Streptococcus pneumoniae induced regulation of cyclooxygenase-2 in human lung tissue

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ABSTRACT

The majority of cases of community acquired pneumonia are caused by *Streptococcus pneumoniae* and most studies on pneumococcal host interaction are based on cell culture or animal experiments. Thus, little is known about infections in human lung tissue. Cyclooxygenase-2 and its metabolites play an important regulatory role in lung inflammation. Therefore, we established a pneumococcal infection model on human lung tissue demonstrating MAP-kinase dependent induction of cyclooxygenase-2 and its related metabolites. In addition to alveolar macrophages and vascular endothelium, cyclooxygenase-2 was up regulated in alveolar type II but not type I epithelial cells which was confirmed in lungs of patients suffering from acute pneumonia. Moreover, we demonstrate the expression profile of all four E prostanoid receptors on the mRNA level and showed functionality of E prostanoid, receptor by cAMP production. Additionally, in comparison to previous studies cyclooxygenase-2/prostaglandin E₂ related pro- and anti-inflammatory mediator regulation was partly confirmed in human lung tissue after pneumococcal infection. Overall, cell-type specific and MAP-kinase dependent cyclooxygenase-2 expression and prostaglandin E₂ formation in human lung tissue may play an important role in the early phase of pneumococcal infections.
INTRODUCTION

Pneumonia belongs to the most common causes of death worldwide [1] and *Streptococcus pneumoniae* (*S. pneumoniae*) is the most frequently isolated pathogen [2]. Vaccines are available but only provide protection against selected serotypes [3]. Moreover, the number of resistant strains has increased [4] and multi drug resistant serotypes have emerged [5]. Thus, it is important to investigate pathogen host interactions as the basis for the design of novel adjunctive treatment approaches.

Most studies on host pathogen-interaction, including pneumococcal infections, were based on cultured and frequently immortalized cell lines or animal experiments [6]. These models represent an integral part of current pneumonia research, but their obvious limitations of the models should be considered [7, 8], and the principal investigation or at least the validation of results in original human material should be performed whenever possible.

Therefore, we established a *S. pneumoniae* ex vivo infection model in human lung tissue, which was obtained from patients undergoing lung resection due to lung cancer.

Cyclooxygenase (COX)-derived generation of prostaglandins (PG) is suspected to play an important regulatory role in the innate immunity of the lung [9]. COX-1 is generally believed to be constitutively expressed, whereas COX-2 is the induced isoform [10]. Induction is caused by a variety of stimuli including bacteria, viruses or cyto-/chemokines [11-14]. They can lead to the activation of mitogen-activated protein kinases (MAPK) which contribute to various important cell functions including the regulation of inflammation. For example, p38 MAPK dependent induction of COX-2 and consecutive PG formation by bacteria has been shown in cultured bronchial epithelium [11-13].

One of the most prominent PGs is PGE2 which has been shown to contribute to the inflammatory response of various cell types [15-18]. PGE2 seems to signal through four distinct G protein-coupled E prostanoid receptors, EP1-EP4 [9].

In this study we used freshly isolated human lung tissue which was infected *ex vivo* with *S. pneumoniae*. We hypothesised that pneumococcal infection may induce COX-2 as well as related formation of arachidonic acid-derived products and investigated the cell-type specific COX-2 expression pattern and the underlying signal transduction pathways. The results clearly establish that in the alveolus, next to alveolar macrophages (AM), COX-2 is predominantly induced in type II, but not type I alveolar epithelial cells (AEC). A similar expression pattern of COX-2 in AEC was also demonstrated in lungs of patients who suffered from acute pneumonia. Inhibition of p38 MAPK or extracellular regulated kinase 1/2 (ERK) abolished the induction of COX-2 and subsequent release of PGE2. In human lung tissue,
activation of EP₄ induced a significant increase of cyclic adenosine monophosphate (cAMP). Overall, this study is to our knowledge the first which establishes the robust effects of pneumococcal infection on prostanoid metabolism in primary human lung tissue.
MATERIALS AND METHODS

For a detailed method description see supplementary material.

