FRONT ROWS OF SCIENCE

Phagocytes transmit *Chlamydia pneumoniae* from the lungs to the vasculature

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ABSTRACT: ***Chlamydia pneumoniae*, a major cause of community-acquired pneumonia, primarily infects the respiratory tract. Chronic infection of nonrespiratory sites, such as the vascular wall, the brain or blood monocytes, requires evasion from the lungs and spreading *via* the bloodstream. The cell types involved in dissemination are insufficiently characterised.**

In this study, New Zealand White rabbits were infected intratracheally with *C. pneumoniae*, and lung manifestation and systemic dissemination were monitored by polymerase chain reaction and immunohistochemistry. Infection of the lungs was characterised by an early phase dominated by granulocytes and a late phase dominated by alveolar macrophages (AM).

Granulocytes, AM and alveolar epithelial cells acted as host cells for chlamydiae, which remained detectable for up to 8 weeks. AM transported the pathogen to the peribronchiolar lymphatic tissue, and subsequently *C. pneumoniae* entered the spleen and the aorta *via* dissemination by peripheral blood monocytes.

In conclusion, *Chlamydia pneumoniae*-infected alveolar macrophages transmigrate through the mucosal barrier, and give the pathogen access to the lymphatic system and the systemic circulation. Infected peripheral blood monocytes are the vector system within the bloodstream and transmit the infection to the vascular wall. This is the first description of granulocytes acting as a reservoir for *Chlamydia pneumoniae* early in infection.
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Chlamydia pneumoniae has been established as the third species of the genus chlamydia and has emerged as the cause of ∼10% of community-acquired pneumonias, and 5% of pharyngitis and sinusitis cases [1]. The seroprevalence in the adult population is as high as 80%, indicating that nearly everybody acquires the infection at least once during their lifetime and that re-infections often occur [2]. *C. pneumoniae* has been associated with several nonrespiratory diseases, such as multiple sclerosis [3], Alzheimer′s disease [4] or coronary heart disease [5]. As no chlamydiae elementary bodies have ever been detected circulating freely throughout the bloodstream, it must be assumed that a cellular vector system transports the obligate intracellular pathogen from the respiratory origin of infection to the vascular wall. Within the bloodstream, monocytes have been shown to harbour viable chlamydiae *in vivo* [6, 7] and to transmit the pathogen to endothelial or smooth muscle cells *in vitro* [8]. In these cells, chlamydiae enter a persistent infection that is refractory to antimicrobial treatment *in vivo* [7]. However, the vector cell, which allows the respiratory pathogen to evade from the lungs, has yet to be identified. Previous animal models have suggested macrophages as possible carrier cells [9]. To further investigate this hypothesis, New Zealand White rabbits were inoculated intratracheally, the cell types infected by *C.*

pneumoniae during the pulmonary manifestation were identified and the dissemination from the lungs to the systemic circulation was followed by immunohistochemistry (IHC) and polymerase chain reaction (PCR).

Materials and methods

Cultivation and purification of Chlamydia pneumoniae

The *C. pneumoniae* strain CV-6, which was used in this study, was previously isolated from a coronary artery plaque and continuously propagated on HEp-2 cells, as described previously [10]. After confluence of the cell sheet, the growth medium, consisting of Eagle′s minimal essential medium (Gibco, Eggenstein, Germany), 10% foetal bovine serum (Biochrom, Berlin, Germany) and nonessential amino acids (Gibco), was replaced by serum-free Eagle′s minimal essential medium supplemented with 1 μg·mL^{-1} cycloheximide (Sigma, Deisenhofen, Germany) and centrifuged at 3,000×g for 45 min. After incubation at 35°C and 5% carbon dioxide for 72 h, the infected monolayers were mechanically disrupted. Host debris was removed by centrifugation at 1,000×g for 10 min and chlamydiae were concentrated by ultracentrifugation of the supernatant at 24,000×g for 1 h. Inclusion

forming units (IFU) were determined by inoculating fresh monolayers and staining with a chlamydia-specific, anti-lipopolysaccharide (LPS) monoclonal fluorescein isothiocyanate (FITC)-coupled antibody (Dako, Ely, England) after 3 days. Mock-infected cells were prepared in the same way and used for inoculation of negative controls.

