Chlamydia pneumoniae (Chlamydia pneumoniae) accelerates the formation of complex atherosclerotic lesions in Apo E3-Leiden mice


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Abstract

Objective: Atherosclerosis is an inflammatory process and is characterised by the presence of T-lymphocytes in the lesions. To study the role of Chlamydia pneumoniae in this process and the effect of infection on T-cell influx, we infected Apo E3-Leiden mice with C. pneumoniae and investigated the effect on lesion development and T-cell influx in atherosclerotic lesions at different time points post infection (pi). Methods: Nine week old mice, fed an atherogenic diet, were either mock-infected or infected with C. pneumoniae and sacrificed at 1, 6 and 9 months pi. Longitudinal sections of the aortic arches of the mice were stained with hematoxylin–eosin for atherosclerotic lesion type and lesion area analysis, or with rabbit-anti-CD3 to detect the presence of T-cells in the atherosclerotic lesions. T-cell influx was expressed as number of T-lymphocytes / lesion area. Results: At 1 month pi, type 1, 2 and 3 lesions were present. At other time points pi, more complex lesion types 4, 5a and 5b were also present. Although infection did not influence the total lesion number or area, we observed an effect of C. pneumoniae infection on lesion type. Infection resulted in a significant shift in lesion formation from type 3 to type 4 (P=0.022) at 6 months pi, and from type 4 to type 5a (P=0.002) at 9 months pi. T-cells were observed at every time point pi. At 1 month pi, a significant increase in T-cell influx in the C. pneumoniae-infected atherosclerotic lesions was observed (P=0.0005). Conclusion: This study shows that C. pneumoniae infection enhances the inflammatory process by increasing T-lymphocytes in the plaque and accelerates the formation of complex lesions.

Keywords: Atherosclerosis; Immunology; Infection/inflammation; Leukocytes; Macrophages

This article is referred to in the Editorial A.C. Van der Wal (pages 178–180) in this issue.

1. Introduction

Cardiovascular complications due to atherosclerosis are the major causes of morbidity and mortality in the Western world. Besides several well known risk factors, such as smoking and hypercholesterolemia [1], infections have been suggested to play an important role in the development of atherosclerosis over the last decades [2–4]. Infectious agents such as Cytomegalovirus, Chlamydia pneumoniae (C. pneumoniae), Helicobacter pylori, and bacteria causing dental infections have all been linked to atherosclerosis [1,5–7]. For C. pneumoniae the association has been demonstrated in sero-epidemiological, experimental, and clinical studies [4,8–15]. However, the mechanism by which C. pneumoniae contributes to atherosclerosis has not been clarified. To understand this mechanism, various animal models, mostly rabbits and mice, have been used [11–14,16]. The progression of atherosclerosis has been reported in New Zealand White rabbits [15], as well as in low-density lipoprotein receptor (LDLR)
knockout mice [11] inoculated with *C. pneumoniae*. In all of these studies, an effect of *C. pneumoniae* infection was only documented on lesion size. However, little is known about the effect of infection on the development and/or progression of lesion types. Since lesion type is an important indicator of the severity of atherosclerosis, we studied the effect of *C. pneumoniae* infection on the type of atherosclerotic lesion by grading the lesions according to the guidelines of the American Heart Association [17,18].

By understanding the atherogenicity of *C. pneumoniae*, insight should be gained into the ability of this micro-organism to cause local infection and inflammation of the vascular wall as characterised by the influx of leukocytes, consisting mainly of monocytes and T-lymphocytes. When T-lymphocytes are activated, they can act as a powerful source of pro-atherogenic cytokines, thereby contributing to the progression of atherosclerotic lesion formation from fatty streaks into complex atherosclerotic plaques [19]. Immunohistochemical studies have demonstrated the presence of T-lymphocytes in human [20,21] and animal [22] atherosclerotic plaques. Activation of T-lymphocytes in the plaque can be established by antigens such as ox-LDL [23–25]. However, *C. pneumoniae* has also been demonstrated to be a good candidate for activating T-cells cultured from the peripheral blood of patients with atherosclerosis as well as T-cells cultured from carotid endarterectomy tissue [20]. Recently, it has even been demonstrated that *C. pneumoniae* can infect lymphocytes of human peripheral blood and mouse spleens as well as a T-lymphocyte cell line [26].