Materials
PGE$_2$ and PGE$_1$ alcohol (PGE$_1$-OH) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). 3-isobutyl-1-methylxanthine (IBMX) and forskolin were purchased from Merck (Darmstadt, Germany). Trichloroacetic acid was obtained from Sigma-Aldrich (Munich, Germany). U0126, SB202190 and NS-398 were purchased from Calbiochem (Merck, Bad Soden, Germany). Tumour necrosis factor (TNF)$\alpha$ and interleukin (IL)-1$\beta$ were obtained from R&D Systems (Wiesbaden, Germany). All other chemicals used were of analytical grade and obtained from commercial sources.

Bacterial strains
Encapsulated $S$. pneumonieae D39 serotype 2 (NCTC7466) (friendly gift from S. Hammerschmidt, University of Greifswald, Germany) was grown as described previously [19].

Human lung tissue
Fresh lung explants were obtained from patients undergoing lung resection at local thoracic surgeries. Additionally, lung tissue samples from 3 patients with acute pneumonia and 1 control patient were randomly selected from routine cases. Written informed consent was obtained from all patients and the study was approved by the ethic committee at the Charité clinic (protocol number EA2/050/08 and EA2/023/07). For infection with $S$. pneumonieae tumour-free normal lung tissue was stamped into small cylinders (~ 8x8x8 mm) and weighed. Specimens were incubated for 24 h in RPMI 1640 with 10% (vol·vol$^{-1}$) heat-inactivated FCS (except for bacterial growth) prior to experimental infection. 200 µL of prepared control or infection medium per 100 mg tissue was injected, thereby assuring thorough stimulation of the tissue. After respective time points lungs were processed for further analysis.

Cfu assay
Human lung tissue was infected with $S$. pneumonieae (10$^2$ cfu·mL$^{-1}$) for 0 h (controlled load directly after infection) and 8 h. Afterwards, specimens were disrupted using the FastPrep-24 homogeniser and the supernatants were plated on Columbia agar. Bacterial colonies were counted and cfu per gram lung tissue were calculated.
**Western blot**

Western blot was performed as described previously [20]. Antibodies used were COX-1, COX-2, ERK2, actin (Santa Cruz Biotechnology, CA, USA), EP4 receptor (Cayman Chemical Co.), phosphorylated ERK or p38 (Cell Signalling, Danvers, USA). ERK2 or actin were used as loading controls. Proteins were visualised by secondary IRDye 800- or Cy5.5-labelled antibodies with an Odyssey infrared scanner (LI-COR Inc., Bad Homburg, Germany).

**Immunohistochemistry and confocal immunofluorescence**

After infection of human lung tissue, specimens were fixed in formalin, embedded in paraffin and processed for histology. Primary COX-2 antibody (Cayman Chemical Co.) was incubated overnight and detected with the Super Sensitive Link Label Detection System (Biogenex, Fremont, CA, USA). Mayer’s hematoxylin (Carl Roth, Karlsruhe, Germany) was used to counterstain nuclei and slides were mounted with Aquatex (Merck). Antibody specificity was verified using blocking peptide. For confocal imaging COX-2 was labelled with Alexa Fluor 488 or 594 (Invitrogen, Darmstadt, Germany; all diluted 1:2000). Type I epithelial cells were detected with anti-caveolin 1 antibody (Santa Cruz Biotechnology), whereas type II pneumocytes were labelled with anti-surfactant protein C precursor (pro-SP-C) (Millipore, Billerica, MA, USA). *S. pneumoniae* was stained with anti-*S. pneumoniae* (kind donation by S. Hammerschmidt, University of Greifswald, Germany) and slides were analysed using a Zeiss Axioskop 2 mot [21] or a Zeiss LSM 780 confocal microscope (Zeiss, Jena, Germany).

**PGE₂ ELISA**

Human lung tissue specimens were infected with *S. pneumoniae* as indicated. PGE₂ was measured in the supernatant according to manufacturer’s instructions (R&D Systems, Minneapolis, USA).

