

Alveolar epithelial cells type II are major target cells for *C. pneumoniae* in chronic but not in acute respiratory infection

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Abstract

Pulmonary presence of *Chlamydia pneumoniae* is associated with acute and chronic infections. We show that unapparent chlamydial infection in four out of 31 chronic obstructive pulmonary disease (COPD) patients (12.9%) is characterized by a significant increase in infected alveolar epithelial cells type II ($18.2 \pm 3.5\%$ vs. 2.3 ± 0.9 ; IHC/ISH) compared to a newly established model of acute chlamydial infection (ACIM) in vital lung specimens from pulmonary lobectomy. Expression of cHSP60 demonstrated pathogen viability and virulence in the ACIM. We conclude that target cells differ in acute and chronic chlamydial infection and suggest the ACIM as a novel tool to analyze the host–pathogen–interactions in acute respiratory infections.

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1. Introduction

The obligate intracellular pathogen *Chlamydia (Chlamydophila) pneumoniae* has been established as a common cause of acute and chronic respiratory diseases. Acute *C. pneumoniae* infections of the upper respiratory tract during adolescence remain frequently unapparent but have been suspected to trigger bronchial asthma [1]. However, *C. pneumoniae* is also a leading cause of community-acquired pneumonia (CAP) and of acute exacerbations of chronic obstructive pulmonary disease (COPD) [2,3]. Chronic colonisation of the lungs with *C. pneumoniae* has been linked to a higher rate of infectious exacerbations in COPD patients and may in addition be a focus of systemic dissemination of the pathogen to the vascular wall [4,5]. Seroepidemiological

studies consistently showed an extremely high seropositivity (60–80% in adults) which proves the ubiquitous presence of the pathogen [6]. Increased antichlamydial IgG and IgA indicated acute exacerbations with *C. pneumoniae* in COPD patients [7]. The chlamydial target cells within the lung in acute and chronic infection have not been described though they are crucial to the resulting pathology. Gaydos et al. [8] showed epithelial and endothelial cells, fibroblasts, smooth muscle cells as well as mononuclear cells to serve as host cells for acute infection in vitro. Animal studies proved bronchial epithelial cells (BEC), alveolar epithelial cells (AEC), and endothelial cells as preferred target cells for acute chlamydial infection [9,10], and convincingly demonstrated the systemic spread of *C. pneumoniae* from the respiratory tract to the vasculature [11,12]. However, mice are not natural hosts for *C. pneumoniae*. We were thus interested in the infection pattern of acute versus chronic *C. pneumoniae* infection in human lungs. As pulmonary tissue samples from patients with acute chlamydial

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pneumonia are obviously not available we developed an ex vivo model of acute chlamydial infection (ACIM), using vital lung specimens from patients undergoing lobectomy due to a solitary pulmonary nodule. This novel model was established according to a previously described model of short term stimulation of soft tissues [13]. The vital lung tissue sections served to analyze the chlamydial infection pattern by immunohistochemical staining (IHC) and in situ hybridisation (ISH). To prove chlamydial viability and functionality in host–pathogen interactions in the ex vivo model we analyzed the expression of the proinflammatory chlamydial heat shock protein 60 (cHSP60) and the major outer membrane protein (MOMP) mRNA, as indicators of chlamydial viability and virulence. In addition, the *C. pneumoniae*-induced release of the 92-kD gelatinase (matrix metalloproteinase-9, MMP-9), which has been shown to be involved in matrix degradation and progressive lung destruction, was analyzed [14]. Lung specimens were treated with formalin or the recently developed HOPE-fixative, which was propagated to enhance DNA-protein preservation and detection [15,16]. As the ex vivo infected lung tissues do not remain vital for the prolonged periods needed to establish a chronic chlamydial infection we comparatively analyzed the infection pattern in known *C. pneumoniae* positive lung specimens from COPD patients who had no signs of acute respiratory infection and underwent surgical lobectomy due to a solitary pulmonary nodule.

