Chronic obstructive pulmonary disease · Inhaled corticosteroids · Non-typeable *Haemophilus influenzae* · Intracellular infection

Abstract

Background: Inhaled corticosteroids (ICS) are widely used in the treatment of obstructive lung diseases. Recent data suggest a higher pneumonia risk in chronic obstructive pulmonary disease (COPD) patients treated with ICS. **Objective:** Since non-typeable *Haemophilus influenzae* (NTHi) is the most common pathogen associated with acute exacerbations of COPD, we investigated the effects of budesonide (BUD) on NTHi-induced inflammation and invasive infection. **Methods:** The alveolar epithelial cell line A549 and specimens of human lung tissue (HLT) were used in our experiments. Intracellular infection was determined by a lysis/culture assay of infected cells. Activated p38 mitogen-activated protein kinase (MAPK) was assessed using Western blotting and immunohistochemistry, expression of toll-like

receptor 2 (TLR2) was determined by PCR, and CXCL-8 levels were measured using ELISA. Immunohistochemistry was used for detection of CXCL-8, platelet-activating factor receptor (PAF-R) and NTHi. **Results:** BUD significantly reduced CXCL-8 secretion in A549 cells and lung tissue infected with NTHi. Furthermore, BUD decreased the expression of PAF-R in HLT and A549 cells. In A549 cells and HLT, BUD inhibited intracellular infection and – synergistically with NTHi – increased the expression of TLR2 (in A549 cells). TLR2 stimulation did not influence the intracellular infection of A549 cells, but p38 MAPK inhibition resulted in a significant reduction of infection. **Conclusion:** The present study adds new insights into the effects of glucocorticoids on pulmonary host defence after NTHi infection. Although the inflammatory response to infection is suppressed by BUD, interestingly, the intracellular infection is also inhibited. This effect seems to depend on the inhibition of p38 MAPK – a key enzyme in many pro-inflammatory pathways – as well as of PAF-R expression.

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Introduction

Patients with chronic obstructive pulmonary disease (COPD) and asthma frequently suffer from episodes of exacerbation during the course of the disease. Treatment of both diseases includes inhaled corticosteroids (ICS) [1]. ICS have been extensively studied for the treatment of COPD patients with frequent exacerbations, demonstrating a reduction of acute exacerbations [2] and an improvement in pulmonary function [3], and they are a mainstay of treatment for patients with asthma [4].

In contrast to the beneficial effects of ICS, recent studies and meta-analyses have yielded evidence that administration of ICS is associated with a higher risk of pneumonia in COPD patients [5–7]. This was observed in randomized trials [8, 9] as well as in large cohort studies irrespective of combinations with long-acting β -agonists and appears to depend on dosage and observation time [7, 9]. On the other hand, an association of ICS use with pneumonia risk was not described in asthma patients [10].

The value of ICS for chronic airway diseases mainly results from their anti-inflammatory effects, including suppression of cytokine and chemokine production and adhesion molecules in airway epithelial cells (AEC). Furthermore, they reduce the production of reactive oxygen species by neutrophil granulocytes in vitro. ICS exert their effects by binding to the glucocorticoid receptor, which in turn either interferes directly or indirectly with genomic or non-genomic processes. In contrast to their immunosuppressive effects, glucocorticoids have also been proven to amplify innate immune mechanisms. Synergistically with non-typeable *Haemophilus influenzae* (NTHi) or lipopolysaccharide, they are able to increase the expression of toll-like receptor 2 (TLR2) [11]. TLR2 is part of the recognition molecule system of innate immunity and is expressed and regulated on pulmonary cells involved in first-line host defence, including monocytes, macrophages and AEC [12]. TLR2 recognizes microbial components of Gram-positive and Gram-negative bacteria and mediates a variety of immune responses [13]. NTHi is the most common bacterial pathogen implicated in COPD exacerbations [14], with newly acquired strains significantly increasing the risk of exacerbations [15]. NTHi is found not only in the airways of patients during exacerbations [14], but also intracellularly in biopsies taken during COPD exacerbations [16]. One receptor mediating the invasion of human epithelial cells is the platelet-activating factor receptor (PAF-R) [17]. Recent studies suggest that NTHi resides within

AEC without replication [18]. It is, moreover, able to colonize diseased lungs [19]. Additionally, NTHi is among the most common pathogens isolated in patients suffering from pneumonia [20].