**Mass spectrometry**

Human lung tissue specimens were infected with *S. pneumoniae* for 16 h and supernatants were analysed for free and lipid matrix bound eicosanoids as described elsewhere [22].

**Quantitative PCR**

Total RNA extraction was done using TRIZOL (Invitrogen). After tissue disruption quantitative PCR (iCycler, 20 µl reaction volume or CFX96, 10 µl reaction volume, BIO-RAD, Munich, Germany) was carried out. For the calculation of EP receptor copy numbers,
plasmids with cloned cDNAs coding for EP-receptors and GAPDH were used as templates to prepare standard curves with defined copy numbers.

**cAMP ELISA**
For cAMP measurement lung tissue was weighed and processed in trichloroacetic acid. Samples were homogenised and cAMP ELISA was carried out according to manufacturer’s instructions (Cayman Chemical Co.).

**Bioplex protein array system**
For determination of cytokine release, human lung tissue was infected with $10^6$ cfu·mL⁻¹ *S. pneumoniae* for 16 h. Supernatants were collected and cytokine release was analysed using the Bioplex Protein Array System with beads specific for TNFα, IL-1β, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1 receptor antagonist (ra), IL-10, IL-15, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), IL-6, IL-8 and macrophage inflammatory protein 1α (MIP-1α) (BioRad, Hercules, CA, USA).

**Statistical methods**
Data are presented as means ± SEM of at least three separate experiments. The one-tailed Wilcoxon signed rank test was used to test for significant differences between means. A value of $p< 0.05$ was considered significant.
RESULTS

**S. pneumoniae induced expression of COX-2 in human lung tissue**

To assure reproducible pneumococcal infection of human lung tissue bacterial growth was measured in 8 lung tissue samples demonstrating strong and regular bacterial replication within 8 h (fig. 1). No bacterial growth was detected in serum-free culture medium alone (data not shown) confirming that growth of pneumococci is dependent on factors released by the human lung tissue.

After infection we observed a strong and time dependent COX-2 protein induction whereas constitutively expressed COX-1 remained unaffected in all human lung tissue samples investigated (fig. 2a (4 samples), b (3 samples)). In preliminary experiments we found that next to the serotype 2 (D39) strain also invasive and non-invasive clinical pneumococcal isolates induced COX-2 expression in a similar pattern (data not shown). In the next step, immunohistochemistry was performed to study the cell-type specific expression of COX-2 revealing positive induction mainly in AEC, AM, and in vascular endothelial cells after pneumococcal infection (10 samples) as well as after TNFα/IL-1β (5 samples) treatment in human lung tissue (fig. 3a). Interestingly, we detected strong COX-2 expression also in AEC at inflammatory sites in lung tissue of 3 patients suffering from acute pneumonia (fig. 3b).

Specificity of COX-2 staining was confirmed by pre-incubation of the corresponding blocking peptide on infected lung sections (data not shown). Double staining of tissue sections with anti-caveolin 1 (type I cells) [23] or pro-SP-C (type II cells) on *S. pneumoniae* infected and TNFα/IL-1β stimulated tissue was used to confirm the AEC type for COX-2 expression.

Notably, despite of the stimulus used, almost all COX-2-positive AEC were clearly labelled for pro-SP-C and thus identified as type II cells, whereas no COX-2 staining was observed in type I cells (fig. 3c and d). Likewise, these observations were confirmed in lung tissue of 3 pneumonia patients (data not shown). Interestingly, COX-2 expression was not only detected in cells directly faced to pneumococci, but also in cells without attachment of pneumococci. (fig. 3e shown in 5 samples).

**COX-2 induced PGE₂ formation is p38 and ERK dependent**

6 human lung tissue samples were infected with *S. pneumoniae* in presence or absence of the COX-2 inhibitor NS-398 and PGE₂ liberation was analysed. PGE₂ levels significantly increased 16 h to 24 h after infection and were abolished by NS-398 (fig. 4a). Previous *in vitro* studies using cultured lung epithelial cell lines [11-13] indicated that COX-2 expression is dependent on MAPK activity. We observed increased p38 phosphorylation in presence of
pneumococci whereas ERK phosphorylation was already detected in uninfected human lung tissue (fig. 4b, 5 samples). Pre-treatment of 7 lung tissue samples with ERK inhibitor U0126 or p38 inhibitor SB202190 abolished COX-2 protein expression (fig. 4c) as well as PGE2 release in the supernatant (fig. 4d).