Experimental animals

A total of 21 female New Zealand White rabbits, including three negative control animals (1,600–2,000 g; Charles River, Kisslegg, Germany), raised and kept under specific pathogen-free conditions, and fed standard rabbit chow, were anaesthetised with a cocktail of atropine (Fresenius, Homburg, Germany), diazepam (Ratiopharm, Ulm/Donautal, Germany), ketamine (Parke-Davis, Berlin, Germany) and rompun (Bayer, Leverkusen, Germany). Animals were inoculated intratracheally in a nontraumatic fashion with 1×10^6–0.5×10^7 IFU *C. pneumoniae* CV-6 in 0.5 mL phosphate-buffered saline through an 8-charière (2.4-mm) catheter, inserted into the trachea, distal to the thyroid cartilage. Twelve animals were inoculated once and sacrificed in batches of two after 12 h, 1 day, 3 days, 7 days, 14 days and 8 weeks. Six animals were inoculated three times at day 0, 3 and 7, and sacrificed in batches of two at 2 weeks, 4 weeks or 8 weeks after the last inoculation. Three control animals were inoculated three times in the same manner with a preparation of mock-infected cells and sacrificed after 3 days, 2 weeks and 4 weeks. Rabbits were sacrificed by intravenous injection of T-61 (Hoechst Roussel Vet, Unterschleissheim, Germany). Lungs, spleen and aorta were removed from the animals. Only single-use instruments were used to prevent cross contamination. One part of the sample was fixed in 10% buffered formalin and embedded in paraffin for histological examination and IHC. The other part was homogenised and deoxyribonucleic acid (DNA) was extracted using Qiagen columns (Qiagen, Hilden, Germany), according to the manufacturer′s instructions. Peripheral blood monocytes (PBMC) were isolated from the blood using Histopaque (Sigma) gradient centrifugation according to standard protocols.

Polymerase chain reaction

The PCR assay used was consistent with Centers for Diseases Control and Prevention (CDC) recommendations for *C. pneumoniae* PCR [11]: 100 µg of extracted rabbit DNA was used for a *C. pneumoniae*-specific nested PCR based on the species-specific HL-l/HR-1 primer pair and on the nested IN-1 (5′ AGTTGAGCATATTCGTGAGG 3′), IN-2 (5′ TTTATTTCCGTGTCGTCCAG 3′) primer pair, which yields a 128-base pair product of the *pst*I fragment, as previously described in detail [12]. Each PCR step contained 32 cycles of 1.5 min at 95°C, 1 min at 55°C and 1.75 min at 72°C. For confirmation and enhancement of sensitivity and specificity, a nonradioactive DNA hybridisation was performed using the digoxigenin-labelled HM-1 oligonucleotide probe [12]. In order to assess the sensitivity of the PCR protocol, ten-fold dilutions of a plasmid containing the target sequence (pGMP, 3475 base-pairs (Promega, Madison, Wisconsin USA)) were used as a template. The sensitivity of nested PCR and hybridisation ranged 1–100 plasmid copies. To disclose the presence of PCR inhibitors in each sample, 1×10^2 copies of the plasmid were added to the genomic DNA and the nested PCR was repeated as described above.

