Journal of Immunotoxicology

ISSN: 1547-691X (Print) 1547-6901 (Online) Journal homepage: https://www.tandfonline.com/loi/iimt20

Exposure to cigarette smoke and *Chlamydia pneumoniae* infection in mice: Effect on infectious burden, systemic dissemination and cytokine responses: A pilot study

Swati Kumar, Tamar A. Smith-Norowitz, Stephan Kohlhoff, Petra Apfalter, Patricia Roblin, Andrei Kutlin, Jack Harkema, Sheung P. Ng, Shannon Doherty-Lyons, Judith T. Zelikoff & Margaret R. Hammerschlag

To cite this article: Swati Kumar, Tamar A. Smith-Norowitz, Stephan Kohlhoff, Petra Apfalter, Patricia Roblin, Andrei Kutlin, Jack Harkema, Sheung P. Ng, Shannon Doherty-Lyons, Judith T. Zelikoff & Margaret R. Hammerschlag (2016) Exposure to cigarette smoke and *Chlamydia pneumoniae* infection in mice: Effect on infectious burden, systemic dissemination and cytokine responses: A pilot study, Journal of Immunotoxicology, 13:1, 77-83, DOI: 10.3109/1547691X.2015.1006346

To link to this article: https://doi.org/10.3109/1547691X.2015.1006346

Journal of Immunotoxicology

http://www.tandfonline.com/iimt
ISSN: 1547-691X (print), 1547-6901 (electronic)
J Immunotoxicol, 2016; 13(1): 77–83

DOI: 10.3109/1547691X.2015.1006346

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Exposure to cigarette smoke and *Chlamydia pneumoniae* infection in mice: Effect on infectious burden, systemic dissemination and cytokine responses: A pilot study

Swati Kumar[1]*, Tamar A. Smith-Norowitz[1]*, Stephan Kohlhoff[1], Petra Apfalter[1], Patricia Roblin[1], Andrei Kutlin[1], Jack Harkema[2], Sheung P. Ng[3], Shannon Doherty-Lyons[4], Judith T. Zelikoff[4]*, and Margaret R. Hammerschlag[1]*

[1]*Department of Pediatrics, State University of New York Downstate Medical Center, Brooklyn, NY, USA,* [2]*Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA,* [3]*DuPont Stine Haskell Research Center, Newark, DE, USA, and* [4]*Department of Environmental Medicine, New York University School of Medicine, Tuxedo, NY, USA*

Abstract

Cigarette smoke exposure has been considered a risk factor for infection with *Chlamydia pneumoniae*. *C. pneumoniae* infection is associated with respiratory tract infection and chronic respiratory disease, which is a serious public health concern. To determine whether prior exposure to cigarette smoke worsens *C. pneumoniae* infection (specifically, increases infectious burden and systemic dissemination) as well as alters cytokine responses in mice, adult female C57BL/6 mice were exposed to either filtered air (FA) or mainstream cigarette smoke (MCS) (15 mg/m^3, total suspended particulates) for 5 days/week for 2 weeks and then infected with *C. pneumoniae* (10^5 IFU) via intratracheal instillation. Mice were euthanized on Days 7, 14 or 26 post-infection (p.i.). Chlamydial burdens in the lungs and spleen were quantified by quantitative PCR (qPCR) and histologic analyses were performed; cytokine levels (TNFα, IL-4, IFNγ) in bronchoalveolar lavage fluid and serum were assayed by enzyme-linked immunosorbent assay (ELISA). The results indicated that: (1) mice exposed to either FA or MCS had similar chlamydial burdens in the lungs and spleen on Days 14 and 26 p.i.; (2) proximal and distal airway inflammation was observed on Day 14 p.i. in both FA and MCS mice, but persisted in MCS mice until Day 26 p.i.; FA exposed mice demonstrated resolution of distal airway inflammation; and (3) MCS mice displayed higher serum levels of IFNγ and IL-4 on Day 26 p.i. These findings indicate that exposure of mice to MCS (at a concentration equivalent to smoking <1 pack cigarettes/day) led to greater *C. pneumoniae*-induced inflammation, as indicated by prolonged inflammatory changes.

Keywords

Chlamydia pneumoniae, cigarette smoke, mice, cytokines

History

Received 28 October 2014
Revised 17 December 2014
Accepted 6 January 2015
Published online 2 February 2015

Introduction

It is well established that cigarette smoke exposure, active smoking and secondhand smoke affects pulmonary function (Mannino et al., 2002; Schwartz et al., 2000), asthma symptoms (Comhair et al., 2011; Mannino et al., 2002) and impairs host immune responses (Ng & Zelikoff, 2008). Even though the general public is aware of the potential risks of smoking, the World Health Organization (WHO) attributes 4.9 million deaths a year to tobacco, a figure expected to rise to more than 10 million by 2030 (Peto & Lopez, 2001).

Prior literature has reported an association between smoking and increased morbidity after both viral and bacterial infections (Arcavi & Benowitz, 2004; Finkela et al., 1969) that might be explained, in part, by either impairment of local/systemic immune mechanisms or enhancement of inflammatory processes involved with production of symptoms. *Chlamydia pneumoniae*, an obligate intracellular pathogen (Hammerschlag, 2004), has been associated with respiratory tract illness, including acute bronchitis, community acquired pneumonia, and chronic obstructive pulmonary disease (Hammerschlag, 2004; Hammerschlag et al., 2009; Kumar & Hammerschlag, 2007); it has also been implicated as a possible trigger for asthma exacerbation (Emre et al., 1994).