**COX-2 expression is not regulated by a positive feedback loop**

In mouse lung fibroblasts using PGE2 stimulation a positive feedback loop was demonstrated by Vichai et al., indicating that COX-2-related PGE2 production further fostered COX-2 expression [24]. Therefore, we measured COX-2 expression after pre-incubation of 5 lung tissue samples with COX-2 inhibitor NS-398 for 1h (fig. 5). However, in human lung tissue *S. pneumoniae* induced COX-2 expression was unaffected by prior inhibition of COX-2 (fig. 5a). Since exposure of 3 lung tissue samples to different doses of PGE2 failed to affect COX-2 protein expression, no positive feedback loop as presented in mouse fibroblasts was found in intact human lung tissue. TNFα/IL-1β stimulation served as positive control (fig. 5b).

**Induction of COX-2 metabolites by *S. pneumoniae***

In principle, a variety of biologically highly active mediators can be produced via COX-2 [25]. Therefore, 5 human lung tissue samples were infected with or without pre-treatment of COX-2 inhibitor NS-398 and mass spectrometry was performed to detect 6-keto PGF1α, thromboxane B2 (TXB2), PGE2, PGH2, PGE2 ethanolamide, PGF2α, and 8-iso-PGE1. An increase after infection and a decrease after NS-398 pre-incubation was measured for PGE2, 6-keto-PGF1α and TXB2 (fig. 6 a-c), whereas other metabolites remained unchanged (data not shown).

**EP receptor expression profile in human lung tissue**

PGE2 signalling is mainly transduced by the prostanoid receptors EP1-4 [9]. Therefore, we investigated their regulation in 3 lung tissue samples after infection with *S. pneumoniae*. Low copy counts of mRNA were detected for EP1-3 whereas higher levels were observed for EP4 (fig. 7a). EP4 receptor expression was also demonstrated on the protein level in 3 lung tissue samples (fig. 7b). However, within the investigated time frame of 18 h – 24 h pneumococcal infection did not change receptor expression levels neither on the mRNA nor on the protein level (fig. 7a, b). Since EP4 activation leads to cAMP induction we analysed receptor functionality by the EP4 agonist PGE1-OH. Selective EP4 activation as well as addition of
PGE\(_2\) or forskolin significantly increased intracellular cAMP levels in 6 human lung tissue samples (fig. 7c).

**COX-2 regulation of S. pneumoniae-induced cytokine release in human lung tissue**

PGE\(_2\) induction has previously been demonstrated to significantly contribute to cyto-/chemokine regulation [15-18]. Therefore, we analysed 5 infected human lung tissue samples for chemo-/cytokine release in presence or absence of COX-2 inhibitor NS-398. Pneumococci significantly induced TNF\(\alpha\), IL-1\(\beta\), PDGF, GM-CSF, IL-17, IL-10, and IL-15 liberation (fig. 8 a-g). Furthermore, IL-1ra was significantly increased, whereas vascular endothelial growth factor, IL-6, IL-8 and macrophage inflammatory protein 1\(\alpha\) levels remained unchanged following pneumococcal infection (data not shown). As expected, COX-2 inhibition reduced PGE\(_2\) in 4 lung tissue samples and resulted in a significant increase of PDGF and slight increases of TNF\(\alpha\) (\(p< 0.09\)) and GM-CSF (\(p< 0.15\)) expression but displayed no effect on other cyto-/chemokines in the same experiments (fig. 8 a-h).
DISCUSSION