Immunohistochemistry

A total of 100 4-µm thin paraffin sections of each sample were prepared to detect chlamydial antigens immunohistochemically using the avidin-biotin peroxidase-complex method (Vectastain ABC Elite; Vector Laboratories, Burlingame, CA, USA). Forty sections each were stained with the CF-2 genus-specific monoclonal antibody (dilution 1:8,000; Washington Research Foundation, Seattle, WA, USA) and with a monoclonal *C. pneumoniae*-specific anti-lipopolysaccharide antibody (dilution 1:500; kindly provided by H. Brade, Research Center Borstel, Borstel, Germany) as primary antibody. An isotype-identical (immunoglobulin (Ig)G2a) monoclonal antibody (anti-human CK 13, DE-K13; Dako, Hamburg, Germany) and mouse ascitic fluid (Sigma) served as negative controls (10 sections each). Consecutive sections of the lungs were stained with a monoclonal anti-rabbit macrophage antibody, RAM 11 (Dako, Germany). A biotinylated rabbit-anti-mouse-IgG (Dako, Germany) was applied as a second antibody, followed by incubation with horseradish peroxidase-labelled streptavidin (Dako, Germany) and detection with diaminobenzidine-solution (Sigma). Finally, slight counterstaining with haematoxylin was performed. Data were interpreted according to the CDC-recommendations for *C. pneumoniae* tissue diagnostics [11]; a sample was considered positive when specific reactivity was observed with both chlamydial antibodies in the absence of reactivity in the negative controls.

In vitro *infection of granulocytes with* Chlamydia pneumoniae

Whole blood obtained from healthy adult female New Zealand White rabbits was layered on a Histopaque® gradient consisting of two layers, Histopaque 1,071 (top) and Histopaque 1,119 (bottom) (Sigma), and centrifuged for 5 min at $300 \times g$, followed by 15 min at $800 \times g$ (without brake). The granulocyte-rich layer of Histopaque 1,119 was collected and washed twice in RPMI 1640 medium (Seromed-Biochrom, Berlin, Germany), and the cells were further fractionated on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient, consisting of layers with densities of 1,105 $g \cdot mL^{-1}$ (85%), 1,100 $g \cdot mL^{-1}$ (80%), 1,093 $g \cdot mL^{-1}$ (75%), 1,087 $g \cdot mL^{-1}$ (70%) and 1,081 $g \cdot mL^{-1}$ (65%). After centrifugation for 25 min at $800 \times g$, the interface between the 80 and 85% Percoll layers was collected and washed twice in RPMI 1640. All procedures were carried out at room temperature. Granulocytes were infected with *C. pneumoniae* elementary bodies, strain CV-6, at a multiplicity of infection of 1, and cultivated in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine, 10 mM HEPES (all Seromed-Biochrom) and with 10% foetal calf serum (Gibco) for 4 days at 35°C. Cytospins were prepared using standard protocols. Cells were fixed with methanol and stained with FITC-conjugated anti-chlamydia LPS monoclonal antibodies (Dako). A total of 1×10^6 infected granulocytes were disrupted by glass beads and passed on one well of a 24-well cluster plate containing 1×10^6 HEp-2 cells. Chlamydiae were cultivated for two passages as described above. Infected HEp-2 cells were fixed with methanol and stained with a FITC-conjugated anti-chlamydia LPS monoclonal antibody.

Results

Histopathology

The *C. pneumoniae* infection of the lungs resulted in an interstitial and alveolar pneumonia with bronchiolitis

that resolved spontaneously after 2–4 weeks. Repeated inoculations did not result in significantly prolonged disease courses. Histological findings exhibited infiltrates of heterophilic granulocytes and mononuclear cells within the interstitium, alveolar space and bronchiolar lumen. Within 12 h after infection, an intrabronchiolar and alveolar infiltrate of granulocytes developed. Later, an interstitial infiltrate consisting of granulocytes and a few mononuclear cells was observed from 24 h to 3 days (fig. 1a). After 3 days, the granulocytic infiltrates within the interstitium, the bronchiolar and alveolar space were replaced by mononuclear cells (fig. 1b). Some animals demonstrated a mild vasculitis and perivasculitis that reached a maximal extent at 3 days post-infection. Perivascular and peribronchiolar lymphatic hyperplasia was observed in all infected animals beginning at day 3 and lasting for the whole period studied. Histological changes were not observed in the spleen and the aorta until the end of the observation period of 8 weeks.

