

## ***Chlamydia pneumoniae* replicates in Kupffer cells in mouse model of liver infection**

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infection, involving cells of the innate immunity such as Kupffer cells, could also trigger pathological immune reactions involving the liver, as observed in human patients with primary biliary cirrhosis.

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**Key words:** *Chlamydia pneumoniae*; Liver infection; Kupffer cells; Hepatocytes; Culture-isolation; Fluorescence *in situ* hybridization; TNF- $\alpha$ ; Primary biliary cirrhosis

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### **Abstract**

**AIM:** To develop an animal model of liver infection with *Chlamydia pneumoniae* (*C. pneumoniae*) in intraperitoneally infected mice for studying the presence of chlamydiae in Kupffer cells and hepatocytes.

**METHODS:** A total of 80 BALB/c mice were inoculated intraperitoneally with *C. pneumoniae* and sacrificed at various time points after infection. Chlamydiae were looked for in liver homogenates as well as in Kupffer cells and hepatocytes separated by liver perfusion with collagenase. *C. pneumoniae* was detected by both isolation in LLC-MK2 cells and fluorescence *in situ* hybridization (FISH). The releasing of TNF- $\alpha$  by *C. pneumoniae* *in vitro* stimulated Kupffer cells was studied by enzyme-linked immunosorbent assay.

**RESULTS:** *C. pneumoniae* isolation from liver homogenates reached a plateau on d 7 after infection when 6 of 10 animals were positive, then decreased, and became negative by d 20. *C. pneumoniae* isolation from separated Kupffer cells reached a plateau on d 7 when 5 of 10 animals were positive, and became negative by d 20. The detection of *C. pneumoniae* in separated Kupffer cells by FISH, confirmed the results obtained by culture. Isolated hepatocytes were always negative. Stimulation of Kupffer cells by alive *C. pneumoniae* elicited high TNF- $\alpha$  levels.

**CONCLUSION:** A productive infection by *C. pneumoniae* may take place in Kupffer cells and *C. pneumoniae* induces a local pro-inflammatory activity. *C. pneumoniae* is therefore, able to act as antigenic stimulus when localized in the liver. One could speculate that *C. pneumoniae*

### **INTRODUCTION**

*Chlamydia pneumoniae* (*C. pneumoniae*) is a common cause of respiratory infections in humans<sup>[1,2]</sup>, and it is also associated with outcomes other than respiratory disease, including coronary heart disease and myocardial infarction<sup>[3,4]</sup>. Systemic disease has also been reported in which *C. pneumoniae* was detected by polymerase chain reaction in lymph nodes and/or liver and spleen<sup>[5]</sup>. In addition, recent reports suggest a possible association of *C. pneumoniae* infection in patients with primary biliary cirrhosis (PBC)<sup>[6]</sup>. This disease is characterized by the presence of anti-mitochondrial autoantibodies and is considered as an autoimmune disease, although precise etiopathogenetic mechanisms remain unknown<sup>[7]</sup>. Infectious agents have been proposed as triggers in susceptible individuals through a mechanism known as molecular mimicry<sup>[8]</sup>. It seems therefore possible that antigens from dissociated or alive microbes in Kupffer cells, in other macrophages and lysed cells can trigger immunologically-mediated disorders of the liver such as those observed in PBC<sup>[9]</sup>.

In intranasally infected mouse, *C. pneumoniae* infection has been shown to spread systemically *via* infected macrophages from the initial infection site, the lung, to other organs, including the spleen and occasionally the liver<sup>[10]</sup>. Here, we report on *C. pneumoniae* infection of the liver in intra-peritoneally infected mice and the involvement of Kupffer cells.