2. Materials and methods

2.1. Acute chlamydial infection model of human lungs

The respiratory isolate *C. pneumoniae* CWL029 (ATCC VR-1310) was grown on HEp-2 (ATCC CLL 23) monolayers as described previously [17]. Vital lung specimens were obtained from five patients who underwent thoracic surgical intervention due to a suspect pulmonary process which in histopathological examinations proved to be non-malignant. A nested-PCR was performed as described below to prove that specimens were initially *C. pneumoniae*-DNA negative. Lung specimens (1 cm³ size) were cultured in RPMI1640 medium (Sigma, Taufkirchen, Germany) at 37 °C and 5% CO₂ for 48 h. To enhance host cell infection with *C. pneumoniae* a centrifugation step was required and 500 µl chlamydial suspensions (10⁷ IFU/ml) were centrifuged (2000g, 30 min) onto the specimens.

2.2. Immunohistochemical staining

Formalin- and HOPE (Hepes–glutamic acid buffer mediated organic solvent protection effect)-treated lung specimens were prepared for IHC as described previ-

ously [11]. For *C. pneumoniae* detection primary antibodies against chlamydial LPS (CF-2; Washington Research Foundation, WA, USA) and chlamydial heat shock protein-60 (cHSP60; Affinity BioReagents, CO, USA) were applied in a dilution of 1:100. Purified anti-MMP-9 IgG antibodies (MMP-9; Serotec, Duesseldorf, Germany) were used for detection of the matrix metalloproteinase-9. Staining procedure was performed by a DAB substrate kit (VectorLabs, CA). For detection of surfactant protein-A as marker of AEC type II, the lung specimens were labeled with a monoclonal antibody PE-10 (Dako, Denmark) and stained with new-fuchsin substrate (Sigma). Quantification of infected cells (infected cells/non-infected cells × 100%) was performed by analysis of 10 high-power-fields (HPF) by two independent investigators (J.R. and D.D.). Negative controls comprised omission of the antibody.

2.3. In situ hybridisation

Lung specimens were treated with the newly developed HOPE-fixative [13,15]. PCR targeting *C. pneumoniae* was performed for specimens included, to determine the preservation of DNA prior to ISH as described below. Hybridization targeting the *C. pneumoniae* specific *Pst*I fragment was carried out overnight in moist chambers at 46 °C. New-fuchsin substrate (Sigma) was used as a chromogen for the alkaline phosphatase reaction, and counterstain was performed with Mayer's hemalum as previously described [13].

2.4. Nested-PCR for detection of *C. pneumoniae* in lung specimens

Nested-PCR for *C. pneumoniae* detection was performed for vital lung specimens from the ACIM and 31 lung specimens from COPD patients undergoing lobectomy due to a suspect pulmonary process and histopathological findings of chronic bronchitis. DNA was purified from lung specimens by lysis and column extraction (Qiagen, Hilden, Germany) by a standard procedure. *C. pneumoniae*-DNA was detected by using HL-1/HR-1 primers that amplify a genomic 437-bp *C. pneumoniae* target sequence of proven species specificity [18,19]. For enhanced specificity, a nested PCR protocol was used with the nested oligonucleotide primer pair IN-1/IN-2 yielding a 128-bp product. For confirmation, nonradioactive DNA hybridization was performed using 4 pmol/mL oligonucleotide HM-1 3'-tailed with digoxigenin-11-dUTP/dATP (Roche Diagnostics, Mannheim, Germany) as the probe.

2.5. RT-PCR of HSP60 and MOMP mRNA expression

RT-PCR was performed from homogenised lung tissues using the NucleoSpin RNA II kit (Macherey-

Nagel, Düren, Germany). PCR amplification for the chlamydial 16sRNA, HSP60 and MOMP was analyzed with the LightCycler® detection system (Roche Molecular Biochemicals). In a dissociation protocol single peaks were confirmed in each of the amplified sequences to exclude nonspecific amplification. For relative quantification chlamydial target genes were correlated to the chlamydial 16sRNA gene as described previously [20].