Immunohistochemistry and polymerase chain reaction

C. pneumoniae was detected by IHC and PCR in the lungs, the spleen, blood monocytes and the aorta, as shown in table 1. *C. pneumoniae* DNA was present continuously in the lungs for 2 weeks in all animals and for 8 weeks in two out of four animals who had acquired asymptomatic chronic respiratory infection. Antigen was detected up to day 7 after single inoculation and for up to 2 weeks after triple inoculation. Four different cell types were identified within the lungs as target cells of a *C. pneumoniae* infection *in vivo*. Until day 3, *C. pneumoniae* could not be detected in granulocytes of the lungs by IHC (fig. 1c). Chlamydial antigen was present in alveolar macrophages (AM), alveolar and bronchiolar epithelium during the entire course of infection (fig. 1d, e and f). Infected macrophages were identified in the lymphatic tissue of the lungs, shown by staining of consecutive sections with a macrophage-specific antibody (fig. 1g and h). When circulating PBMC were tested
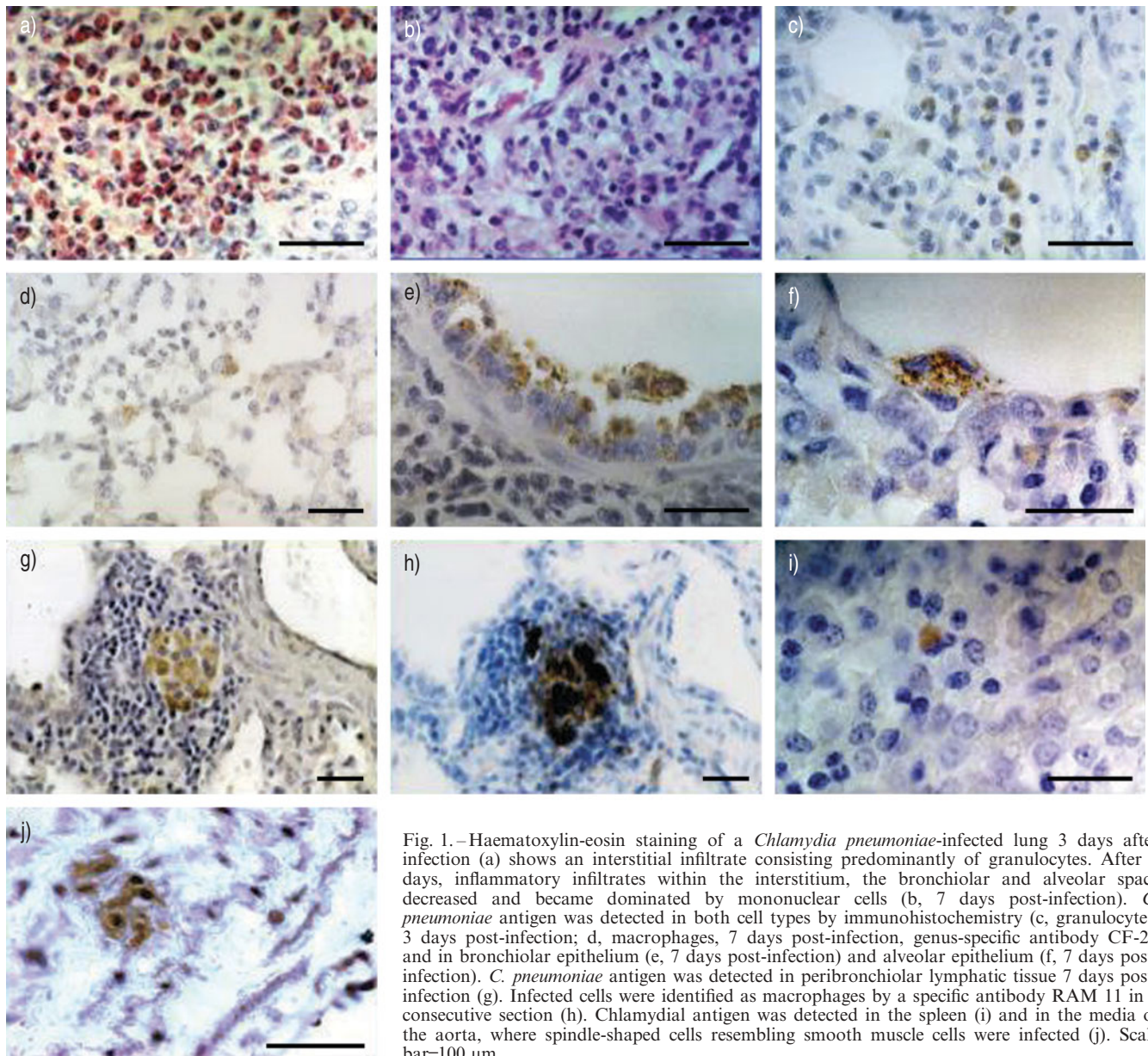