In this study, the role of *C. pneumoniae* in atherosclerotic lesion progression was studied using Apo E3-Leiden mice. Mice were infected with *C. pneumoniae* and sacrificed at different time points post infection (pi) to analyse lesion progression in the aortic arch and main branchpoints. To understand the possible mechanism by which *C. pneumoniae* accelerates atherosclerotic lesion progression, we studied inflammation of the vascular wall by determining the influx of T-lymphocytes into the atherosclerotic lesion.

### 2. Methods

#### 2.1. Mice

Sixty-four transgenic Apo E3-Leiden mice on a C57Bl/6J background were bred at the Central Animal Facility at Maastricht University [27]. For the study, both males and females were used and randomly divided over all groups. All mice were specific-pathogen-free (SPF) and were housed under standard conditions.

This study was approved by the Institutional Committee for the Welfare of Laboratory Animals of Maastricht University.

#### 2.2. *C. pneumoniae*

*C. pneumoniae* strain TWAR 2043 (ATCC VR-1355) was cultured in HEp-2 cells as previously described [28]. Bacterial titers were determined by titration in HEp-2 cells and staining with monoclonal antibody RR 402 (Dako, Denmark) followed by fluorescein-isothiocyanate (FITC)-conjugated rabbit-anti-mouse (Dako). Titers were expressed as inclusion-forming units per milliliter (IFU/ml).

#### 2.3. Experimental design

At the age of 5 weeks, the mice received a high-fat cholesterol (HFC) diet containing 15% cacao butter, 0.5% cholate, 1% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil, and 4.7% cellulose (Hopefarms, Woerden, The Netherlands). Since it has been demonstrated that multiple infections, the age of the animal at the time of inoculation and the interval between inoculations are all critical factors in mice for enabling *C. pneumoniae* to promote atherosclerosis [11,14,29,30], we decided to inoculate our mice twice with *C. pneumoniae* using different time intervals between inoculations. All mice received the first infection by intraperitoneal injection of 6×10^7 IFU *C. pneumoniae* at the age of 9 weeks (Fig. 1). Control mice were injected with sterile phosphate-buffered saline (PBS, mock infection). Since no differences in the

<table>
<thead>
<tr>
<th>Age of mice</th>
<th>5 weeks</th>
<th>9 weeks</th>
<th>11 weeks</th>
<th>13 weeks</th>
<th>GROUP 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29 weeks</td>
<td>33 weeks</td>
<td>41 weeks</td>
<td>45 weeks</td>
<td>GROUP 2</td>
</tr>
</tbody>
</table>

**Fig. 1.** Design of animal experiment, showing the different mice groups analysed in this study, the inoculation time points and time points of sacrifice.
dissemination of \textit{C. pneumoniae} after intraperitoneal or intranasal inoculation have been reported \cite{31}, we selected intraperitoneal infection for our mice. In group 1, mice received a second inoculation at the age of 11 weeks and were sacrificed at the age of 13 weeks, 1 month after the first infection (Fig. 1). In group 2, mice were inoculated for the second time at the age of 29 weeks and were sacrificed at the age of 33 weeks, 6 months after the first infection. Finally, mice in group 3 received a second inoculation at the age of 41 weeks and were sacrificed at the age of 45 weeks, 9 months after the first infection.