2.6. MMP-9 immunoassay

MMP-9 protein in the supernatants of *C. pneumoniae* infected and non-infected lung specimens was quantified by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions.

2.7. Statistical analysis

All data are presented as means \pm SEM. Statistics were performed with non-parametric tests. For independent samples the Student's *t* test was used. *p* values <0.05 were considered statistically significant. Calculations were carried out with Statistica™ for Windows (version 5), 1997.

3. Results

3.1. Detection of *C. pneumoniae* in the acute chlamydial infection model

Vital lung specimens could be infected with *C. pneumoniae* for at least 48 h. After 48 h infection was predominantly detected in alveolar macrophages (AM) by IHC for chlamydial LPS (Fig. 1(a)). Thus, $30.0 \pm 9.6\%$ of AM were infected in the early phase of pulmonary infection in comparison to $4.2 \pm 1.6\%$ of AEC, Figs. 1(b) and (c), with the infection equally distributed between AEC type I and II (Table 1). BEC were sporadically infected ($<1\%$; Fig. 1(d)), and infection of bronchial smooth muscle cells was not detected. Results for IHC staining were confirmed by ISH for *C. pneumoniae* on lung specimens of the ACIM treated with the novel HOPE-fixative. After 48 h *C. pneumoniae* infection was detected in $39.6 \pm 9.8\%$ of AM (Fig. 1(e)), and $3.6 \pm 1.1\%$ of AEC.

3.2. Expression of chlamydial HSP and MOMP in the ACIM

Chlamydia pneumoniae infected lung tissues from the ACIM were analyzed with respect to chlamydial heat shock protein-60 (HSP60) and major outer membrane protein (MOMP) mRNA and protein expression.