Fig. 1. – Haematoxylin-eosin staining of a *Chlamydia pneumoniae*-infected lung 3 days after infection (a) shows an interstitial infiltrate consisting predominantly of granulocytes. After 3 days, inflammatory infiltrates within the interstitium, the bronchiolar and alveolar space decreased and became dominated by mononuclear cells (b, 7 days post-infection). *C. pneumoniae* antigen was detected in both cell types by immunohistochemistry (c, granulocytes, 3 days post-infection; d, macrophages, 7 days post-infection, genus-specific antibody CF-2), and in bronchiolar epithelium (e, 7 days post-infection) and alveolar epithelium (f, 7 days post-infection). *C. pneumoniae* antigen was detected in peribronchiolar lymphatic tissue 7 days post-infection (g). Infected cells were identified as macrophages by a specific antibody RAM 11 in a consecutive section (h). Chlamydial antigen was detected in the spleen (i) and in the media of the aorta, where spindle-shaped cells resembling smooth muscle cells were infected (j). Scale bar=100 μm.

Table 1. – Detection (+) of *Chlamydia pneumoniae* by immunohistochemistry (IHC) and polymerase chain reaction (PCR) in various organs after single and triple intratracheal inoculation of New Zealand White rabbits

	Lung		PBMC	Spleen		Aorta	
	IHC	PCR	PCR	IHC	PCR	IHC	PCR
Single inoculation							
12 h	+/+[#]	+/+	+/+	-/-	-/-	-/-	+/+
1 day	+/+	+/+	-/+	+/-	+/-	-/-	-/+
3 days	+/+	+/+	-/-	-/-	-/-	-/-	+/+
7 days	+/+	+/+	+/+	-/-	-/-	-/-	+/+
2 weeks	-/-	+/+	+/+	-/+	-/-	+/-	+/+
8 weeks	-/-	+/-	-/-	-/-	-/-	-/-	-/-
Triple inoculations[¶]							
2 weeks	+/+	+/+	+/-	+/-	-/-	-/-	+/-
4 weeks	-/-	-/-	-/-	-/-	-/-	-/-	+/-
8 weeks	-/-	-/+	-/-	-/-	-/-	-/-	-/-

PBMC: peripheral blood monocytes. [#]: two animals at each time point (animal 1/animal 2); [¶]: time after last of three inoculations (days 0, 4, 7).

by PCR, chlamydial DNA was present for up to 2 weeks. Samples of the spleen were occasionally positive for *C. pneumoniae* by PCR or IHC (fig. 1i). There was a poor correlation between the two methods. Chlamydial DNA was detected in the aorta for up to 4 weeks. Chlamydial antigen was demonstrated by IHC in only one sample 2 weeks post-infection (fig. 1j).

In vitro *infection of granulocytes*

Immunofluorescence staining for *C. pneumoniae* of *in-vitro*-infected rabbit granulocytes showed intracellular presence of *C. pneumoniae* LPS 4 days after inoculation (fig. 2a). The number of infected cells increased from ∼20% after 2 days to ∼90% after 4 days, as uninfected cells underwent apoptosis. Infection with *C. pneumoniae* delayed apoptosis of granulocytes (data not shown). Chlamydial viability was proven by subcultivation on HEp-2 cells. Typical inclusions were then detected in ∼5% of the HEp-2 cells in the second passage, proving retained chlamydial viability after a granulocyte passage (fig. 2b).