TLR2 signalling has been shown to be essential for host defence in mouse models of *Haemophilus* infection. In this regard, it has been shown that p38 mitogen-activated protein kinase (MAPK) plays an important role in the regulation of TLR2 expression and of airway inflammation in COPD in general [21].

Due to the frequent use of ICS in COPD treatment, and the accumulating evidence from clinical trials about increased infection risks under this therapy, we investigated the effects of ICS on the NTHi-induced inflammatory response and invasive infection in cell culture and in a human lung tissue (HLT) infection model.

Methods

Cell Culture

The A549 cell line (DSMZ) was used in our experiments. Cells were cultured in DMEM High Glucose medium (PAA Laboratories GmbH) supplemented with 10 μ g/ml gentamicin (Sigma-Aldrich) in 175-cm² flasks (Greiner Bio-One); 10% fetal calf serum (FCS; PAA) and HEPES buffer (PAA; S11-001) were added as well. For the experiments, cells were harvested and counted, and 500,000 cells were seeded in 6-well cell culture plates (Greiner Bio-One). The cells were incubated overnight in 2 ml RPMI 1640 medium (PAA) supplemented with 5 ml of 200 mM L-glutamine (PAA), 5 ml of MEM NEAA (PAA) and 10% FCS. For the experiments, the medium was removed, and the experiments were carried out in RPMI 1640 medium with L-glutamine and NEAA. In all the experiments, incubation was always conducted at 37°C, 95% air and 5% CO₂. As a negative control, in all the experiments, 1 well was pre-incubated with 900 μ l of the medium only.

Lung Tissue

HLT was taken after informed consent from patients (mean age 63 years; 3 males, 1 female) without underlying chronic or acute lung diseases undergoing lung resections because of pulmonary nodules as previously described [22]. The study was approved by the Ethics Committee of the University of Lübeck and is in compliance with the Declaration of Helsinki. Tumour-free pieces were taken at least 5 cm away from the tumour front. Pieces of 0.3–0.4 g were cultured for 24 h in RPMI 1640 medium (Biochrome AG) containing 10% FCS in 48-well plates (Nunc) at 37°C and 5% CO₂.

NTHi Culture and Infection

For infection, the NTHi strain Rd KW20 was used. It was isolated in 2002 from a COPD patient suffering from invasive pneumonia. NTHi was grown overnight on PVX plates (bioMérieux). For infection, bacteria were harvested and suspended in sterile 0.9% NaCl solution. To obtain the desired multiplicity of infection (MOI), turbidity was measured, and a McFarland (McF) value of 4 for an MOI of 200 colony-forming units (CFU)/cell and an McF

value of 0.5 for an MOI of 20 CFU/cell were adjusted. One hundred microlitres of each NTHi suspension was then added to the wells already containing 900 µl of the medium used for pre-incubation. As a negative control, 100 µl of sterile saline solution was added instead of the solution containing NTHi. For the ex vivo infection experiments of HLT, specimens were incubated with 500 µl NTHi suspension (10^7 CFU/ml).

Treatment Protocols

The ICS budesonide (BUD, Pulmicort 0.5 mg/2 ml; AstraZeneca) was diluted in cell culture medium, and the A549 cells were pre-incubated with 900 µl of the ICS solutions for 1 h. To achieve concentrations similar to lung tissue concentrations following therapeutic application, we used concentrations of 10^{-6} and 10^{-8} M according to previous data [23].

SB 203580 was used at a concentration of 20 µM; A549 cells were pre-incubated with the inhibitor for 1 h before infection. Pam3Cys-SKKKK (EMC Microcollections, Cat. No. L2000) was used as a ligand for TLR2. The cells were stimulated with 100 nM of Pam3 simultaneously with the NTHi infection. The dilution was carried out in the same cell culture medium as used in the infection experiments. WEB-2086 (Santa Cruz Biotechnology) was used as a PAF-R antagonist according to the manufacturer's instructions. Cells were pre-incubated at two different concentrations for 1 h before infection with NTHi.

Enzyme-Linked Immunosorbent Assay

Supernatants were collected, aliquoted and stored at -70°C . Measurement of CXCL-8 levels was performed using commercially available ELISA kits (Human CXCL8/IL-8 DuoSet; R&D Systems).