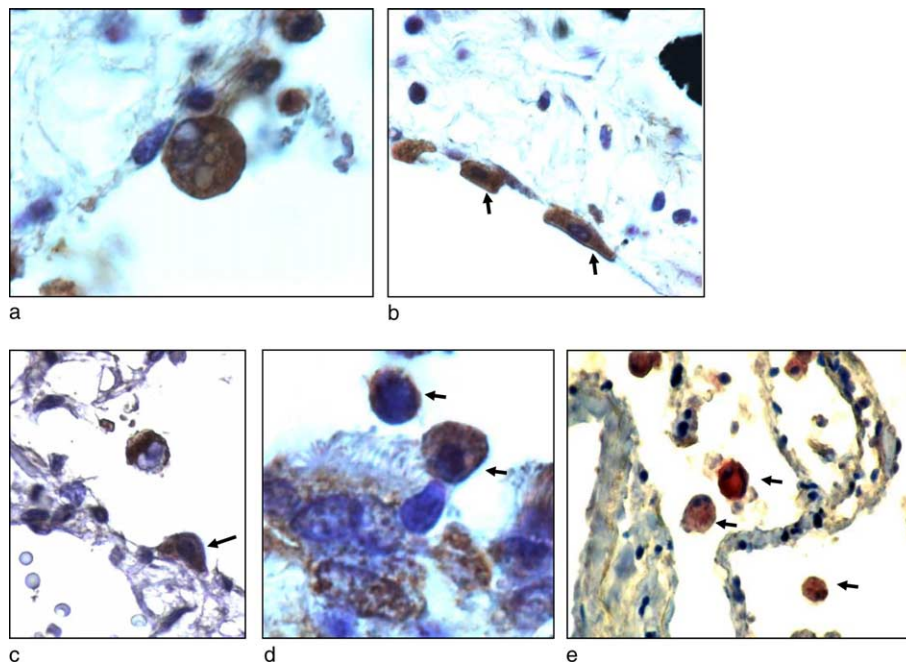


Fig. 1. Detection of *C. pneumoniae* infection in the newly developed acute chlamydial infection model (ACIM). (a)–(d) show immunohistochemical (IHC) staining of chlamydial LPS in vital lung specimens 48 h after infection with *C. pneumoniae*. Chlamydial infection was detected in alveolar macrophages (AM, a), alveolar epithelial cells type I (AEC, b; arrow) and type II (c, arrow), and sporadically in BEC ($<1\%$; (d) also showing infected AM, arrow). In situ hybridisation (ISH) of *C. pneumoniae* infected lung specimens, which were treated with the new HOPE-fixative, confirmed alveolar macrophages as the primary host cells for *C. pneumoniae* infection in the ACIM (e).

Table 1

Comparison of the chlamydial target cells in the acute chlamydial infection model ($n = 5$) and persistent chlamydial infection in COPD patients ($n = 4$) by immunohistochemistry (IHC) of chlamydial LPS and in situ hybridisation (ISH) in HOPE-treated lung specimens (mean \pm SD)

Host cell population	Host cells infected (%)			
	Acute chlamydial infection model (ACIM)		Persistent chlamydial infection in COPD patients	
	IHC	ISH	IHC	ISH
Alveolar macrophages	30.0 \pm 9.6%	39.6 \pm 9.8%	27.5 \pm 6.8%**	29.8 \pm 6.7%**
Bronchial epithelial cells	<1%	<1%	<1%**	<1%**
Alveolar epithelial cells type I	1.9 \pm 0.7%	0.8 \pm 0.4%	4.6 \pm 0.9%**	3.7 \pm 1.5%**
Alveolar epithelial cells type II	2.3 \pm 0.9%	2.8 \pm 0.7%	18.2 \pm 3.5%*	19.3 \pm 5.9%*

Comparison between *C. pneumoniae* detection (IHC and ISH) in COPD patients versus the ACIM correlated to different cell types.

* $p < 0.01$.

** Not significant.

Chlamydial HSP60 mRNA expression was significantly enhanced in acute *C. pneumoniae* infected lung specimens ($p < 0.05$), whereas the expression of MOMP mRNA remained unchanged during the 48 h observation period of the ACIM (Fig. 2(a)). Expression of cHSP60 was detected in parallel by IHC in *C. pneumoniae*-positive AM after 48 h (Fig. 2(b)).

3.3. Expression of MMP-9 in the ACIM

Infection of lung specimens with *C. pneumoniae* enhanced the release of MMP-9 after 24 and 48 h without reaching statistical significance. Compared to non-infected lung specimens chlamydial infection resulted in a 1.3-fold (24 h) and 1.5-fold (48 h) increase of MMP-9 protein levels (Fig. 3(a)). IHC proved alveolar macrophages as the predominant source of MMP-9 expression in non-infected and *C. pneumoniae* infected lung specimens of the ACIM after 48 h (Fig. 3(b)).

3.4. Detection of *C. pneumoniae* in lung specimens of COPD patients

To compare the findings of our newly established ACIM with a more chronic infection we analyzed formalin and HOPE-treated lobectomy lung specimens from COPD patients without clinical signs of acute respiratory infection. Four lung specimens out of 31 (12.9% infection rate) proved to be *C. pneumoniae*-DNA positive as detected with nested-PCR (data not shown).

3.5. Pulmonary chlamydial infection pattern in COPD patients

Lung specimens positive for chlamydial DNA were analyzed by IHC of chlamydial LPS to evaluate the infection pattern of chlamydial infection in COPD patients. As observed in the ACIM, chlamydial infection was primarily detected in AM. 27.5 \pm 6.8% of AM stained positive for *C. pneumoniae* LPS (Fig. 4(a)). In