## MATERIALS AND METHODS

### Animal infection

The animals used in the studies were adult (10-11 wk old) Balb/c mice (Morini, S. Polo D'Enza, Italy). Animals anaesthetized with Ketamine, were inoculated intraperitoneally with purified<sup>[11]</sup> *C. pneumoniae* elementary body (EB) suspension<sup>[11]</sup>. Infected animals received 0.1 mL of organism suspension: the inoculum preparation contained  $2.0 \times 10^7$  inclusion-forming units (IFU) of EBs. At d 2, 7, 10, and 20 after infection, anaesthetized animals were sacrificed. The protocol was approved by the ethical committee of the University of Bologna.

### *C. pneumoniae* isolation from the liver

Ten animals were tested at each time point: i.e. at 2, 7, 10 and 20 d after infection. The liver was removed, weighed and homogenized in a mortar to obtain a 10% (wt/vol) suspension in cold sucrose phosphate-glutamic acid (SPG) buffer. Tissue suspensions were centrifuged at  $300 \times g$  for 10 min at 4°C to remove coarse debris. The clarified homogenates (200  $\mu$ L) were inoculated in duplicate onto LLC-MK2 cells (a continuous cell line derived from Rhesus monkey kidney tissue, used to isolate chlamydiae) seeded into plastic individual wells of a 24-well plate, incubated at 37°C for 72 h in chlamydial growth medium (Eagle's minimal essential medium supplemented with 10% heat inactivated fetal calf serum, containing 2 mmol/L glutamine, 5 mg/L glucose and 1 ng/L cycloheximide) and then fixed in methanol. Chlamydial inclusions were visualized by immunofluorescence.

### Isolation of Kupffer cells and hepatocytes

To isolate Kupffer cells and hepatocytes, animals, infected as above, were anaesthetized with Ketamine and sacrificed 2, 7, 10 and 20 d after infection: 10 animals were tested at each time. Kupffer cells and hepatocytes were harvested and separated following the procedure of Smedsrød and Pertoft<sup>[12]</sup> with minor modifications, as previously described<sup>[13,14]</sup>. Briefly, the liver was perfused with 30 mL of calcium- and magnesium-free Hanks' balanced salt solution (BSS) followed by Hanks' (BSS) containing 0.05% collagenase (type IV; Sigma) for 10 min. The liver was then excised and the cells dispersed in calcium- and magnesium-free Hanks' (BSS). The cells were then centrifuged at  $50 \times g$  at 4°C for 2 min, in a Beckman J6B centrifuge (Beckman Instrument, Palo Alto, Calif.). The non-parenchymal cell-enriched supernatant was centrifuged at  $800 \times g$  for 10 min, the pellet resuspended in 40 mL of PBS, and portions of 10 mL were layered on top of preformed two-step Percoll gradient (the bottom cushion with a density of 1.066 g/mL and an osmolality of 310 mOsm; the overlying cushion with a density of 1037 g/mL and an osmolality of 300 mOsm), and centrifuged at  $400 \times g$  for 15 min at 4°C. Purified non-parenchymal cells enriched in Kupffer cells extended throughout the lower Percoll cushion. The pellets consisted of erythrocytes, non-parenchymal liver cells, and other small white cells. The Kupffer cells-enriched fraction was diluted in PBS and centrifuged at  $800 \times g$  for 10 min. The resulting pellet was resuspended in culture medium (RPMI 1640 with 10% fetal calf serum) at a concentration of  $1.0 \times 10^6$  cells/mL. A 0.5-mL portion of cell suspen-

sion was added to 8-well culture plate (Lab-Tek, Nalge Nunc International, Naperville, IL, USA). Kupffer cells were selected by allowing them to adhere for 2 h at 37°C in an atmosphere with 50 mL/L CO<sub>2</sub>. After nonadherent cells were removed by gentle washing, adherent cells were incubated in RPMI 1640. More than 95% of adherent cells were esterase-positive. The purity of Kupffer cell preparations was also validated using the FITC labelled anti-mouse F4/80 monoclonal antibody (Tebu-bio, Magenta, Italy). Hepatocytes were obtained by differential centrifugation of the initial suspension of mouse liver cells followed by an additional purification step, consisting of sedimentation of the cells through a single cushion of Percoll (density 1.08 g/mL). The hepatocytes were seeded on fibronectin-coated slides and grown in RPMI 1640 with 10% fetal calf serum. Kupffer cells and hepatocytes were also separated from uninfected animals.