Invasion Assay

To evaluate the invasion of epithelial cells by NTHi, an invasion assay was used as described by Isberg and Falkow [24] and others [25]. After 24 h of infection, the supernatants were removed and the wells were washed twice with phosphate-buffered saline (PBS; Gibco). Then, the cells were incubated with the same fresh cell culture medium as used for infection, supplemented with 100 µg/ml gentamicin to kill the remaining extracellular bacteria. After 1 h, the medium was removed, the wells were washed again 3 times with PBS, and a sample of the last washing solution was plated on a PVX agar plate to make sure the extracellular bacteria were successfully killed. Sequentially, cells were lysed using 1% saponin (Riedel-de Haën) in PBS to release the intracellular bacteria. After adequate dilution with PBS, NTHi was plated onto PVX agar and incubated. The next day, the colonies were counted.

Detection of Cytotoxicity

For detection of cytotoxicity, lactate dehydrogenase (LDH) was measured in the cell culture and tissue supernatants according to the manufacturer's instructions (Roche).

Quantitative Real-Time PCR

Quantitative real-time PCR was used in order to assess the changes in TLR2 expression caused by ICS and NTHi. As a first step, RNA was isolated after 4.5 h of infection, using the NucleoSpin RNA II Kit (Roche), and then transcribed into cDNA. The primers used were purchased from TIB Molbiol. The following primers were used: hTLR2 sense 5'-GCC AAA GTC TTG ATT

GAT TGG; hTLR2 antisense 5'-TTG AAG TTC TCC AGC TCC TG (347 bp); 18S right 5'-CCT CCA ATG GAT CCT CGT TA, and 18S left 5'-AAA CGG CTA CCA CAT CCA AG. PCR was performed using a LightCycler Detection System (Roche). The measured hTLR2 levels were evaluated as a ratio against 18S levels to account for different quantities of cDNA in each sample.

Western Blot

Changes in p38 MAPK activity were determined using Western blotting. After 1 h of pre-incubation either with BUD or the PAF-R antagonist followed by 24 h of infection with NTHi (MOI = 200), the supernatants were removed and the cells were lysed and stored at -30°C . Before performing Western blotting, the lysates were denatured at 95°C . Immunodetection of phosphorylated p38 MAPK was performed with specific antibodies (Cell Signaling Technology). β -Actin was used as a loading control.

Immunohistochemical Staining

Primary antibodies [phosphorylated p38 MAPK, rabbit anti-human, Cell Signaling Technology; *H. influenzae*, mouse monoclonal (clone 1079-457), www.antikoerper-online.de; PAF-R, rabbit anti-human, Novus] were applied at a dilution of 1:100 as described elsewhere [26]. Identification of cell types was performed morphologically by lung pathologists.

Statistical Analysis

For statistical analysis, SPSS 17.0 was used. All data are displayed as means ($\pm$ SEM). Comparisons between absolute and relative values were made using the Wilcoxon signed-rank test. ANOVA with Bonferroni's correction for multiple comparisons was performed on each data set. A significant result was defined by a p value <0.05 .

Results

CXCL-8 Expression in A549 Cells and HLT Is Induced by NTHi Infection and Reduced after Pre-Incubation with BUD

A549 cells were infected with two different concentrations of NTHi. Both concentrations (20 and 200 CFU/cell) elicited inflammation in the cell culture, as shown by a significant increase in CXCL-8 levels after 24 h of infection. To test the anti-inflammatory capacity of BUD, we incubated the cells for 1 h prior to infection with two different concentrations of BUD (10^{-8} and 10^{-6} M). According to previous data, a concentration of 10^{-8} M is likely to represent concentrations in the distal respiratory tract during inhalation therapy [23]. Both concentrations significantly decreased CXCL-8 levels (fig. 1a). A cytotoxic effect was excluded using the LDH cytotoxicity detection assay.