Discussion

In this study, the pulmonary infection and systemic dissemination of *C. pneumoniae* are described in a rabbit model. Its presence in the lungs, lung-associated lymphatic tissue, the spleen and the vasculature were studied, and the cell types involved in the course of infection were identified.

The pulmonary manifestation was histologically characterised by interstitial, intra-alveolar and intrabronchiolar infiltrates of granulocytes and mononuclear cells. It could be divided into an early and a late phase: during the first 3 days of infection, granulocytes dominated the number of macrophages; the later course was characterised by heavy infiltrates of macrophages, while only a few granulocytes were present. *C. pneumoniae* antigen was present in both cell types. The authors of this study wanted to establish whether the chlamydial antigen in granulocytes represented living organisms and, hence, it was shown for the first time that *C. pneumoniae* can infect and survive within granulocytes, as shown by cultural recovery from infected granulocytes *in vitro*. This suggests that granulocytes are involved in the dissemination of the infection and that the granulocyte-mediated
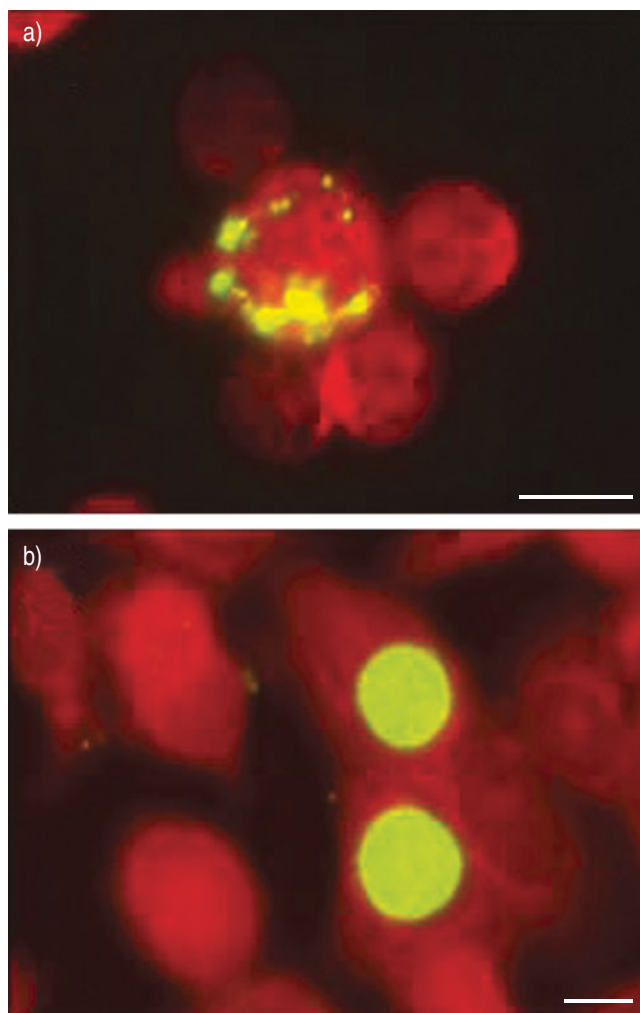

Fig. 2. – Immunofluorescence stain of *Chlamydia pneumoniae*-(anti-lipopolysaccharide monoclonal anitbody) infected rabbit granulocytes 2 days after infection *in vitro* (a). Viability was proven by passing infected granulocytes on HEp-2 cells and immunofluorescence staining of the second passage (b). Scale bar=10 μm.