COX-2 derived metabolites are important regulators of inflammation [25]. Studies using cultured lung cell lines [11-13] and mice [26, 27] indicated a prominent immunomodulatory role of COX-2 in pneumonia. Since there is virtually no study having systematically analyzed the biology of COX-2 in the inflamed human lung we used an *ex vivo* model of pneumococcal infection [28] of freshly isolated peripheral human lung tissue to explore cell-type specific COX-2 regulation and function. An important advantage of our model system is that the different lung cell types are still organized in the unique lung architecture and cell-specific behavior can therefore be studied. Resident cells including AM are still present in the tissue and are capable of contributing to the observed response as shown previously in a similar model [28]. However, this model does not allow for investigations of aspects of immunity like the recruitment of immune cells from the blood. Moreover, the disconnection of lung tissue from the blood stream, oxygen supply and gas exchange can only partly be compensated by adaption of cell culture conditions. As a consequence, e.g. cell death by apoptosis and necrosis might influence the inflammatory response. Therefore, a timeframe up to 24 h has been used where neither LDH release nor increase of caspase-3 activation was observed (data not shown). Furthermore, the infection route of our infection model varies from the natural one since bacteria are directly injected into the lung tissue, allowing bacteria to bypass the way from trachea to the alveolus. Although this method does not reflect the natural infection route it nevertheless reproducibly allows studying the direct interaction of bacteria with the alveolar compartment revealing the inflammatory response of the resident cell population. However, the validity of the results obtained from that model should be compared with samples from pneumonia patients whenever possible which could also be performed in the present study. Additionally, the availability of such human lung tissue samples is limited leading to small experimental sample sizes. This is why robust biological signals are required for statistically significant results. We found significant and tissue dependent growth of *S. pneumoniae* in human lungs which induces the expression of the COX-2/PGE$_2$/cAMP axis thereby partly regulating bacteria induced cyto-/chemokine expression. The panel of mediators induced is comparable to studies having investigated *in vitro* infected bronchial epithelial cell lines [29].

The results of this study established that pneumococci are capable of inducing strong expression of COX-2 whereas COX-1 was constitutively expressed and remained unaffected in the infected lung. Immunohistochemistry revealed COX-2 expression in AM, AEC and the vascular endothelium. The relevance of these findings was further supported by the positive
staining of AEC in lungs of patients with acute pneumonia. Interestingly, within the alveolar epithelium, induction of COX-2 was almost exclusively seen in type II but not in type I AEC. Since TNFα/IL-1β exposure of lung tissue produced the same pattern, it appears reasonable to assume that type II cells are in general important alveolar epithelial pacemakers of inflammation. The pro-inflammatory environment after pneumococcal infection or cytokine treatment fostered COX-2 expression also in alveolar macrophages and lung endothelium. Apart from these cell types we occasionally observed COX-2 positive stromal cells, which were neither caveolin 1 nor pro-SP-C positive. Notably, a comparable expression pattern of COX-2 was seen in cynomolgus monkeys with acute severe pneumonia in vivo [30]. We detected *S. pneumoniae* in areas where no COX-2 positive cells were found and vice versa. This finding suggests that COX-2 induction is not necessarily related to cellular attachment of *S. pneumoniae*. Factors released in the supernatant either from pneumococci or from the lung tissue might foster COX-2 expression which is supported by our data from TNFα/IL-1β stimulated lungs also showing strong COX-2 expression in the same cell types.

In infected lung tissue the chemical inhibitor NS-398 almost completely blocked the release of PGE₂, a major COX-2 product. In addition, we observed production of 6-keto-PGF₁α and TXB₂. However, no release of other prostanoids was found in our experiments. As evidenced by previous studies, PGE₂ seems to be the major prostanoid produced by human lung cells [31]. Since PGE₂ produced by lung cells down regulates neutrophil responses it may dampen the pro-inflammatory potential of neutrophils thereby preventing epithelial damage [31].

*In vitro* experiments have previously established that induction of COX-2 upon bacterial infection is dependent on MAPK [11-13]. In line with the study of Xu et al. [28] we observed activation of p38 MAPK during pneumococcal infection in human lungs, whereas ERK was already activated in control lungs. Inhibition of both kinases suppressed *S. pneumoniae* induced COX-2 induction and subsequent PGE₂ release in our human lung infection model. In some studies PGE₂ was shown to regulate COX-2 expression in a positive feedback loop, whereas other studies even reported a repressive effect of PGE₂ [24]. In human lungs neither inhibition of COX-2 nor the direct stimulation with different doses of PGE₂ had any effect on COX-2 demonstrating that the feedback loop is most probably not present in human lung tissue.