In an ex vivo HLT model which more closely reflects the situation in vivo, we could demonstrate that BUD was similarly able to significantly reduce the CXCL-8 secre-

Fig. 1. Effect of BUD on inflammation and cell death in NTHi-infected A549 cells (**a, b**) and HLT (**c–e**). **a, b** Levels of CXCL-8 (**a**) and LDH (**b**) measured in supernatants of A549 cells. A549 cells were infected with different numbers of NTHi. The MOI were 200 (high) and 20 CFU/cell (low). Cells were incubated with one of two different concentrations of BUD prior to infection for 1 h. The control was neither treated with BUD nor infected. Samples were collected 24 h after infection. **a** $n = 6$ (BUD only $n = 3$); $^+ p < 0.05$

(NTHi low), $^* p < 0.05$ (NTHi high), infected assays pre-treated with BUD vs. assays only infected. **b** $n = 6$; $^+ p < 0.05$ (NTHi low), $^* p < 0.05$ (NTHi high). **c** CXCL-8 secretion in HLT after pre-incubation with two different concentrations of BUD prior to infection with NTHi (10^6 CFU/ml) for 24 h. $n = 4$; $^+ p < 0.05$ (NTHi low), $^* p < 0.05$ (NTHi high). **d, e** Representative immunohistochemistry of CXCL-8 in NTHi-infected HLT without (**d**) and with BUD pre-treatment (**e**). All values are displayed as means $\pm$ SEM.

tion ($n = 4$, $p < 0.05$; fig. 1c) and expression (fig. 1d, e) mediated by NTHi infection.

Invasion of A549 Cells and HLT

To investigate whether BUD treatment influences the invasion of epithelial cells, the intracellular invasion by NTHi after 24 h of infection was examined. Again, we

used two different concentrations of bacteria (200 and 20 CFU/cell) and ICS.

BUD significantly reduced bacterial invasion by 40% in A549 cells at both concentrations (10^{-6} and 10^{-8} M, $p < 0.01$; fig. 2a). A direct antibiotic effect of the preparation of BUD used was excluded by plating NTHi diluted in RPMI 1640 with and without BUD at 0.01 μ M on PVX

Fig. 2. Pre-treatment with BUD reduces cell invasion by NTHi. **a** Relative amounts of intracellular CFU in A549 cells after 24 h of infection. CFU counted in cells pre-treated for 1 h with BUD were compared with those counted in untreated cells. Two different concentrations of BUD and two different MOI (high: 200 CFU/cell; low: 20 CFU/cell) were used. $n = 7$; $+ p < 0.05$ (NTHi low), $* p < 0.05$ (NTHi high). All values are displayed as means $\pm$ SEM. **b, c** Representative immunohistochemistry of NTHi without (**b**) or with BUD pre-treatment (**c**) (n = 4). **d, e** Representative immunohistochemistry of PAF-R without (**d**) or with BUD pre-treatment (**e**) in NTHi-infected HLT (n = 4). **f, g** Representative immunohistochemistry of PAF-R without (**f**) or with BUD pre-treatment (**g**) in A549 cells (n = 4).

agar. After 24 h, the colonies were counted, and no difference could be detected (data not shown). In addition, reduced invasion of NTHi into HLT induced by BUD was demonstrated by immunohistochemistry (fig. 2b, c). This effect was most clearly observed in alveolar macrophages.

Concomitantly, the expression of PAF-R as an important receptor for adherence of NTHi was evaluated. A bright membranous and intracellular expression of PAF-R was detected in alveolar macrophages as well as epithelial cells after NTHi infection using HLT (fig. 2d), where-

Fig. 3. BUD enhances TLR2 expression in A549 cells infected with NTHi. Fold change in TLR2 mRNA levels in A549 cells treated with two different concentrations of BUD for 1 h prior to infection with NTHi (high MOI: 200 CFU/cell; low MOI: 20 CFU/cell) for 4 h. The mRNA levels of pre-treated and infected cells were compared with levels measured in untreated and infected cells. Additionally, pre-treated and uninfected cells were compared with untreated and uninfected cells (BUD only). $n = 7$; # $p < 0.05$ (BUD only), * $p < 0.05$ (NTHi low), + $p < 0.05$ (NTHi high). All values are displayed as means $\pm$ SEM.

as pre-incubation with BUD led to a decreased expression of PAF-R (fig. 2e). A549 cells showed a down-regulation of PAF-R after pre-incubation with BUD as well (fig. 2f, g).