antimicrobial effector mechanisms are not able to prevent establishment of pulmonary infection but rather promote it. Granulocytes do not re-enter the systemic circulation and have a short life span, however, they can transmit the infection to epithelial cells or AM, which are host cells for *C. pneumoniae* [13] and are able to phagocytose infected granulocytes. Moreover, AM have been shown to migrate through the mucosal barrier [14]. Apparently, granulocytes can act as a kind of Trojan horse for *C. pneumoniae* in the early stage of infection, granting access to the AM, which arrive later and can disseminate the pathogen. Accordingly, *C. pneumoniae*-infected macrophages were detected in the lung-associated lymphatic tissue, which suggests that the infection of macrophages is the mechanism by which *C. pneumoniae* bypass the mucosal barrier and gain access to the systemic circulation. This idea is supported by the detection of *C. pneumoniae* DNA in the majority of the PBMC samples. Experiments by others have confirmed the role of macrophages as vector cell. Macrophages were adoptively transferred by intraperitoneal injection from infected to uninfected mice, and *C. pneumoniae* DNA was later detected in the lungs, thymus, spleen or abdominal lymph nodes [9]. However, primary intraperitoneal infection does not occur naturally.

In this study, the dissemination into the spleen was studied and the pathogen was detected in low numbers and frequency. As the morphology of the infected cells resembles macrophages, the current authors speculate that the macrophages that acquired the infection in the lungs migrated through the lymphatic system into the circulation and finally got sequestered into the spleen. *C. pneumoniae* DNA was further detected in the majority of aortic samples and antigen was also detected by IHC in one sample, where few cells of the media harboured the pathogen. The cell type could not be determined but morphology was consistent with a phagocyte or a smooth muscle cell, cell types that have previously been shown to act as target cells in the vasculature [15]. Here, PCR was clearly more sensitive than IHC. The result again substantiates the idea of monocytes/macrophages acting as a vector system for the systemic spread of *C. pneumoniae*.

C. pneumoniae antigen and genomic DNA were tested for in parallel. PCR was more sensitive in comparison to IHC, as only two spleen samples positive by ICH were negative by PCR. Triple infection did not result in a significantly more severe or longer course of infection, a single intatracheal inoculation was sufficient to establish infection for up to 8 weeks.

It is remarkable that other pathogens like *Bacillus anthracis* use a similar strategy to invade the systemic circulation. The bacteria infect AM, germinate and escape from the phagosome into the cytosol [16]. They are carried through the mucosal barrier by the host cell. Due to a reduced viability of infected macrophages, the bacteria emerge into the bloodstream, multiply and cause the characteristics of an anthrax infection. *C. pneumoniae* elementary bodies have never been detected circulating freely in the bloodstream. Thus, the pathogen depends on a host cell to reach an atherosclerotic plaque and to infect the vascular wall. It is not know whether the same infected macrophage that migrated from the lungs into the blood stream is able to further invade an atherosclerotic plaque. The macrophages might be trapped in the spleen where *C. pneumoniae* can infect other blood cells like monocytes or lymphocytes. These cells have been shown to be infected with *C. pneumoniae in vivo* and are able to invade an atherosclerotic plaque [17–19].

Rabbit models for *Chlamydia pneumoniae* infection have been described previously [20, 21]. The authors of the present study focused on the cell types involved in the infection and dissemination. While granulocytes have been described in cell infiltrates of *Chlamydia pneumoniae*-infected animals before [21], it has been shown here for the first time that they are target cells for *Chlamydia pneumoniae in vivo* and that they can be productively infected *in vitro*. The importance of AMs for the spread of *Chlamydia pneumoniae* into the lymphatic tissue and the systemic circulation has been further demonstrated, showing that a vascular infection can result from a respiratory challenge with the pathogen.