PGE₂ exerts its effects through four different E prostanoid receptors (EP₁-EP₄) which show a cell type-specific expression [9] and, previously, we have demonstrated that *Moraxella catarrhalis* infection led to an up-regulation of EP₂ and EP₄ protein in bronchial epithelium [13]. Additionally, in *S.pneumoniae* infected mice deficiency of EP₃ was beneficial for
bacterial clearance and survival underlining the role of EPs in bacterial infection [32]. It is well established that activation of EP receptors by PGE₂ subsequently changes the level of intracellular cAMP which finally might contribute to the inflammatory reaction [9]. However, neither EP receptor expression nor cAMP generation has been shown in pneumococcal infected human lung tissue so far. Therefore, we investigated EP mRNA expression demonstrating low EP₁ - EP₃ and high levels for EP₄. Although, pneumococcal infection of peripheral lung tissue did not alter constitutive EP₄ receptor expression neither on mRNA nor on protein level, specific receptor agonists for EP₄ induced a significant increase of cAMP in the lung tissue which might influence the regulation of inflammatory mediators after *S. pneumoniae* infection.

Several studies have demonstrated strong cyto-/chemokine induction after infection with pro-inflammatory stimuli or even with *S. pneumoniae in vitro* as well as *in vivo* [15-18, 29, 33]. Likewise, pneumococcal infection of human lung tissue also revealed significant induction of TNFα, IL-1β, GM-CSF, PDGF, IL-10, IL-1ra, IL-15, and IL-17. However, other expected mediators like IL-6, IL-8, macrophage inflammatory protein 1α, and vascular endothelial growth factor failed to be induced indicating either cell-specific expression in the bronchial compartment which is not present in this model or differences in the mediator regulation in human lung tissue per se. Furthermore, it has been shown that COX-2/PGE₂ may contribute to the inflammatory control by inducing IL-10, or inhibiting GM-CSF, TNFα, IL-12, or PDGF expression *in vitro* [15-18, 34]. In line with these studies COX-2 inhibition led to a significant induction of PDGF [18] but only slight increases for TNFα [16, 34] and GM-CSF [15]. Since the effects of PGE₂ have been shown in isolated cell types of different origin small or counteracting regulation of cytokines in the alveolar compartment might not be measureable in sum signals from whole lung tissue. Moreover, another possibility of the slight effects measured for COX-2 inhibition might be that PGE₂ is primarily directed against invading immune cells which are not represented in this organ model.

In summary, using peripheral human lung tissue infected *ex vivo* with *S. pneumoniae* we demonstrated MAPK-related induction of COX-2 and subsequent PGE₂ release. In the alveolus COX-2 is induced in type II, but not type I alveolar epithelial cells as well as in AM and vascular endothelium. In human lung tissue, activation of EP₄ increased cAMP. PGE₂ production by lung cells may regulate immune cell function and contribute to the control of inflammatory mediator production in pneumococcal pneumonia of the human lung. Further studies using human lung tissue are needed to understand the complexity of lung
inflammation, in particular, the role of type II pneumocytes and to verify results obtained with cell lines and animal models.
SUPPORT STATEMENT

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STATEMENT OF INTEREST

The authors have no financial conflict of interest.