There have been few definitive animal studies performed exploring the association between cigarette smoke exposure and subsequent *C. pneumoniae* infection. Although experimental mouse models of cigarette smoke exposure and infectious agents (i.e. *Pseudomonas aeruginosa*, *Mycoplasma pneumoniae, Pneumocystis murina*) have been described (Christensen et al., 2008; Drannick et al., 2004; Martin et al., 2006), a more relevant model of cigarette smoke exposure and increased *C. pneumoniae* burden has yet to be reported. In addition, little is known about regulation of *C. pneumoniae*-associated cytokine responses subsequent to cigarette smoke exposure. Thus, the objective of our study was to investigate the relationship between exposure to mainstream cigarette smoke and subsequent *C. pneumoniae* infection using a mouse model.

Since exposure to cigarette smoke is an environmental factor implicated in the development of atopy, respiratory infections and

*Swati Kumar and Tamara A. Smith-Norowitz contributed equally to the paper. Judith Zelikoff and Margaret Hammerschlag were co-senior authors.

Address for correspondence: Tamar A. Smith-Norowitz, PhD, Associate Professor, Pediatrics, Department of Pediatrics, SUNY Downstate Medical Center, Box 49, 450 Clarkson Ave., Brooklyn, New York 11203, USA. Tel: 718-270-1295. Fax: 718-270-3289. E-mail: tamar.smith-norowitz@downstate.edu

Abbreviations

CVD, cardiovascular disease;
COPD, chronic obstructive pulmonary disease;
FA, filtered air;
MCS, mainstream cigarette smoke;
p.i., post-infection;
TSP, total suspended particulates;
IFU, inclusion forming units;
qPCR, quantitative polymerase chain reaction;
ELISA, enzyme-linked immunosorbent assay;
WHO, World Health Organization;
CO, carbon monoxide;
PBS, phosphate buffered saline;
RB, reticulate bodies;
EB, elementary bodies;
Ct, cycle thresholds;
TNFα, tumor necrosis factor-α;
IFNγ, interferon-γ;
IL-4, interleukin-4;
TGFβ, transforming growth factor-β;
TLR, Toll-like receptor;
ETS, environmental tobacco smoke;
MIF, microimmunofluorescence;
EIA, enzyme immunoassay;

asthma (Comhair et al., 2011; Mannino et al., 2002; Schwartz et al., 2000), the data here raise the possibility that (1) *C. pneumoniae* infection may also be a risk factor for chronic respiratory illness and (2) there may be some evidence of synergy between cigarette smoke and infection.

Materials and methods

Preparation and maintenance of chlamydial stocks

Mycoplasma-free *C. pneumoniae* isolate CWL-029 [ATCC VR-1310]) (ATCC; Manassas, VA) was grown in HEp-2 cells (ATCC, CCL 23) to a titer of 1.4×10^9 inclusion forming units (IFU)/ml, as previously described by Roblin et al. (1992). Chlamydial stocks were stored at −80 °C until used for infection of mice.

Animals

C57BL/6 mice (female, 4–6-week-old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in polycarbonate cages (with corncob bedding) in a temperature (20–23 °C) and humidity (~55% RH) controlled room with a 12-h light/dark cycle. Mice were provided food (Purina 5001 lab chow) and tap water *ad libitum*. Mice were acclimated for at least 1week prior to use. Weight, clinical observations and semi-quantitative feeding activity were recorded for these mice over the course of the study to ensure there was no overt toxicity. Mice weighed 15–20 g at the start of the study. All animal procedures were conducted under an animal protocol approved by the New York University Institutional Animal Care and Use Committee (IACUC).

Smoke generation and exposure

Mainstream cigarette smoke was generated, as described by Ng et al. (2006) from the burning of filtered 1R3F cigarettes (Kentucky Tobacco Research & Development Center, Lexington, KY) using an automated cigarette smoke generation system (Baumgartner-Jaeger CSM 2070, CH Technologies Inc., Westwood, NJ). Reference cigarettes were stored long-term at 4–7 °C (55% RH); 24 h prior to use, cigarettes were relocated to a humidor and stored at 20–23 °C (55% RH). The continuous smoking machine was adjusted to load and light 4–5 cigarettes simultaneously, each of which produced 2-s puffs of 35 ml volume/puff under the control of an automatically regulated piston pump that cycled once per minute. Smoke was diluted 90% prior to introduction into the exposure chamber. Filtered dilution air entered the bottom of the generation chamber and the output was introduced into the top of the chamber. Mice ($n = 5$/group) were exposed (whole-body) to MCS or FA in polycarbonate cages with wire mesh tops. Cages were rotated among three racks in the chamber to assure even smoke distribution to all animals. Chamber levels of carbon monoxide (CO) and TSP were monitored throughout the exposure. Particle samples were collected from exposure chambers every hour (for the entire duration of exposure) on Pallflex Emfab filters (Pall Corporation, East Hills, NY); mean TSP levels were determined gravimetrically from filters weighed before and after sampling. Chamber CO levels were measured continually over the entire 4-h exposure period, as described by Ng and Zelikoff (2008) using a 48C CO Analyzer (Thermo Environmental Instruments Inc, Franklin, MA).

Animal exposure conditions

After acclimatization, mice were exposed to filtered room air or to 15 mg/m^3 (TSP) mainstream cigarette smoke for a 4-h exposure period, 5 days/week for 2 weeks. Five mice were used for each exposure group (i.e. FA or MCS) for culture and PCR analysis for each timepoint. An additional three mice per exposure group were used for histology and another three were exposed to cigarette smoke, but not infected to determine any effect of cigarette smoke (alone) on lung pathology. After the 2 week FA- or MCS-exposure, mice were then infected with *C. pneumoniae*.

Infection with C. pneumoniae

Immediately prior to infection, the concentrated chlamydial stock was thawed and diluted in phosphate-buffered saline (PBS, pH 7.5) to achieve a final concentration of 10^5 IFU in a final delivery volume of 50 µl. Mice were sedated with isofluorane and then infected by intratracheal instillation with the 50 µl aliquot of organism-bearing solution.