References

1. Grayston JT. Infections caused by *Chlamydia pneumoniae* strain TWAR. *Clin Infect Dis* 1992; 15: 757–761.
2. Saikku P. Epidemiology of *Chlamydia pneumoniae* in atherosclerosis. *Am Heart J* 1999; 138: S500–S503.
3. Gieffers J, Pohl D, Treib J, *et al.* Presence of *Chlamydia pneumoniae* DNA in the cerebral spinal fluid is a common phenomenon in a variety of neurological diseases and not restricted to multiple sclerosis. *Ann Neurol* 2001; 49: 585–589.
4. Balin BJ, Gerard HC, Arking EJ, *et al.* Identification and localization of *Chlamydia pneumoniae* in Alzheimer's brain. *Med Microbiol Immunol* 1998; 187: 23–42.
5. Grayston JT, Campbell LA. The role of *Chlamydia pneumoniae* in atherosclerosis. *Clin Infect Dis* 1999; 28: 993–994.
6. Boman J, Soderberg S, Forsberg J, *et al.* High prevalence of *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells in patients with cardiovascular disease and in middle-aged blood donors. *J Infect Dis* 1998; 178: 274–277.
7. Gieffers J, Fullgraf H, Jahn J, *et al. Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. *Circulation* 2001; 103: 351–356.
8. Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA, Quinn TC. Replication of *Chlamydia pneumoniae in vitro* in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun* 1996; 64: 1614–1620.
9. Moazed TC, Kuo CC, Grayston JT, Campbell LA. Evidence of systemic dissemination of *Chlamydia pneumoniae via* macrophages in the mouse. *J Infect Dis* 1998; 177: 1322–1325.
10. Maass M, Bartels C, Engel PM, Mamat U, Sievers HH. Endovascular presence of viable *Chlamydia pneumoniae* is a common phenomenon in coronary artery disease. *J Am Coll Cardiol* 1998; 31: 827–832.
11. Dowell SF, Peeling RW, Boman J, *et al.* Standardizing *Chlamydia pneumoniae* assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). *Clin Infect Dis* 2001; 33: 492–503.
12. Maass M, Bartels C, Kruger S, Krause E, Engel PM, Dalhoff K. Endovascular presence of *Chlamydia pneumoniae* DNA is a generalized phenomenon in atherosclerotic vascular disease. *Atherosclerosis* 1998; 140: S25–S30.
13. Redecke V, Dalhoff K, Bohnet S, Braun J, Maass M. Interaction of *Chlamydia pneumoniae* and human alveolar macrophages: infection and inflammatory response. *Am J Respir Cell Mol Biol* 1998; 19: 721–727.
14. Harmsen AG, Muggenburg BA, Snipes MB, Bice DE. The role of macrophages in particle translocation from lungs to lymph nodes. *Science* 1985; 230: 1277–1280.
15. Davidson M, Kuo CC, Middaugh JP, *et al.* Confirmed previous infection with *Chlamydia pneumoniae* (TWAR) and its presence in early coronary atherosclerosis. *Circulation* 1998; 98: 628–633.
16. Guidi Rontani C. The alveolar macrophage: the Trojan horse of Bacillus anthracis. *Trends Microbiol* 2002; 10: 405–409.
17. Kaul R, Uphoff J, Wiedeman J, Yadlapalli S, Wenman WM. Detection of *Chlamydia pneumoniae* DNA in CD3+ lymphocytes from healthy blood donors and patients with coronary artery disease. *Circulation* 2000; 102: 2341–2346.
18. Heinemann M, Susa M, Simnacher U, Marre R, Essig A. Growth of *Chlamydia pneumoniae* induces cytokine production and expression of CD14 in a human monocytic cell line. *Infect Immun* 1996; 64: 4872–4875.
19. Kaukoranta-Tolvanen SS, Teppo AM, Laitinen K, Saikku P, Linnavuori K, Leinonen M. Growth of *Chlamydia pneumoniae* in cultured human peripheral blood mononuclear cells and induction of a cytokine response. *Microb Pathog* 1996; 21: 215–221.
20. Fong IW, Chiu B, Viira E, Fong MW, Jang D, Mahony J. Rabbit model for *Chlamydia pneumoniae* infection. *J Clin Microbiol* 1997; 35: 48–52.
21. Moazed TC, Kuo C, Patton DL, Grayston JT, Campbell LA. Experimental rabbit models of *Chlamydia pneumoniae* infection. *Am J Pathol* 1996; 148: 667–676.