### In vitro infection of Kupffer cells and hepatocytes by *C. pneumoniae*

When required, Kupffer cells from uninfected animals were obtained as above described and seeded into 8-well culture plate ( $1.5 \times 10^5$  cells/well) and cultured in RPMI 1640 medium for 24 h at 37°C in an atmosphere with 5% CO<sub>2</sub>. Cultures of Percoll-separated hepatocytes were likewise established from uninfected animals by seeding on fibronectin and grown in RPMI 1640 medium, in 8-well culture plate (Lab-Tek). Kupffer cells and hepatocytes were then infected with *C. pneumoniae* EBs ( $5 \times 10^5$  inclusion forming units/mL) and examined for inclusion formation and infectivity at various times after infection by culturing of infected preparations in LLC-MK2 cells.

### Detection of viable chlamydiae

To detect the presence of viable chlamydiae in separated Kupffer cells and hepatocytes either from infected animals or from *in vitro* infected Kupffer cells and hepatocytes. Both Kupffer cells and hepatocytes were resuspended in chlamydial growth medium, sonicated and clarified by low speed centrifugation. The supernatant was then inoculated onto LLC-MK2 cells. Chlamydial inclusions were detected by immunofluorescence.

### Detection of *C. pneumoniae* by immunofluorescence assay (IFA)

The detection of *C. pneumoniae* by IFA was performed by fixing separated Kupffer cells and hepatocytes or LLC-MK2 cells with methanol for 10 min at room temperature. Inclusions were then visualized using fluorescein-conjugated *C. pneumoniae* species-specific (Chlamydia-cell, Cellabs PTY LTD Brookvale, Australia) monoclonal antibody for 35 min at room temperature. Slides were observed under a Zeiss UV microscope.

### Detection of *C. pneumoniae* 16S RNA by fluorescence in situ hybridization (FISH)

The *C. pneumoniae* 16S rRNA detection by FISH was performed by fixing mouse Kupffer cells and hepatocytes within the Lab-Tek culture plates with 2% paraformaldehyde for 30 min at 4°C and prior to FISH, cells were dehydrated with increasing concentrations of ethanol (50%, 80% and 100%). The *Chlamydia*-specific probes used in this

**Table 1** Isolation of *C. pneumoniae* from liver homogenates of 40 intraperitoneally infected mice at various days after infection

Days after infection	<i>n</i> (pos)/ <i>n</i> (tested)
2	0/10
7	6/10
10	4/10
20	0/10

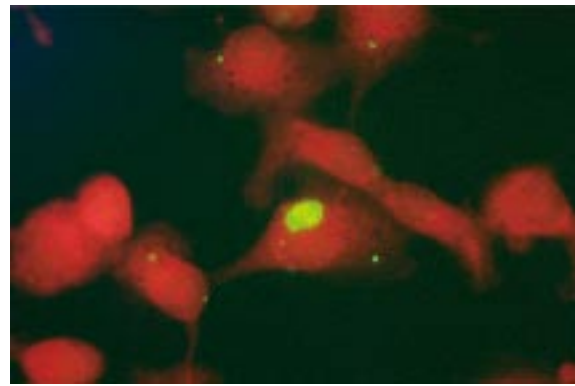
**Table 2** Detection of *C. pneumoniae* in isolated Kupffer cells and hepatocytes by FISH, IFA and culture, performed in 40 intraperitoneally infected mice at various days after infection

Days after infection	<i>C. pneumoniae</i> by FISH and IFA in:		<i>C. pneumoniae</i> isolation from:	
	Kupffer cells <i>n</i> (pos)/ <i>n</i> (tested)	Hepatocytes <i>n</i> (pos)/ <i>n</i> (tested)	Kupffer cells <i>n</i> (pos)/ <i>n</i> (tested)	Hepatocytes <i>n</i> (pos)/ <i>n</i> (tested)
2	0/10	0/10	0/10	0/10
7	6/10	0/10	5/10	0/10
10	4/10	0/10	4/10	0/10
20	0/10	0/10	0/10	0/10