Organ recovery and lung lavage

FA- or MCS-exposed *C. pneumonia*-infected mice (and their uninfected counterparts) were euthanized using overdose with SleepAway (0.3 ml diluted 1:10 in PBS) on Days 7, 14 or 26 post-infection. At necropsy, the spleens from each mouse were removed, weighed and placed in MagNA Lyser Green Beads (Roche Applied Science, Penzberg, Germany) containing 1 ml of chlamydia transport medium. Spleens were removed before the lungs to prevent any potential carryover of chlamydial DNA from highly-positive infected lungs to the spleen. The lungs were then removed *en bloc* and bronchoalveolar lavage fluid (BALF) collected and processed to yield an acellular fluid, as previously described (Blum et al. 2014). The BALF was then stored at −80°C for later use. Both lungs from each mouse were placed into a single tube; the spleen from the same mouse was put into a second tube. Lungs and spleens were then transported on dry ice to SUNY Downstate Medical Center (Brooklyn, NY) within 4 h of harvest and partitioned. Some portions were to be used for analysis of chlamydial burdens and other portions for PCR; the latter were frozen at −80 °C until needed.

Histopathology

An additional set of mice was euthanized on Days 14 and 26 post-infection for histologic analyses (a day before or after sacrifice timepoints for culture/PCR). The tissues were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin wax. Sections (5-μm thick) were prepared and stained with hematoxylin and eosin (H&E; Richard-Allan Scientific, Kalamazoo, MI) for blind analyses by a trained pathologist at SUNY Downstate Medical Center.

Isolation of C. pneumoniae

With the samples of spleen or lung to be analyzed for chlamydial levels, the tissues were homogenized in D-PBS (Dulbecco's phosphate-buffered saline) (GIBCO, Grand Island, NY). For the lung samples, the remaining BALF fluid/cells (after recording volumes removed for analyses of cytokines [see below]) were added back to their corresponding parent homogenate. Detection of *C. pneumoniae* present was then carried out by centrifuging each homogenate suspension at 500 x g for 10 min, recovering the supernatants (and recording the volumes) and then inoculating the *C. pneumonia*-bearing fluid onto HEp-2 monolayers in 96-well microtiter plates as described by Roblin et al. (1992). Confirmation of bacteria was performed using genus-specific fluorescein-conjugated *Chlamydia* monoclonal antibody (Pathfinder *Chlamydia* Confirmation System; Kallestad Diagnostics, Chaska, MN).

Real-time PCR (qPCR)

Frozen lung and spleen containing MagNA Lyser Green Beads (Roche Diagnostics Corp., Indianapolis, IN) were thawed, loaded onto a pre-cooled rotor while placed on the cooling block and homogenized in the MagNA Lyser Instrument (Roche Diagnostics) at 6800 rpm (for 60 s) as per manufacturer instructions. Volumes of homogenates corresponding to 25 mg of lung and 10 mg of spleen were transferred into 1.5 ml Eppendorf tubes in duplicate for tissue culture and PCR studies. DNA extraction was carried out using the QIAmp DNA Mini Kit (Qiagen Inc., Valencia, CA) and nucleic acid eluted in 50 μl volumes. PCR reactions were performed using a LightCycler real-time instrument (Roche Molecular Diagnostics, Indianapolis, IN). Each run consisted of study specimens, one extraction control, two positive controls (one low and one medium) and a 'no template' control. Amplification was performed in 25 μl reaction volumes consisting of 5 μl nucleic acid template, $MgCl_2$ at 4 mM, each of the primers (at 250 nM) and 200 nM TaqMan probe. Primer and probe sequences targeted a highly conserved region in the *ompA* gene amplifying an 85 bp region (Apfalter et al., 2003). The analytical performance characteristics of the assay have been previously published (Apfalter et al., 2003). Background inhibition experiments were performed by spiking tissue homogenates with serial dilutions of quantified chlamydial elementary bodies (EB) to determine any inhibition by mouse tissues prior to analyzing the study specimens. The cycling protocol used was 50 °C × 2 min followed by 95 °C × 10 min and then 45 cycles of 95 °C for 5 s, 65 °C for 10 s and 72 °C for 10 s. Quantitation of chlamydial DNA in each sample was achieved from standard curves using purified, previously quantified chlamydial nucleic acid. Cycle thresholds (Ct) of ≤ 40 were considered positive.

Detection of IL-4, IFNγ and TNFα in BALF and serum

BALF and serum were collected from mice on Days 7, 14 and 26 p.i. and stored at −80 °C until assayed. IL-4, IFNγ and TNFα were assayed in BALF samples (Days 14 and 26 p.i.) and IL-4 and IFNγ were assayed in serum samples (Days 14 and 26 p.i.). Commercial cytokine ELISA kits (BioLegend, San Diego, CA) for mouse TNFα IFNγ and IL-4 were used according to manufacturer protocols. Briefly, wells of plates pre-treated with coating antibody to each of the three cytokines were loaded with appropriate standards or biological samples (in duplicate) and then incubated at room temperature for 2 h, then washed 3-times with kit-provided wash buffer, treated with detection antibodies and then incubated at room temperature for 1 h, washed 4-times with wash buffer and then provided detection substrate reagent and incubated at room temperature for 30 min. The reaction in each well was stopped by the addition of 2 N H_2SO_4 and the absorbance then read within 15 min using a Model Elx800 automated plate reader (BioTek Instruments, Wisnooski, VT); optical density (OD) measurements were taken at 450 nm. The sensitivity of the assay was determined by calculating 2 SD above the mean of 10 replicate readings of negative control wells incubated with buffers only. The minimum detection limit for each cytokine was: TNFα < 3 pg/ml; IFNγ < 1 pg/ml; and IL-4 < 5 pg/ml.