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REFERENCES


Figure 1. Growth of *S. pneumoniae* in human lung tissue. Human lung tissue was infected with *S. pneumoniae* (D39, $10^2$ cfu·mL$^{-1}$) and colony forming units (cfu) were determined at 0 h and 8 h. Data are means ±SEM of 8 different samples. **: $p<0.01$ versus 0 h of growth.
Figure 2. Regulation of cyclooxygenase (COX)-1 and -2 by *S. pneumoniae* in human lung tissue. Human lung tissue was infected for 24 h a) or the indicated time periods b) with $10^6$ cfu·mL$^{-1}$ *S. pneumoniae*. COX-1 and COX-2 protein expression was analysed by Western blot. Representative experiments from 4 a) and 3 b) different samples are shown.
Figure 3  a) Human lung tissue was stimulated with *S. pneumoniae* or TNFα/IL-1β. Immunohistochemical staining revealed COX-2 expression (indicated by red colour) in alveolar epithelial cells (AEC) (open arrowheads), alveolar macrophages (black arrowheads) and endothelial cells (grey arrowheads) in *S. pneumoniae* as well as in TNFα/IL-1β stimulated tissue, whereas no staining was observed in lungs stimulated with medium only (control). b) In tissues of patients with acute pneumonia COX-2 was also found to be expressed in AEC (open arrowheads). Acute inflammation can clearly be seen by the infiltration of neutrophils into the alveolar space (black asterisks). Black arrowheads indicate stained distal bronchial epithelium (in patient 2). Healthy, non-inflamed control tissue was obtained from patients who underwent tumour resection. No COX-2 expression could be found in control tissue. Prior incubation of the primary antibody with its corresponding blocking peptide abolished staining indicating antibody specificity (data not shown). c) Multi-colour immunofluorescent COX-2 (green channel) and caveolin 1 (red channel) double staining of *S. pneumoniae* and TNFα/IL-1β stimulated tissue and subsequent confocal imaging revealed no co-localization of COX-2 and caveolin 1 expression. This implies that COX-2 is not induced in type I AEC. Representative cells are indicated by white arrowheads in each channel. d) In contrast, both *S. pneumoniae* and TNFα/IL-1β stimulated lungs revealed COX-2 expression (green channel) in pro-SP-C positive cells (red channel) indicating COX-2 expression in type II AEC. Representative cells with co-localisation of COX-2 and pro-SP-C expression (yellow in merged channels) are indicated by white arrowheads in each channel. Merged panels demonstrate lung structure by using differential interference contrast. e) Colonies of *S. pneumoniae* (green channel, open arrowheads) were detected in the alveolar space adjacent to COX-2 (red channel, white arrowheads) positive type II AEC as well as attached to alveolar macrophages (white asterisk). Merged panels demonstrate lung structure by using differential interference contrast microscopy. Representative figures of 10 *S. pneumoniae* infected lungs, of 5 TNFα/IL-1β stimulated lungs and of 3 different patients with acute pneumonia are shown in a-d) Representative of 5 separate experiments is shown in e).
Figure 4. Time dependent cyclooxygenase (COX)-2, extracellular-signal regulated kinase 1/2 (ERK) and p38 MAP kinase mediated induction of prostaglandin E₂ (PGE₂) by *S. pneumoniae*. a) and b) Human lung tissue was infected with $10^6$ cfu·mL⁻¹ *S. pneumoniae* or pre-treated with selective COX-2 inhibitor (NS-398, 10 µM, 1 h) and infected for indicated time periods. a) Time dependent PGE₂ release was inhibited by COX-2 inhibition as measured by ELISA. b) Western blots demonstrate activation of p38 MAPK by *S. pneumoniae*, whereas ERK phosphorylation was already visible in uninfected tissue. c) and d) Tissue was pre-treated for 1 h with ERK MAPK inhibitor (U0126 10 µM) or p38 MAPK inhibitor (SB202190 10 µM) and infected with $10^6$ cfu·mL⁻¹ *S. pneumoniae* for 8 h. COX-2 c) and PGE₂ d) induction was suppressed by MAPK inhibitors as shown by Western blot and ELISA, respectively. Data presented are means ± SEM of 6 a) and 7 d) different samples. Representatives of 5 and 7 blots are shown in b) and c), respectively. *: $p<0.05$ versus control; **: $p<0.01$ versus control; #: $p<0.05$ versus infected tissue without pre-incubation with inhibitor; ##: $p<0.01$ versus infected tissue without pre-incubation with inhibitor.
Figure 5. Western blot of *S. pneumoniae* induced cyclooxygenase (COX)-2 shows no feedback regulation a) Human lung tissue was infected with $10^6$ cfu·mL$^{-1}$ *S. pneumoniae* or pre-treated with selective COX-2 inhibitor (NS-398 10 µM, 1 h) prior to infection. COX-2 induction was analysed by Western blot showing no inhibition of COX-2 expression. b) Lung tissue was incubated for 24 h with the indicated concentrations of PGE$_2$ or with tumour necrosis factor (TNF)$\alpha$ and interleukin (IL)-1$\beta$ (100/10 ng·mL$^{-1}$). Representatives of 5 a) and 3 b) blots are shown.