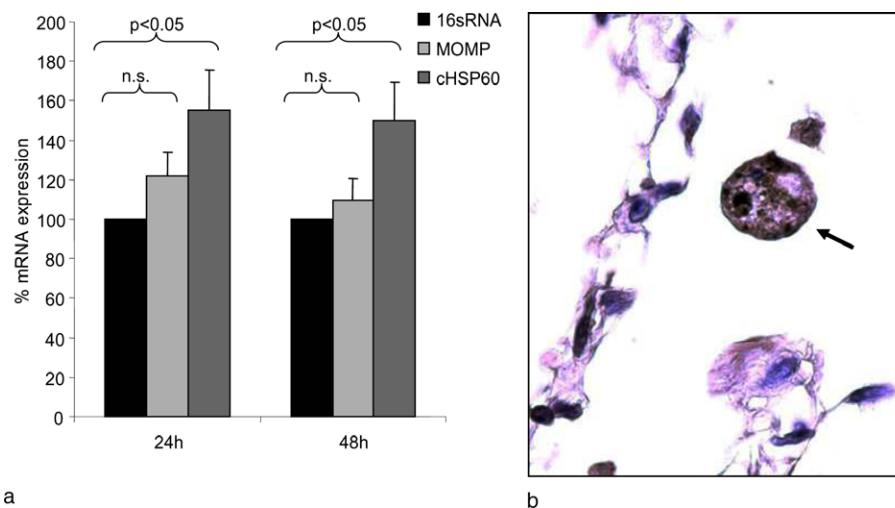


Fig. 2. Homogenised lung tissues from the ACIM showed significantly enhanced cHSP60 mRNA expression 24 and 48 h after *C. pneumoniae* infection (a, RT-PCR; $p < 0.05$, $n = 5$) whereas MOMP mRNA expression remained unchanged. Expression of cHSP60 was predominantly detected in alveolar macrophages (b, arrow) 48 h after chlamydial infection.

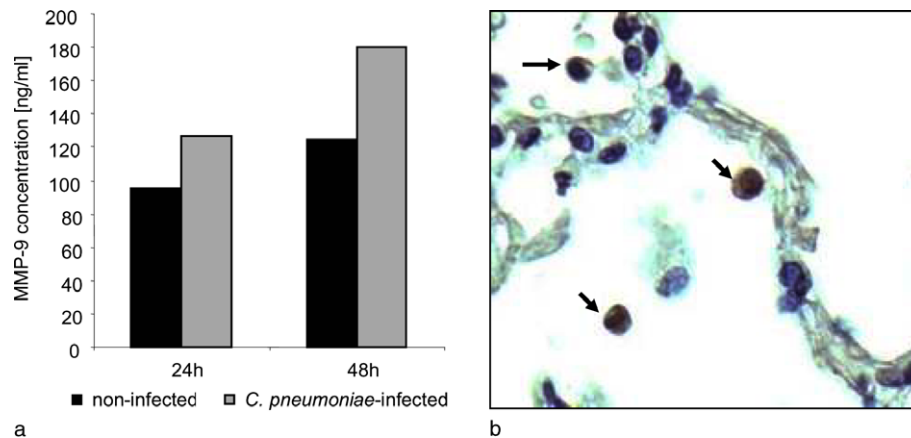


Fig. 3. Protein expression of MMP-9 in *C. pneumoniae* infected and non-infected lung specimens of the ACIM was analyzed by ELISA after 24 h and 48 h (a). MMP-9 expression in chlamydially infected (b) and non-infected specimens was predominantly detected in alveolar macrophages by IHC staining of HOPE-treated lung specimens after 48 h (representative experiment, $n = 3$).