Results

C. pneumoniae dissemination in organs in the absence of MCS exposure

To establish the *C. pneumonia* infection model, pilot experiments ($n = 3$ mice/group) were performed using a range of *C. pneumoniae* inoculation doses (10^4–10^6). For subsequent experiments, the 10^5 dose was used because reliably positive lung infections could be achieved without overt toxicity as compared with using the lower and higher concentrations, respectively. On Day 10 post-infection, all three *C. pneumoniae* doses produced a dose-dependent response/infection in the lungs and titers were detected by PCR and cell culture; titers exhibited a 10-fold increase with increasing inoculation doses (Table 1). On Day 10 p.i., spleens tested positive for *C. pneumoniae*, but infection

Table 1. *C. pneumoniae* dissemination in mice prior to cigarette smoke exposure.

	Inoculation dose					
	PCR (particles/5 μl)*			Culture (IFU/ml)		
Organ/mouse number	10^4	10^5	10^6	10^4	10^5	10^6
Lung 1	2.3×10^2	6.8×10^3	3.2×10^4	5.0×10^2	2.5×10^3	8.0×10^5
Lung 2	2.8×10^2	7.0×10^2	1.7×10^4	5.0×10^2	1.0×10^3	1.0×10^5
Lung 3	3.3×10^2	9.9×10^3	6.4×10^4	5.0×10^2	1.0×10^3	5.0×10^5
Spleen 1	negative	negative	2.1×10^1	negative	negative	2.5×10^3
Spleen 2	negative	negative	1.9×10^1	negative	negative	negative

Detection (PCR or cell culture) of *C. pneumoniae* (10^4–10^6 inoculation dose) in lungs and spleen of C57BL/6 mice sacrificed Day 10 p.i.
*Particles refer to chlamydial DNA (both EB and reticulate bodies (RB), detected by PCR).

Table 2. *C. pneumoniae* dissemination in lungs/spleens of FA- or MCS-exposed mice that were sacrificed 7, 14 or 26 days p.i.

FA			MCS		
Day	Culture	PCR	Day	Culture	PCR
Lungs					
7	+ (5/5)	7.1×10^3	7	+ (4/5)	2.9×10^3
14	+ (4/5)	2.8×10^3	14	+ (4/5)	1.4×10^3
26	+ (4/5)	1.4×10^3	26	+ (5/5)	5.9×10^2
Spleens					
7	− (5/5)	$< 1.1 \times 10^1$	7	− (5/5)	$< 1.1 \times 10^1$
14	− (5/5)	0	14	− (5/5)	1.5×10^{-1}
26	− (5/5)	1.0×10^{-1}	26	− (5/5)	1.0×10^{-1}

Detection (cell culture/PCR) of *C. pneumoniae* in lungs or spleens of C57BL/6 mice ($n = 5$/group) infected (10^5 organisms/dose) and then exposed daily to fresh air (FA) or mainstream cigarette smoke (MCS) for 2 weeks and then euthanized at 7, 14 or 26 days p.i. Values shown are mean *C. pneumoniae* particles in five mice (lung or spleen)/group.

was observed only in those mice intratracheally instilled with 10^6 *C. pneumonia*. Bacterial titers were detected by PCR (3/3 mice) and tissue culture (1/3 mice), thus confirming dissemination of *C. pneumoniae* from the lungs to the spleens of mice infected with 10^6 infectious organisms.

C. pneumoniae dissemination in organs after MCS/FA exposure

Day 7 p.i.

The mean number of *C. pneumoniae* particles/5 µl template in the lungs of FA- and MCS-exposed mice were 7.1×10^3 and 2.9×10^3, respectively (Table 2). In the spleen, the values were $< 1.1 \times 10^1$ and 1.1×10^1, respectively (Table 2). Bacterial loads were higher in the lungs compared with in the spleens.

Days 14 and 26 p.i.

The mean number of *C. pneumoniae* particles in the lungs of FA-exposed mice was 2.8×10^3 on Day 14 p.i. and 1.4×10^3 on Day 26 p.i. (Table 2); levels in the lungs of MCS-exposed mice were 1.4×10^3 and 5.9×10^2, respectively, at those timepoints. Spleens tested positive in the FA-exposed mice on both Days 14 and 26 p.i. (0 and 1.0×10^{-1}, respectively), whereas in MCS-exposed mice, more spleens demonstrated positive Chlamydial titers (1.5×10^{-1} and 1.0×10^{-1}, respectively, at those timepoints).

Exposure to cigarette smoke followed subsequently by *C. pneumoniae* infection produces pulmonary pathology

C. pneumoniae-infected mice exposed either to FA or MCS and then sacrificed 14 days p.i. had similar mild-to-moderate peri-bronchiolar inflammatory cell infiltration consisting of primarily large and small lymphocytes in both the proximal and distal sections of the left lung lobe. In some areas, the cellular inflammatory response extended to proximal alveolar ducts and adjacent alveoli (minimal to mild alveolitis) in centriacinar regions of the lung. The severity of the inflammatory lesion was greatest in the proximal transverse lung section at the generation 5 level of the main axial airway, compared to that seen in the distal lung section (generation 11 of main axial airway) (data not shown).