2.4. Tissue handling

At sacrifice, the mice were anesthetized with a weight-adjusted dose of pentobarbital \cite{32} (Nembuta \textsuperscript{®}, Sanofi Sante, Maassluis, The Netherlands) and blood was collected from the left ventricular apex for assessment of lipid profiles and anti-\textit{C. pneumoniae} antibody titers. The arterial tree was perfused with PBS followed by 1.85% PBS-buffered formaldehyde, both containing 0.1 mg/ml sodium-nitroprusside (Merck, Darmstadt, Germany), through a catheter placed in the left ventricular apex. The arterial tree was removed, fixed overnight in 3.7% PBS-buffered formaldehyde and embedded in paraffin. Four micrometer thick longitudinal sections were consecutively cut, stained and analysed \cite{18}.

2.5. Evaluation of atherosclerotic lesions

Since all lesion types were found in the aortic arch \cite{18}, lesion analysis was restricted to this segment of the arterial tree. Only aortic arches with an intact morphology (brachiocephalic trunk, left and right common carotid artery and left subclavian artery) were used.

Longitudinal, coronal, consecutive sections of the entire arch were prepared. Every fifth section was hematoxylin–eosin (HE)-stained to evaluate lesion type, size and number. Since atherosclerotic lesion development progresses over time, thereby increasing the size of the aortic arch, more sections were collected from the aortic arches of groups 2 (33 weeks of age) and 3 (45 weeks of age) than from arches of group 1 (13 weeks of age). Five HE-stained sections from each aortic arch were analysed in group 1, while in groups 2 and 3, 11–15 sections per aortic arch were analysed. The type of atherosclerotic lesion was determined according to the guidelines of the American Heart Association (Table 1) \cite{17}. The size of the lesions was determined using a microscope coupled to a computer-assisted morphometry system (Quantimet 570, Leica, The Netherlands). For every lesion, the mean area was calculated and total lesion area was calculated as the sum of all mean areas. All sections were analysed by impartial investigators (inter-observer variability <10%), who were blinded with respect to the presence or absence of \textit{C. pneumoniae} infection as well as to the time point of sacrifice.

2.6. Detection and evaluation of T-lymphocytes in atherosclerotic lesions

In order to detect T-lymphocytes in the atherosclerotic lesions, a rabbit-anti-CD3\textsuperscript{+} polyclonal antibody was used (Dako). After a blocking step with bovine serum albumin (BSA)/PBS 2% for aspecific binding, slides were incubated with the CD3\textsuperscript{+} polyclonal antibody for 60 min at a dilution of 1:200, followed by a second-step incubation with a biotinylated swine-anti-rabbit IgG (1:1000, Dako) for 30 min. Sections were then labelled with an alkaline phosphatase coupled ABC reagent (Dako) for 30 min. Sections were then labelled with an alkaline phosphatase coupled ABC reagent (Dako) for 30 min. Alkaline phosphatase activity was visualised using Fast-red (Sigma, St. Louis, MO, USA). Rabbit-anti-rat IgG (Dako) was used as a negative control. For positive controls, tissue sections from mouse spleens were used. T-lymphocytes in the atherosclerotic lesion were counted and expressed as the number of T-lymphocytes in the plaque divided by the total plaque area. The investigator was blinded to the

| Table 1 |
| Classification of atherosclerotic lesion types according to the Stary classification defined in 1995 |

<table>
<thead>
<tr>
<th>Lesion type</th>
<th>Nomenclature</th>
<th>Main histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1 Initial lesion</td>
<td>Initial lesion</td>
<td>Isolated macrophage foam cells, adaptive thickening intima (smooth muscle cells)</td>
</tr>
<tr>
<td>Type 2 Fatty streak</td>
<td>Fatty streak</td>
<td>Intracellular lipid accumulation</td>
</tr>
<tr>
<td>Advanced lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 3 Intermediate lesion</td>
<td>Intermediate lesion</td>
<td>Type 2 changes with small extracellular lipid pools</td>
</tr>
<tr>
<td>Type 4 Atheroma</td>
<td>Atheroma</td>
<td>Type 2 changes with lipid core</td>
</tr>
<tr>
<td>Type 5a Fibroatheroma</td>
<td>Fibroatheroma</td>
<td>Fibrotic cap surrounding a lipid core</td>
</tr>
<tr>
<td>Type 5b Calcified lesion</td>
<td>Calcified lesion</td>
<td>Fibrotic cap surrounding a lipid core, calcifications</td>
</tr>
<tr>
<td>Type 6 Complicated lesion</td>
<td>Complicated lesion</td>
<td>Cap disruption, hematoma-hemorrhage, thrombus</td>
</tr>
</tbody>
</table>
presence or absence of *C. pneumoniae* infection as well as to the time point of sacrifice.