BUD Increases TLR2 mRNA Levels in A549 Cells Infected with NTHi

As TLR play an important role in innate immunity and TLR2 is known to be regulated by NTHi and glucocorticoids, we examined the effect of *Haemophilus* infection with or without BUD pre-incubation on TLR2 expression using an MOI of 20. Our findings demonstrate that incubation with BUD increases the expression of TLR2 mRNA 4- to 10-fold compared to NTHi infection without prior ICS treatment, irrespective of the infection dose ($p < 0.05$; fig. 3).

TLR2 Co-Stimulation Demonstrates No Effect on NTHi Invasion of A549 Cells in Contrast to Blockade of p38 MAPK

To determine whether additional TLR2 stimulation is involved in the reduction of the invasive infection of epi-

thelial cells, we conducted an experiment with simultaneous NTHi infection (MOI = 20 CFU/cell) and Pam3 stimulation. As shown in figure 4a, no significant difference in intracellular CFU after 24 h of infection was observed between cells with and those without additional TLR2 stimulation.

We found a strong expression of p38 MAPK in HLT after NTHi infection mainly in alveolar macrophages, which was markedly reduced after BUD coincubation (fig. 4b, c). In order to further characterize the effects of p38 MAPK down-modulation on bacterial invasion of AEC, we blocked p38 MAPK by the specific p38 inhibitor SB 203580 prior to NTHi infection of A549 cells. Incubation of the cells with 20 μ M of SB 203580 for 1 h prior to infection led to a strong reduction of intracellular CFU after 24 h ($p < 0.05$; fig. 4a).

p38 MAPK Activity Is Inhibited by BUD and PAF-R Antagonist

Pre-incubation of A549 cells with BUD at the same concentrations as used in the previous experiments inhibited the phosphorylation of p38 MAPK after infection with NTHi significantly (fig. 5). Since NTHi infection of respiratory cells has been linked to PAF-R, we additionally evaluated if PAF-R plays a role in the activation of p38 MAPK using a PAF-R antagonist. As shown in figure 5a, pre-incubation of A549 cells with this inhibitor also led to a reduced amount of phosphorylated p38 MAPK after infection with NTHi (MOI = 200).

Discussion

In the present study, we demonstrate divergent effects of ICS on NTHi-induced innate immune responses and infection rates. BUD significantly reduced CXCL-8 expression in NTHi-infected A549 cells and HLT. In contrast, BUD increased the NTHi-induced expression of TLR2 in the presence of NTHi on alveolar epithelial cells. Interestingly, ICS treatment led to a significant reduction of intracellular infection, which was associated with reduced p38 MAPK activation. The same effect was seen after targeted inhibition of p38 MAPK. Furthermore, p38 MAPK activation following infection seems to be reduced by blocking of PAF-R. Interestingly, BUD led to decreased expression of PAF-R, which is known to play an important role in adherence and invasion of NTHi. Thus, different mechanisms may contribute to the reduced invasiveness of NTHi under BUD treatment; both effects may contribute, but it remains to be shown whether these

Fig. 4. Effect of TLR2 stimulation and p38 inhibition on cell invasion by NTHi. **a** Relative amounts of intracellular CFU in A549 cells after 24 h of infection. Intracellular CFU in cells co-stimulated with Pam3 or pre-treated with 20 μM of SB 203580 were compared with CFU in cells without additional treatment. $n = 7$ (Pam3), $p = \text{n.s.}$; $n = 6$ (SB 203580), $* p < 0.05$. All values are displayed as means $\pm$ SEM. **b, c** Immunohistochemistry of phosphorylated p38 staining in ex vivo NTHi-infected HLT without prior treatment (**b**) or with 1 h of pre-incubation with BUD (**c**).

Fig. 5. Effect of BUD and PAF-R antagonist (PAF-A) on activation of p38. Western blot using specific antibodies against phosphorylated p38 MAPK (p-p38) in A549 cells, with β -actin as the loading control. Cells were infected with NTHi (MOI: 200 CFU/cell) either without prior treatment or after 1 h of incubation with BUD ($n = 5$) or a PAF-A ($n = 2$). Two different concentrations of BUD (10^{-8} and 10^{-6} M) and PAF-A (10^{-7} and 10^{-5} M) were used. **a** Western blot of p-p38 and β -actin as loading control in A549 cells. **b** Relative amounts of p-p38 in A549 cells after 24 h of infection with or without pre-treatment with BUD. The control assay was neither infected nor pre-treated. $n = 5$; $* p < 0.05$, infected assays pre-treated with BUD were compared to assays only infected. All values are displayed as means $\pm$ SEM.

are independent mechanisms or whether p38 activation is directly modulated by blocking PAF-R.