However, MCS-exposed mice (26 days post-infection) exhibited peri-bronchiolar inflammatory cell infiltration involving both large-diameter proximal bronchioles and smaller-diameter pre-terminal bronchioles in both the proximal and distal lung sections (Figure 1B). In one mouse in this group there remained a mild

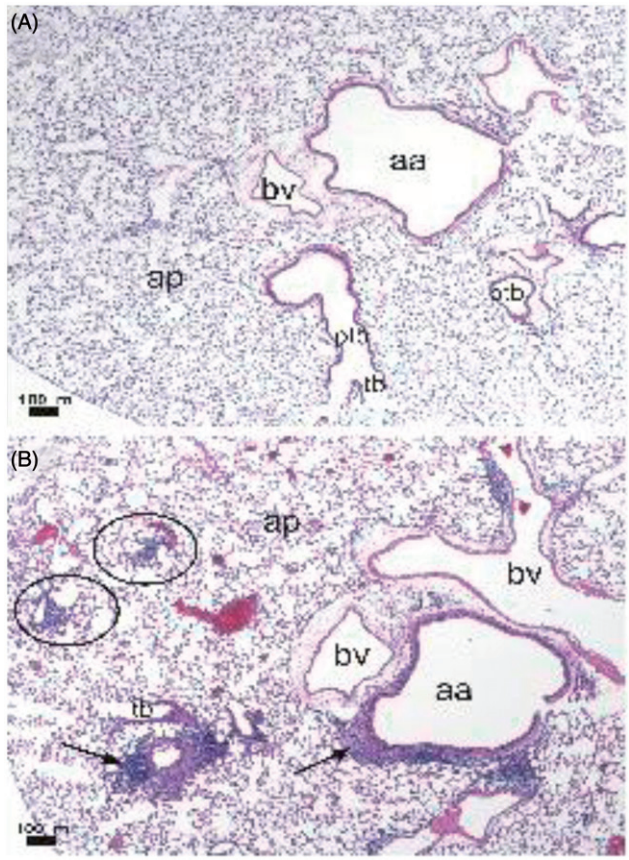

Figure 1. Lung histology on Day 26 p.i. Samples were isolated from FA- or MCS- exposed mice infected with *C. pneumoniae* (10^5 IFU). (A) FA-exposed mouse and (B) MCS-exposed mouse. Arrow/circles indicate lymphocytic infliltrates, aa = alveolar acinus, tb = terminal bronchiole, ptb = pre-terminal bronchiole. Sample shown is a representative sample.

inflammatory response in a few proximal alveolar ducts (centriacinar alveolitis), even at the later post-infection time point. In contrast, the inflammatory lesions in *C. pneumonia*-infected mice exposed only to FA were much less extensive and restricted to peri-bronchiolar infiltrates composed of lymphocytes and plasma cells surrounding large-diameter proximal bronchioles (Figure 1A). In addition, inflammatory airway lesions were present only in the proximal transverse lung sections at this later post-infection timepoint. Uninfected FA- or MCS-exposed mice did not exhibit any lung pathology at the same time points.

Cytokine levels

Bronchoalveolar lavage fluid

Levels of IL-4, IFNγ and TNFα were measured in BALF obtained from *C. pneumonia*e-infected, MCS- or FA-exposed mice on Days 14 and 26 p.i. (Figure 2A). Detectable levels of IL-4 and IFNγ were present in all treatment groups on Day 14 p.i., but levels of IFNγ and IL-4 were slightly lower in FA-exposed uninfected mice compared with other groups (Figure 2A). Levels of TNFα were not detected on Day 14 p.i. in any of the groups. However, measurable levels of TNFα were observed on Day 26 p.i. in the FA- or MCS-exposed *C. pneumoniae*-infected mice (Figure 2A). Levels of TNFα were not detected in uninfected mice on either Day 14 or 26 p.i.

Serum

Levels of IL-4 and IFNγ were measured in serum obtained from *C. pneumoniae*-infected MCS- or FA- exposed mice on Days 14

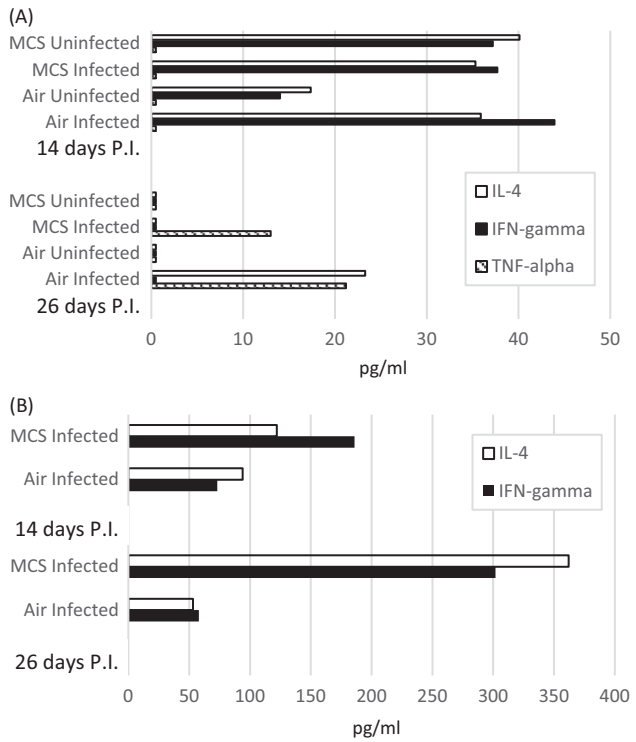

Figure 2. Cytokine levels in BALF or serum obtained from MCS- or air-exposed mice infected with *C. pneumoniae*. (A) Mean IL-4, IFNγ TNFα levels (pg/ml) in BALF from MCS- or air-exposed mice ($n = 2$–3/group) infected with *C. pneumoniae* and uninfected mice. Mice were euthanized on Days 14 or 26 p.i. White bar: IL-4; black bar: IFNγ; speckled bar: TNFα. (B) Mean IL-4 or IFNγ levels (pg/ml) in serum of MCS- or air-exposed mice ($n = 3$/group) infected with *C. pneumoniae*. Mice were euthanized on Days 14 or 26 p.i. Data shown are mean $\pm$ SD and are expressed as pg/ml. White bar: IL-4; black bar: IFNγ.