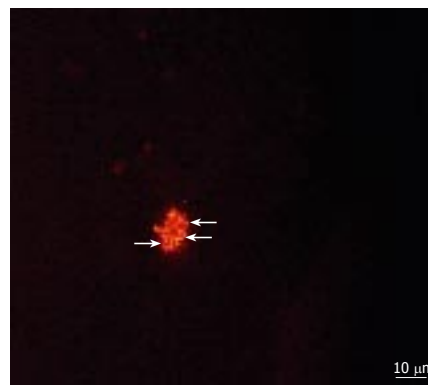
study have been previously described by Poppert *et al.*<sup>15]</sup> and deposited in ProbeBase (<http://www.microbial-ecology.de/probebase/index.html>). Cpn-0974 (5'-AAGTCCAG-GTAAGGTCCT-3') was the species-specific 16S rRNA-targeted oligonucleotide probe that was 5' end labelled with Cy3 fluorochrome (Tib Molbiol, s.r.l., Genova, Italy) giving a red-orange signal. For Kupffer cells and hepatocytes hybridization, a 10 µL aliquot of the hybridization buffer (0.9 mol/L NaCl, 20 mol/L Tris-HCl pH 8, 0.01% SDS and from 0% to 30% formamide) containing 5 pmol of each fluorescent probe was applied to each well of Lab Tek culture plate. After 1 h of incubation in a moist chamber at 46°C in the dark, the slides were washed for 15 min in pre-heated washing buffer containing 20 mmol/L Tris-HCl pH 8, from 0.9 mol/L to 0.15 mol/L NaCl (depending on formamide concentration) and 0.01% SDS. The slides were air dried and mounted with Citifluor AF1 (Citifluor Ltd, London, United Kingdom). The slides were viewed under an epi-fluorescence microscope (Eclipse E600, Nikon) equipped with a super high pressure mercury lamp and Plan Fluor DLL 10 ×, 40 ×, 100 × objectives. Epi-fluorescence filter G-2A was used to analyse Cy3 signal at a magnification of 1000 ×. Photomicrographs were taken using a DXM-1200 digital camera (Nikon, Japan) and image processing was performed with ACT-1 for DXM-1200 software.

### TNF-α production by Kupffer cells

To evaluate the TNF-α induction in Kupffer cells infected by *C. pneumoniae* *in vitro*, Kupffer cells, purified as above reported, were added ( $5 \times 10^5$ ) to each well of an 8-well culture plate (Lab-Tek). After 2 h at 37°C, non-adherent cells were removed by washing three to five times with RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine. Cells were stimulated for 6 h with either LPS (10 mg/L) (Sigma) or viable *C. pneumoniae* at a ratio of 100 EBs/cell. The supernatants were then harvested and tested by enzyme-linked immunosorbent assay (ELISA) kit for TNF-α (Bender MedSystems GmbH, Wien, Austria),



**Figure 1** IFA for the detection of *C. pneumoniae* antigens in separated Kupffer cells. 1000 ×. Kupffer cells separated from intraperitoneally infected mice sacrificed 7 d after infection were tested by immunofluorescence assay (IFA) with specific fluorescein-conjugated monoclonal antibodies. A positive Kupffer cell is clearly evident for the presence of an apple-green intracytoplasmic bacterial inclusion. The inclusion represents an intracytoplasmic bacterial microcolony.



**Figure 2** FISH for *C. pneumoniae* 16S rRNA in separated Kupffer cells. *In situ* hybridization was performed on Kupffer cells separated from intraperitoneally infected mice, sacrificed 7 d after infection. An infected Kupffer cell is evidenced with Cpn-0974 specific probe: the granular inclusion stained red.