Type II pneumocytes are the predominant cell type of the alveolar epithelium and are thought to play an important role in pulmonary host defence [27], as they express recognition receptors of innate immunity such as TLR2 [12]. During NTHi infection, the maximum of epithelial

layer penetration is reached after 24 h, and the pathogens are then shielded from antibodies and antibiotics [28]. The mechanisms of invasion are not fully understood. Some studies demonstrated macropinocytosis as one mechanism [29]; further data suggested receptor-mediated mechanisms of cell invasion [17] or internalization via lipid rafts [18]. Swords et al. [25] showed that binding to

PAF-R initiates the internalization of NTHi. Lipooligosaccharide (LOS), or more precisely its phosphorylcholine (CHoP) portion, plays a major role in cell invasion via PAF-R, as NTHi strains with CHoP-containing LOS have been shown to invade bronchial epithelial cells more efficiently than strains without CHoP. Furthermore, treating the cells with a PAF-R antagonist led to a significant reduction in invasion by NTHi expressing high amounts of CHoP, while only little inhibition could be reached when a strain without CHoP was used [25]. In our study, inhibition of PAF-R expression by BUD was associated with a reduced invasion of HLT by NTHi. Previous data have shown that PAF-R expression on epithelial cells is reduced by fluticasone, leading to impaired NTHi invasion [17].

To further investigate the molecular mechanisms mediating NTHi invasion, we studied the effect of p38 MAPK inhibition on epithelial cell invasion. The p38 MAPK cascade is an essential part of many cellular functions such as the response to infectious agents and the cellular response to heat or osmotic shock. p38 kinases are activated in the respiratory tract in response to inflammatory signals such as cigarette smoke, microbial antigens and reactive oxygen species. They are involved in many pro-inflammatory pathways such as increased expression of cyclooxygenase-2 and prostaglandin E₂, activation of cytokines/chemokines and promotion of inflammatory cell influx. Via binding to their receptor, glucocorticoids are able to inhibit p38 MAPK by inducing MKP-1, which in turn results in de-phosphorylation of p38 [30]. It has been shown that this inhibition of p38 MAPK can lead to reduced expression of inflammatory mediators and human β -defensin 2 (hBD 2) [30], but increased expression of TLR2 [31]. Conversely, increased expression of p38 contributes to corticosteroid insensitivity in COPD [32].

Our results reveal that p38 is involved in the invasion of epithelial cells by NTHi, since blocking of this MAPK results in a strong reduction of intracellular infection. Thus, the inhibitory effect of ICS on NTHi invasiveness may at least partially be due to their inhibition of p38 MAPK.

Why is suppression of p38 MAPK associated with decreased invasiveness of respiratory pathogens? Activation of this enzyme has been shown to promote transepithelial migration of *Streptococcus pneumoniae* and *H. influenzae* in polarized respiratory epithelial cells [33]. This finding could explain the reduced intracellular infection after inhibition of p38 expression. Previous studies have demonstrated that stimulation of PAF-R results in activation of p38 MAPK. Our findings suggest a possible par-

ticipation of the p38 MAPK cascade in PAF-R signalling, since treatment of A549 cells with a PAF-R antagonist prior to infection seems to lead to reduced p38 activation during infection with NTHi.

A further explanation for the relationship between p38 blockade and reduced intracellular infection is a reduced PAF-R transcription due to p38 inactivation, since it is known that the PAF-R promoter gene contains an NF- κ B binding site. However, in our experiments, cells were infected shortly after treatment with glucocorticoids or p38 inhibition, respectively. But an involvement of p38 in the regulation of PAF-R cannot be ruled out. In contrast to our findings of reduced NTHi invasiveness following BUD treatment, it has been shown that BUD is able to enhance intracellular infection with *Pseudomonas aeruginosa* in mice [34]. *P. aeruginosa* enters lung epithelial cells via a lipid raft-dependent pathway [35]. Taken together, these data suggest that the different mechanisms of entry employed by pulmonary pathogens are influenced differently by ICS. Additional studies are needed to further characterize glucocorticoid effects on different mechanisms of epithelial cell invasion by respiratory pathogens.