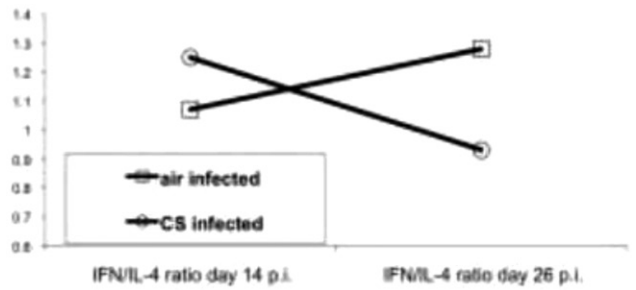

Figure 3. IFNγ:IL-4 ratio in serum obtained from FA- or MCS-exposed mice infected with *C. pneumoniae*. Days 14 or 26 p.i. $n = 3$/group. Square box: air-exposed, *C. pneumoniae*-infected mice. Circle: MCS-exposed, *C. pneumoniae*-infected mice.

and 26 p.i. (Figure 2B). We found that mice infected with *C. pneumoniae* and that had undergone MCS exposure had higher levels of IFNγ on Days 14 and 26 p.i. as compared with the FA-exposed counterparts and higher levels of IL-4 on Day 26 p.i. compared with these FA-exposed hosts. However, on Day 26 p.i., MCS-exposure in combination with *C. pneumoniae* infection led to even higher levels of IL-4 and IFNγ compared with FA-exposed mice.

In addition, the IFNγ:IL-4 ratio decreased ($\sim$30%) only in MCS-exposed *C. pneumoniae* infected mice from Day 14 to Day 26 p.i. (Figure 3). However, in FA-exposed *C. pneumoniae*-infected mice the IFNγ:IL-4 ratio increased ($\sim$40%) from Day 14 to Day 26 p.i.

Discussion

The present study demonstrates an association between cigarette smoke exposure and more severe inflammation with subsequent *C. pneumoniae* infection. Our results suggest that the long-lasting effect of combined cigarette smoke exposure and *C. pneumoniae* lung infection can cause prolonged small airway inflammation.

It is well established that *C. pneumoniae* causes acute respiratory infections (pneumonia and bronchitis) in both children and adults (Grayston, 1988). Our general finding that mice exposed to MCS had *C. pneumoniae* burdens (in lungs and spleen) no different from that seen in their control counterparts could suggest the lack of a link between smoking and *C. pneumoniae* burden. However, in contrast to the findings here, earlier studies demonstrated an association between cigarette smoking and elevated *C. pneumoniae* antibody titers (Karvonen et al., 1994). However, serological studies have been hampered by the heterogeneity of the methods, variable criteria for positivity, borderline significant results, and controls, which might be arbitrarily selected to indicate a seropositive test (Gupta & Camm, 1997). Serology has also been found to have a poor predictive value for documenting acute and chronic infection when compared to cell culture and/or PCR (Karvonen et al., 1994; Kumar & Hammerschlag, 2007). It remains unclear whether an elevated antibody titer is a reliable marker for *C. pneumoniae* infection or is merely serologic cross-reactivity (Gupta & Camm, 1997). Thus, the discrepancy between the studies here and those published previously could be due to the use of different and, possibly, less accurate methodologies to assess *C. pneumoniae* burdens in the lungs and spleen.

In this study, a significant difference was found between proximal and distal airway inflammation observed on Day 14 p.i. in both FA- and MCS-exposed mice, which persisted only in MCS-exposed mice until Day 26 p.i. This finding indicates that smoking could increase the risk for prolonged *C. pneumoniae* infection and that the ability of mice pre-exposed to cigarette smoke to resolve *Chlamydia*-related lung lesions may be compromised. Further research is necessary to identify mechanisms that could explain the observation between *C. pneumoniae* and smoke exposure and to rule out other extraneous variables. It has been suggested that smoking destroys lung and bronchial immunity and increases pulmonary IL-4 levels; cellular immunity is suppressed, thus allowing *C. pneumoniae* to localize in the lung (von Hertzen et al., 1998).

Current findings have attempted to explain the pathogenesis of *C. pneumoniae* infection as an invasion of the lung, which is aided by smoking (Ohshima & Bartsch, 1994; Redecke et al., 1998); it has been speculated that TNFα, IL-1β and IL-8, which are secreted by monocytes and macrophages, can cause damage to the lung and DNA, resulting in either carcinogenesis or genetic damage. Studies by Fan et al. (1998) reported that *C. pneumoniae* infection might cause irregular apoptosis in tissues; however, apoptosis was not assayed in the present study.

Little is known about the cellular or molecular factors/modalilties (i.e. cytokines) that link *C. pneumoniae* infection and smoking to tissue damage and inflammatory changes. Thus, our study also focussed on cytokine patterns observed in MCS-exposed mice infected later with *C. pneumoniae*. Our findings found no differences in serum cytokine levels (IFNγ, IL-4) between all groups on Day 14 post-infection; however, MCS-exposed mice displayed higher levels of IFNγ and IL-4, compared with FA exposed mice, on Day 26 p.i. In addition, the IFNγ:IL-4 ratio decreased only in MCS-exposed *C. pneumoniae* infected mice from Day 14 to Day 26 p.i., perhaps suggesting a bias towards T-helper (T_H)-2 cell-type responses. Studies by Snapper and Paul (1987) demonstrated that IL-4 and IFN-γ reciprocally

regulate polyclonally-stimulated IgE production *in vitro* in mice; however, the IL-4:IFNγ ratio has not been reported in mice infected with *C. pneumoniae*. In our mouse model, the observation that serum cytokines were not altered from a pro-inflammatory to an anti-inflammatory state when exposed to cigarette smoke and *C. pneumoniae* is of interest and might be clinically relevant. Potential explanations for these findings could be that prolonged cigarette smoke exposure might play a role in release of both T_H1- and T_H2-type cytokines by T-cells. The complex interaction between cigarette smoke exposure, *C. pneumoniae* infection and cytokines may influence susceptibility to respiratory illness; the pathogenesis of such disorders is likely to be multifactorial. However, the relevance of the cytokines that are affected by smoking and *C. pneumoniae* is under estimated and, in general, has not been adequately addressed.