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TNF$\alpha$/IL-1$\beta$ - - - +
Figure 6. Induction of cyclooxygenase (COX)-2 metabolites by *S. pneumoniae*. Human lung tissue was pre-incubated with selective COX-2 inhibitor (NS-398 10 µM) for 1 h before infection with 10^6 cfu·mL⁻¹ *S. pneumoniae* for 16 h. a) Prostaglandin E₂ (PGE₂), b) 6-keto Prostaglandin F₁α (6-keto PGF₁α) and c) thromboxane B₂ (TXB₂) release in the supernatant was measured using mass spectrometry. Data presented are means ± SEM of 5 different samples. *: p<0.05 versus control; #: p<0.05 versus infected tissue without pre-incubation with NS-398. N.d.=not detected.
Figure 7. E prostanoid (EP) receptor expression pattern in human lung tissue. Human lung tissue was infected for 18 h a) or 24 h b) with $10^6$ cfu·mL$^{-1}$ *S. pneumoniae*. a) EP$_{1-4}$ mRNA was analysed using qPCR demonstrating highest expression levels for EP4. b) EP$_4$ protein was detected by Western blot showing no induction after bacterial infection. Antibody specificity was verified using corresponding blocking peptides. c) Human lung tissue was pre-treated with non-specific phosphodiesterase inhibitor IBMX (20 µM) for 2 h and stimulated with specific EP$_4$ receptor agonist (prostaglandin E$_1$ alcohol (PGE$_1$-OH) 10 µM), Prostaglandin E$_2$ (PGE$_2$) (10 µM) or forskolin (20 µM) for 30 min. cAMP formation was determined by ELISA demonstrating clear increase in human lung tissue. Data are means ± SEM of 3 a) and 6 c) different samples. Representative of 3 blots is shown in b). *: $p<0.05$ versus control.
a) Normalized copies EP receptors

\[ S. \ pneumoniae \quad - \quad + \quad - \quad - \quad - \quad + \]


b) EP\(_4\) actin

\[ S. \ pneumoniae \quad - \quad + \quad - \quad + \]

Corr. EP Blocking peptides - - + +

c) cAMP pmc\(\) g lung tissue\(^{-1}\)

\[ \begin{array}{c}
PGE_2-OH \quad - \quad + \quad - \quad - \\
PGE_2 \quad - \quad - \quad + \quad - \\
Forskolin \quad - \quad - \quad - \quad + \\
\hline
\text{IBMX} & \\
\end{array} \]
Figure 8. *S. pneumoniae* induces pro-and anti-inflammatory cytokines in human lung tissue. Human lung tissue was pre-treated with and without selective cyclooxygenase (COX)-2 inhibitor (NS-398 10 µM) for 1 h and then infected with $10^6$ cfu·mL$^{-1}$ *S. pneumoniae* for 16 h. a) Tumour necrosis factor (TNFα), b) interleukin (IL-1β), c) granulocyte macrophage-colony stimulating factor (GM-CSF), d) platelet-derived growth factor e) IL-10, f) IL-15, and g) IL-17 release in the supernatant was measured using Bioplex cytokine assay demonstrating a slight increase for TNFα and a significant increase for platelet-derived growth factor after COX-2 inhibition. h) Prostaglandin E$_2$ (PGE$_2$) release in the same samples was used as positive control. Data presented are means ±SEM of 5 a)-g) and 4 h) different samples.*: $p<0.05$ versus control; #: $p<0.05$ versus infected tissue without pre-incubation with NS-398.