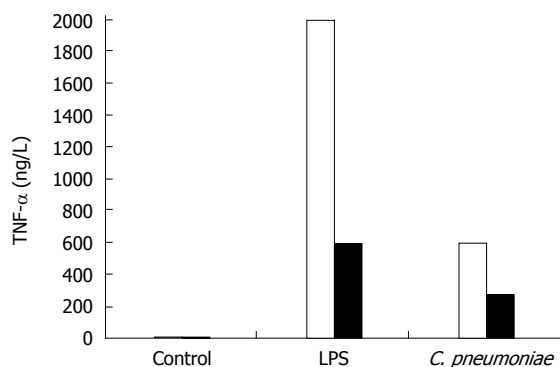
according to the manufacturer's protocol. When required, preincubation of material with polymyxin B (10 mg/L) (Sigma) was used to abrogate the effect of LPS.

## RESULTS

Four groups of infected animals (10 for each group) were sacrificed 2, 7, 10 and 20 d following intraperitoneal infection by *C. pneumoniae* and isolation of viable chlamydiae was performed from liver homogenates. The peak of *C. pneumoniae* isolation from the liver was obtained on d 7 after infection, thereafter the infectivity decreased, all the animals became negative by d 20 of infection (Table 1).

In order to verify whether *C. pneumoniae* was able to infect *in vivo* Kupffer cells and/or hepatocytes, purified liver cells were tested by IFA, to detect chlamydial antigens and by FISH to detect chlamydial rRNA. Four further groups of infected animals (10 for each group) were sacrificed 2, 7, 10, and 20 d after infection, respectively, then isolated Kupffer cells and hepatocytes were analysed. Positive Kupffer cells were observed in preparations obtained on d 7 and 10 (Table 2), both by IFA and FISH, thus confirming the results obtained by culture. Kupffer cells purified 20 d after infections were negative. Positive cells showed





**Figure 3** TNF- $\alpha$  release by Kupffer cells following *in vitro* exposure to alive *C. pneumoniae*. Kupffer cells were stimulated for 6 h with alive *C. pneumoniae*. Control LPS was used at 10 mg/L. Stimulation was performed with (black) or without (white) polymyxin B at concentration of 10 mg/L.

cytoplasmic apple-green inclusions by IFA (Figure 1) and red granular inclusions by FISH (Figure 2). The number of positive cells was 10/100 in preparations obtained 7 d after infection, and 5/100 in preparations obtained 10 d after infection. The preparations from animals sacrificed 20 d after infection were negative. The passage of the content of Kupffer cells obtained from IFA- and FISH-positive animals induced chlamydial inclusion formations in LLC-MK2 cells. Hepatocytes were always negative by IFA and FISH and by culture in LLC-MK2 cells.

The *in vitro* infection of purified mouse Kupffer cells and hepatocytes by *C. pneumoniae* was followed by FISH and by culture: Kupffer cells were positive for the presence of inclusions detectable by 12 h of infection. Sub-culturing Kupffer cells in LLC-MK2 mono-layers three days after infection allowed re-isolation of *C. pneumoniae*. Hepatocytes infected *in vitro* by *C. pneumoniae* and studied by IFA and FISH was negative 12, 24, and 48 h after infection. Sub-culturing hepatocytes in LLC-MK2 cells three days after infection did not allow re-isolation of *C. pneumoniae*, demonstrating that *C. pneumoniae* is unable to infect hepatocytes.

*In vitro* stimulation of Kupffer cells by alive *C. pneumoniae* elicited cellular responses resulting in the production of TNF- $\alpha$  (Figure 3). Pretreatment of chlamydiae with polymyxin B, which binds and inactivates LPS<sup>[16]</sup>, substantially diminished the ability of bacteria to stimulate Kupffer cells (Figure 3).