The cytokine response elicited by *C. pneumoniae* may contribute significantly to an abnormal immune response in susceptible individuals resulting in inflammatory symptoms and/or persistence of infections. Our laboratory and others have noted a potent pro-inflammatory response in peripheral blood mononuclear cells and respiratory epithelial cells induced by *C. pneumoniae* (Dzhindzhikhashvili et al., 2013; Roblin et al., 1996). Previous work suggests that monocytes may contribute not only to dissemination of the bacterium, but also to immunoreactive mechanisms following infection with C. *pneumoniae* (Heinemann et al., 1996); however, these cells were not evaluated in the current study.

While assessing the strengths of this study which include relevance for public health and the use of an animal model that can inhale and deposit CS-associated particles ($\sim 1\,\mu m$) in the deep lung similar to the human situation, potential limitations of this study must also be considered, including small study/sample size. Future larger scale studies are needed to confirm the findings of this pilot study.

While our data do not directly address asthma, the finding of prolonged and sustained inflammation following exposure to both MCS and *C. pneumoniae* may be relevant to chronic respiratory diseases. Previous studies in our laboratory demonstrated a clear relationship between prenatal exposure to cigarette smoke and elevated incidence of transplanted tumors and reduction of cytotoxic T-lymphocyte activity in juvenile male mice offspring (Ng et al., 2009), possibly through changes in T-regulatory cells (Ng et al., 2013). Regulatory T-cells as well as plasmacytoid dendritic cells are necessary determinants that help regulate *C. pneumoniae* infection-induced allergic sensitization in mice (Crother et al., 2011).

Earlier studies in our laboratory reported that exposure to cigarette smoke throughout gestation could act in combination with pregnancy-associated changes to upregulate immune responses, potentially compromising fetal tolerance (Vancza et al., 2009). In human studies, Gill et al. (2014) demonstrated the effect of low-level environmental tobacco smoke (ETS) exposure on asthma control, lung function and inflammatory biomarkers in children with asthma and concluded that school-age children with persistent asthma may be exposed to ETS, even if their parents insist they do not smoke in the home. Those authors also found an association between ETS exposure and higher urinary leukotriene E_4 concentrations in inhaled corticosteroid-treated children, but not in montelukast-treated children.

To our knowledge, the current study is the first study that describes an association between cigarette smoke exposure and worsening of *C. pneumoniae* infection. Our data demonstrate that smoking could play a unique role in regulating immune responses within the respiratory system during *C. pneumoniae* infection. Our findings also provide a strong rationale for investigating the impact of smoking and *C. pneumoniae* interaction in the inflammatory environment. Given the considerable public concern regarding the negative effects of cigarette smoking and the use of alternative smoke and smokeless tobacco products on health, potential intervention strategies targeting prevention, as well as testing and treatment strategies for *C. pneumoniae*, should be a top priority through public health efforts.

Acknowledgements

We thank Kevin B. Norowitz, M.D. (SUNY Downstate Medical Center, Department of Pediatrics) for his critical reading of the manuscript, editing of the figures and fruitful discussions. We also thank Jared Ditkowsky, MS2 (SUNY Downstate Medical Center) for help with graphics.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Apfalter, P., Barousch, W., Nehr, M., et al. 2003. Comparison of a new quantitative *ompA*-based real-Time PCR TaqMan assay for detection of *Chlamydia pneumoniae* DNA in respiratory specimens with four conventional PCR assays. *J. Clin. Microbiol.* 41:592–600.

Arcavi, L., and Benowitz, N. L. 2004. Cigarette smoking and infection. Arch. Intern. Med. 164:2206–2216.

Blum, J. L., Rosenblum, L. K., Grunig, G., et al. 2014. Short-term inhalation of cadmium oxide nanoparticles alters pulmonary dynamics associated with lung injury, inflammation, and repair in a mouse model. *Inhal. Toxicol.* 26:48–58.

Christensen, P. J., Preston, A. M., Ling, T., et al. 2008. *Pneumocystis murina* infection and cigarette smoke exposure interact to cause increased organism burden, development of airspace enlargement and pulmonary inflammation in mice. *Infect. Immun.* 76:3481–3490.

Comhair, S. A., Gaston, B. M., Ricci, K. S., et al. 2011. Detrimental effects of environmental tobacco smoke in relation to asthma severity. *PLoS One* 6:e18574.

Crother, T. R., Schroder, N. W., Karlin, J., et al. 2011. *Chlamydia pneumoniae* infection induced allergic airway sensitization is controlled by regulatory T-cells and plasmacytoid dendritic cells. *PLoS One* 6:e20784.

Drannick, A. G., Pouladi, M. A., Robbins, C. S., et al. 2004. Impact of cigarette smoke on clearance and inflammation after *Pseudomonas aeruginosa* infection. *Am. J. Respir. Crit. Care Med.* 170:1164–1171.

Dzhindzhikhashvili, M. S., Joks, R., Smith-Norowitz, T., et al. 2013. Doxycyline suppresses *Chlamydia pneumoniae*-mediated increases in ongoing IgE and IL-4 responses by peripheral blood mononuclear cells of patients with allergic asthma. *J. Antimicrob. Chemother.* 68: 2363–2368.