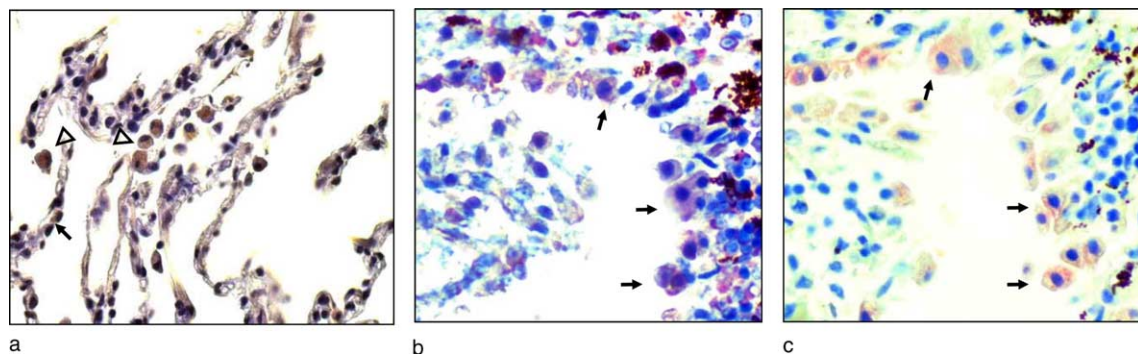


Fig. 4. Lung specimens from COPD patients without clinical signs of respiratory infection undergoing lobectomy were treated with HOPE-fixative. IHC staining of chlamydial LPS consistently showed chlamydial infection of alveolar macrophages (AM; a, triangle) and alveolar epithelial cells (AEC, arrow) only in the *C. pneumoniae*-DNA positive lung specimens. Chlamydial infection of AEC was more frequently detected in unapparent infections of COPD patients than in the ACIM. Adjacent lung sections were analyzed to prove chlamydial infection of AEC type II in COPD patients. In *C. pneumoniae*-DNA positive lung specimens more than 80% of the *C. pneumoniae*-positive AEC (b, arrow; ISH) were identified as the surfactant protein-A positive AEC type II (c, arrow; IHC).

contrast to the findings of the acute pulmonary infection model detection of *C. pneumoniae*-positive AEC was significantly increased to $22.8 \pm 4.4\%$ ($p < 0.05$). Table 1 compares the chlamydial infection from the ACIM to chronic chlamydial infection in COPD patients by IHC and ISH. To discriminate whether chronic chlamydial infection is observed in type I or type II AEC we performed a surfactant protein-A (SP-A, Fig. 4(c)) staining in adjacent lung sections of *C. pneumoniae*-positive (ISH, Fig. 4(b)) lung areals. Chlamydial infection was mainly observed (>80%) in SP-A positive cells (IHC), showing the morphologic characteristics of AEC type II.

4. Discussion

The obligate intracellular pathogen *C. pneumoniae* has been established as a common cause of acute infections of the upper and lower respiratory tract in

adults. Its natural capability to persist within host cells predisposes this pathogen to contribute to chronic inflammatory changes of the lungs. Several studies have shown an increased seroprevalence of *C. pneumoniae* in patients with COPD [21,22], but less is known about the host cells during acute and chronic chlamydial infection within the pulmonary compartment. Therefore, we have developed an ACIM to analyze the infection pattern in acute infection of vital human lung sections. Chlamydial infection was predominantly found in alveolar macrophages (AM), and to a lesser extent in AEC within 48 h. Surprisingly, only few BEC were infected. To exclude the possibility of non-specific staining due to peroxidase activity in respiratory cells we compared the findings of the IHC of chlamydial LPS with ISH of *C. pneumoniae* in lung specimens treated with the recently developed HOPE-fixative [13,15]. This novel fixation method proved to conserve genomic and proteomic structures in soft tissues with high reliability [13,15]. Compared to