## DISCUSSION

Chlamydiae are obligate intracellular bacteria parasitizing eukaryotic cells. Chlamydiae replicate in the cytoplasm of infected cells within an inclusion that does not fuse with lysosomes<sup>[17]</sup>. Within the genus *Chlamydia*, various species preferentially infect different target cells and cause a variety of diseases all ultimately due to inflammatory responses. In particular, *C. pneumoniae* has been associated with respiratory human infections and with the development of atherosclerosis and cardiovascular disease<sup>[18-21]</sup>. *C. pneumoniae* has been also associated with sarcoidosis and it was detected in 4 of 38 liver biopsies from autopsy specimens of patients with sarcoidosis<sup>[22]</sup>. In a rabbit model of *C. pneumoniae* in-

fection, three of 10 infected rabbits had evidence of *C. pneumoniae* elementary bodies in their livers by immunocytochemical staining and *C. pneumoniae* was cultured in two of them<sup>[23]</sup>. In a hamster model of *C. pneumoniae* infection, similarly *C. pneumoniae* was isolated from the liver of the infected animals<sup>[24]</sup>. These studies altogether indicate that *C. pneumoniae* can potentially persist in liver tissues.

More recently, Abdulkarim *et al*<sup>[6]</sup> reported the presence of *C. pneumoniae* antigens and rRNA in liver tissue of patients with end-stage primary biliary cirrhosis, suggesting a potential role of *C. pneumoniae* in the etiology and pathogenesis of the disease, in contrast with serological data by Liu *et al*<sup>[25]</sup> that do not support the concept that *Chlamydia pneumoniae* can be a triggering or causative agent in PBC.

To get further insight into a possible role of *C. pneumoniae* in liver related pathologies, evidence was to be produced as to the possible localization and/or replication of *C. pneumoniae* in parenchymal and not parenchymal liver cells, mainly in Kupffer cells, where chlamydiae could act as a trigger for cellular immunologically-mediated alterations. Kupffer cells constitute the largest population of fixed tissue macrophages found in the body, and blood-clearance and elimination of bacteria taken up by the liver are widely attributed to Kupffer cells. Sometimes microorganisms can survive or even multiply in Kupffer cells, as observed in *Leishmania* and *Salmonella typhi* infections in mice and humans<sup>[26,27]</sup>. In this study, we have described a mouse model of *C. pneumoniae* infection where the organism demonstrated the ability to infect and to multiply in Kupffer cells both *in vivo* and *in vitro*, and to trigger the release of the proinflammatory cytokine TNF- $\alpha$ , by these macrophages. In our animal model, mice eliminated *C. pneumoniae* by d 20 of infection and a chronic infection was not established. However, it is well known that persistence, for as yet unpredictable reasons, is a frequent case of human chlamydial infections<sup>[28]</sup>.

PBC is a chronic liver inflammatory disease whose etiology and pathogenesis remain still unknown. A wide range of data suggest an autoimmune pathogenesis for the disease<sup>[29,30]</sup>, mostly based on the presence of anti-mitochondrial autoantibodies and autoreactive T cells directed against autoantigens. Despite the fact that the autoimmune reaction is directed against ubiquitous mitochondrial autoantigens, the ensuing damage involves primarily biliary epithelial cells<sup>[7]</sup>. Infectious agents have been proposed as triggers in susceptible individuals through a mechanism known as molecular mimicry<sup>[8]</sup>. However, over the past years several studies failed to demonstrate a specific chronic microbial<sup>[8,31]</sup>, viral<sup>[32,33]</sup> or bacterial<sup>[34-36]</sup> infection. Recently, it has been proposed that an aberrant innate immune response to infections has the potential to initiate the development of autoimmunity<sup>[37]</sup>. The ability of *C. pneumoniae* to survive and to replicate in Kupffer cells, as documented in this study, demonstrates the ability of this bacterium to escape, under certain conditions, the defence mechanisms of natural immunity. This characteristic can favour, as suggested by Liu *et al*<sup>[25]</sup>, the production of highly immunogenic substrates and/or provide an inflammatory microenvironment to enhance the self-reactivity of pathogenic T lymphocytes, or can activate pre-existing autoreactive cellular repertoire.

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