Emre, U., Roblin, P. M., Gelling, M., et al. 1994. The association of *C. pneumoniae* infection and reactive airway disease in children. *Arch. Pediatr. Adolesc. Med.* 148:727–732.

Fan, T., Lu, H., Hu, H., et al. 1998. Inhibition of apoptosis in *Chlamydia*-infected cells: Blockade of mitochondrial cytochrome c release and caspase activation. *J. Exp. Med.* 187:487–496.

Finkela, J. F., Sandifer, S. H., and Smith, D. D. 1969. Cigarette smoking and epidemic influenza. *Am. J. Epidemiol.* 90:390–399.

Gill, R., Krishnan, S., and Dozor, A. J. 2014. Low-level environmental tobacco smoke exposure and inflammatory biomarkers in children with asthma. *J. Asthma* 51:355–359.

Grayston, J. T. 1988. TWAR: A newly-discovered *Chlamydia* organism that causes acute respiratory tract infections. *Infect. Med.* 5:215–248.

Gupta, S., and Camm, A. J. 1997. Chronic infection in the etiology of atherosclerosis-the case for *Chlamydia pneumoniae*. *Clin. Cardiol.* 20: 829–836.

Hammerschlag, M. R. 2004. *Chlamydia trachomatis* and *Chlamydia pneumoniae* infections in children and adolescents. *Pediatr. Rev.* 25: 43–51.

Hammerschlag, M. R., Kohlhoff, S. A., and Darville, T. 2009. *Chlamydia pneumoniae* and *Chlamydia trachomatis*. In: *Sequelae And Long-Term Consequences Of Infectious Diseases* (Fratamico, P. M., Smith, J. L., and Brogden, K. A., Eds.) Washington, DC: American Society for Microbiology, pp. 27–52.

Heinemann, M., Susa, M., Simnacher, U., et al. 1996. Growth of *Chlamydia pneumoniae* induces cytokine production and expression of CD14 in a human moncytic cell line. *Infect. Immun.* 64:4872–4875.

Karvonen, M., Tuomilehto, J., Pitkaniemi, J., et al. 1994. Importance of smoking for *Chlamydia pneumoniae* seropositivity. *Int. J. Epidemiol.* 23:1315–1321.

Kumar, S., and Hammerschlag, M. R. 2007. Acute respiratory infection due to *Chlamydia pneumoniae*: Current status of diagnostic methods. *Clin. Infect. Dis.* 44:568–576.

Mannino, D. M., Homa, D. M., and Redd, S. C. 2002. Involuntary smoking, and asthma severity in children: Data from the third national health and nutrition examination survey. *Chest* 122:409–415.

Martin, R. J., Wexler, R. B., Day, B. J., et al. 2006. Interaction between cigarette smoke and *Mycoplasma* infection: A murine model. *COPD* 3: 3–8.

Ng, S. P., and Zelikoff, J. T. 2008. The effects of prenatal exposure of mice to cigarette smoke on offspring immune parameters. *J. Toxicol. Environ. Health* 71:445–453.

Ng, S. P., Conklin, D. J., Bhatnager, A., et al. 2009. Prenatal exposure to cigarette smoke induces diet-and sex-dependent dyslipidemia and weight gain in adult murine offspring. *Environ. Health Perspect.* 117: 1042–1048.

Ng, S. P., Silverstone, A. E., Lai, Z. W., and Zelikoff, J. T. 2006. Effects of prenatal exposure to cigarette smoke on offspring tumor susceptibility and associated immune mechanisms. *Toxicol. Sci.* 89:135–144.

Ng, S. P., Silverstone, A. E., Lai, Z. W., and Zelikoff, J. T. 2013. Prenatal exposure to cigarette smoke alters later-life anti-tumor cytotoxic T-lymphocyte activity via possible changes in T-regulatory cells. *J. Toxicol. Environ. Health* 76:1096–1110.

Ohshima, H., and Bartsch, H. 1994. Chronic infections and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis. *Mutat. Res.* 305:253–264.

Peto, R., and Lopez, A. D. 2001. Future worldwide health effects of current smoking patterns. In: *Critical Issues in Global Health* (Koop, C. E., Pearson, C. E., and Schwarz, M. R., Eds. San Francisco, CA: Jossey-Bass.

Redecke, V., Dalhoff, K., Bohnet, S., et al. 1998. Interaction of *Chlamydia pneumoniae* and human alveolar macrophages: Infection and inflammatory response. *Am. J. Respir. Cell Mol. Biol.* 5:721–727.

Roblin, P. M., Dumornay, W., and Hammerschlag, M. R. 1992. Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. *J. Clin. Microbiol.* 30:1968–1971.

Roblin, P. M., Kutlin, A., and Hammerschlag, M. R. 1996. Production of IL-4 and IL-6 in HEp-2 cells infected with *Chlamydia pneumoniae*. *Infect. Dis. Obstet. Gynecol.* 4:195–196.

Schwartz, J., Timonen, K. L., and Pekkanen, J. 2000. Respiratory effects of environmental tobacco smoke in a panel study of asthmatic children and symptomatic children. *Am. J. Respir. Crit. Care Med.* 161: 802–806.

Snapper, C. M., and Paul, W. E. 1987. IFNγ and B-cell stimulatory factor reciprocally regulate immunoglobulin isotype production. *Science* 236: 944.

Vancza, E. M., Ng, S. P., Harkema, J. R., and Zelikoff, J. T. 2009. The role of parity status on cigarette smoke-induced modulation of anti-tumor immune mechanisms. *J. Immunotoxicol.* 6:94–103.

von Hertzen, L., Kaprio, J., Koskenvuo, M., et al. 1998. Humoral immune response to *Chlamydia pneumoniae* in twin discordant for smoking. *J. Intern. Med.* 244:227–234.