IHC, detection of *C. pneumoniae* in AM by ISH was moderately (10%) increased in HOPE-fixed sections. Interestingly, both techniques did not show *C. pneumoniae* positive bronchial smooth muscle cells in our ACIM. Infection of the lung specimens with *C. pneumoniae* altered the transcriptional activity of chlamydial (cHSP60) and host cell (MMP-9) genes in the ACIM. Acute chlamydial infection in the ACIM was accompanied by enhanced expression of chlamydial HSP60, whereas the expression of the chlamydial MOMP remained unchanged. This is in line with findings from Haranaga et al. [23] who found an increase in chlamydial HSP60 mRNA expression 36 h after chlamydial infection of murine alveolar macrophages, and confirms the predominant role of alveolar macrophages in acute respiratory infection with *C. pneumoniae* on a molecular level. The *C. pneumoniae* induced release of MMP-9 indicated that the newly established ACIM also permits to evaluate direct interactions between *C. pneumoniae* and the pulmonary compartment. IHC staining proved alveolar macrophages as the primary source for MMP-9 expression, a process that has been implicated in the pathogenesis of pneumonia [24,25] as well as in the progression of the COPD through aggravated matrix destruction and increased elastolytic activity [26].

COPD patients have been described to be frequently chronically infected with *C. pneumoniae* [4,7]. Thus, we collected lobectomy specimens from patients with morphological signs of chronic bronchitis in order to compare the infection pattern of chronic chlamydial infection with that found in the ACIM. *C. pneumoniae*-DNA was detected by nested-PCR in four out of 31 lung specimens. Detection of *C. pneumoniae* by nested-PCR was previously evaluated in bronchoalveolar lavage fluid in hospitalized patients with CAP on the base of clinical characteristics [27]. Chlamydial infection was predominantly found in AM and AEC type II, as proven by surfactant protein-A (SP-A) staining of adjacent lung sections. In contrast to the ACIM, chlamydial infection of AEC type II was significantly increased from $2.3 \pm 0.9\%$ up to $18.2 \pm 3.5\%$ in persistent *C. pneumoniae* infected human lungs. Sunil and colleagues [28] recently argued that type II AEC may play a central role in the pulmonary inflammatory response, and that the induction of proinflammatory cytokines, reactive oxygen intermediates as well as eicosanoids through respiratory irritants has been underestimated so far. To our knowledge this is the first study that defines AEC type II as host cells of chronic chlamydial infection in COPD patients. Forthcoming studies will show whether persistence of *C. pneumoniae* in human lungs is due to primary infection of AEC type II during acute infection or sequentially transmitted from infected alveolar macrophages. Chronic *C. pneumoniae* infection of AEC type II may contribute to the progress of lung destruction in COPD patients as chlamydial infection of AEC type II

stimulates the release of surfactant protein-A [29], which is known to trigger the secretion of MMP-9 from alveolar macrophages [30].

Previous studies suggested pulmonary *C. pneumoniae* infection to be present in 13% of autopsy lung specimens from patients with unknown clinical history [31], and in 100% of lung tissues from COPD patients and patients who underwent lobectomy for bronchial carcinoma, with infection rates of 29–54% in alveolar macrophages [32]. Whereas the authors of the latter study pointed out that IHC was more sensitive than PCR in the detection of *C. pneumoniae* in formalin-fixed lung-tissues, our data indicated entire correspondence between *C. pneumoniae*-DNA detection in formalin-treated lung specimens and IHC/ISH in HOPE-treated lung sections. Mygind et al. [33] likewise found a good correlation between PCR detection of *C. pneumoniae* and IHC in paraffin-embedded formalin-fixed lung tissues in experimentally infected mice.

Taken together, our ex vivo ACIM opens up new options to investigate interactions between *C. pneumoniae* and pulmonary host cells in the phase of infection, and allows to modify environmental factors within the pulmonary compartment. Based on our findings on the chlamydial infection pattern in the ACIM, further investigations concerning antibiotic treatment and interactions between host (human pulmonary compartment) and pathogen (*C. pneumoniae*, and others) are warranted in the context of the innate immune response. Furthermore, treatment of lung specimens with the recently developed HOPE-fixative facilitates the analysis of genomic and proteomic structures of bacteria and human host cells, and depicts an additional tool in the detection of respiratory pathogens in human lung tissues. The future identification of reliable markers to distinguish acute from persistent chlamydial infection of the lungs seems to be rewarding and requires further investigations.

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