The previously described stimulatory effect of ICS on TLR2 expression in the presence of NTHi was confirmed in our study. The toll-like receptor family, as known so far, consists of 12 members, of which 11 are present in human cells. As reviewed by Hippenstiel et al. [36], TLR1, 2, 4–6 and 10 are present on the outer cell surface. TLR2, 4 and 5 have been shown to be present in human AEC [36]. TLR2 expression on human type II alveolar cells was described by our group [12]. Treatment with inflammatory cytokines such as IFN- γ and TNF- α is able to enhance the expression of TLR2 and 4 in respiratory cells, whereas dexamethasone treatment alone only enhances the expression of TLR2 [37]. Likewise, infection with NTHi leads to elevated TLR2 expression, but not TLR4 expression, in human epithelial cells [31]. In the mouse lung, TLR2 and 4 contribute to the host defence against NTHi. TLR4 recognizes NTHi LOS and seems to be important for early bacterial clearance [38], whereas the main ligands for TLR2 are bacterial lipoproteins such as the outer membrane proteins OMP 2 and 6. TLR2 knockout mice showed significantly higher bacterial loads than wild-type mice 10 days after infection [38]. During chronic infection with NTHi, TLR2 knockout mice showed an attenuated adaptive immune response [39].

TLR2 mediates host responses to Gram-positive and Gram-negative bacteria [13, 36], including an immune reaction to NTHi [13]. The effects mediated by activation

of TLR2 include an elevated production of pro-inflammatory cytokines such as CXCL-8 and antimicrobial peptides. hBD 2 is produced in epithelial cells of the human lungs upon stimulation of TLR2 and 4 by lipopolysaccharide [40]. hBD 2 is able to reduce the viability of several types of bacteria including NTHi. We sought to determine whether an enhanced presence of TLR2 on the cell surface is also able to influence the invasiveness of NTHi infection, but additional stimulation of TLR2 using the TLR2 ligand Pam3 did not cause any significant changes in the intracellular amount of pathogens. Thus, it seems unlikely that the increased expression of TLR2 caused by pre-treatment with BUD contributes directly to the host defence against intracellular infection with NTHi. Furthermore, treatment with BUD leads to decreased activation of p38, which has been shown to play a crucial role in the mediation of a variety of immune responses against invading pathogens via activation by TLR2 [13]. Interestingly, the augmentation of TLR2 expression following cytokine treatment does not increase NF- κ B or p38 activity, suggesting alternative signalling pathways of TLR2 in this setting [41]. In concert with our findings, TLR2 stimulation leads to induction of the antimicrobial activity of cell culture supernatants, which is greatly enhanced by cytokine pre-treatment, whereas addition of dexamethasone abolishes this effect completely [41].

How can our findings of a reduced invasiveness of NTHi under ICS treatment in the experimental setting be reconciled with the clinical observation of an increased pneumonia risk under ICS? Several explanations are conceivable:

- 1 Pneumonia was not well characterized in randomized clinical trials and most COPD cohorts [5, 6, 9]; thus, it is possible that only a subgroup of bacterial or non-bacterial pathogens is responsible for the increased infection risk under ICS
- 2 We tested only for intracellular NTHi infection; the overall favourable prognosis of pneumonia in ICS-treated patients [8] could suggest that the increased risk may mainly be due to non-invasive infections
- 3 In clinical trials, the incidence of pneumonia under ICS treatment increased with time; the acute infection model used in this study may not capture the risk related to prolonged treatment, but nevertheless, our data indicate that the observed effects are not simply due to the anti-inflammatory properties of ICS which were rapidly seen in our acute infection model
- 4 The underlying condition (COPD, not asthma) may determine the ICS-related pneumonia risk; further studies using samples of patients in different stages of COPD are warranted

In summary, our findings add new, unexpected insights into the effects of glucocorticoids on pulmonary host defence after NTHi infection. ICS seem to exert not only anti-inflammatory effects in this context. We could show that these effects occur at concentrations of ICS which are likely achieved during inhalation therapy. The relevance of the promotional effect of p38 MAPK and of PAF-R on invasive infection should be further explored.

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