### 2.7. Detection of *C. pneumoniae* antigens and anti-*C. pneumoniae* antibodies

In order to detect *C. pneumoniae* antigens, a *Chlamydia* genus-specific mouse monoclonal antibody, CF-2 (Washington Research Foundation, Seattle, WA, USA), was used [33]. This monoclonal is directed against chlamydial lipopolysacharide. For each mouse, a representative section with the highest lesion number and the most advanced lesion type, as analysed by the HE staining, was used for *C. pneumoniae* detection. The primary antibody was probed with goat-anti-mouse IgG conjugated to peroxidase (Dako, Glostrup, Denmark). To visualise the antibody binding, 3,3′-diaminobenzidine (DAB, Sigma) was used. Normal mouse ascitic fluid (Sigma–Aldrich, Zwijndrecht, The Netherlands) was used as negative control. Tissue sections from mock-infected mice were used as controls. For positive controls, sections of *C. pneumoniae*-infected HEp-2 cells were used, which were embedded in agarose, fixed overnight in 3.7% PBS-buffered formaldehyde and embedded in paraffin.

Anti-*C. pneumoniae* antibodies were measured with the indirect micro-immunofluorescence (MIF) technique using mouse plasma sample dilutions of 1:10 and 1:100 on antigen-coated slides (Labsystems, Helsinki, Finland). Goat-anti-mouse IgG conjugated to FITC (Sigma) was used as the secondary antibody. The presence of anti-*C. pneumoniae* antibodies was determined by two independent observers.

### 2.8. Assessment of lipid profile

Total plasma cholesterol and triglyceride concentrations were determined by a standard cholesterol oxidase method performed on a Beckman Synchron CX System [12].

### 2.9. Statistical analysis

The difference in lesion type between the *C. pneumoniae* and mock group was compared and analysed with the Chi-square test. Differences in lesion number and size were analysed with the Mann–Whitney *U*-test. To determine differences in T-cell influx in the lesions the Mann–Whitney *U*-test was used. Plasma lipids of the *C. pneumoniae* - and mock-infected groups were compared using Student’s two-tailed *t*-test. *P*<0.05 was considered statistically significant. Data are expressed as mean±S.E.M.

### 3. Results

To investigate the effect of *C. pneumoniae* infection on atherosclerotic lesion type, number and total lesion size, 64 Apo E3-Leiden mice were infected, sacrificed and analysed. Seven mice died before reaching the point of sacrifice, leaving 57 mice for analysis. The cause of death was unknown.

#### 3.1. *C. pneumoniae* infection leads to more severe atherosclerotic lesions

Atherosclerotic lesions were found in the *C. pneumoniae*-infected group and in the mock-infected group. The type of lesion was analysed according to the histological criteria given in Table 1, which are based on the Stary classification defined in 1995 [17].

<table>
<thead>
<tr>
<th>Time post infection</th>
<th>Inoculation (No. of mice)</th>
<th>Lesion type*</th>
<th>Lesion area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td><em>C. pneumoniae</em> (8)</td>
<td>1 2 3 4 5a 5b</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td></td>
<td>Mock (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 months</td>
<td><em>C. pneumoniae</em> (7)</td>
<td>0.38±0.18 2.13±0.79 2.00±0.57 – – – 0.12±0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mock (8)</td>
<td>0.25±0.17 0.75±0.34 1.50±0.45 – – – 0.08±0.01</td>
<td></td>
</tr>
<tr>
<td>9 months</td>
<td><em>C. pneumoniae</em> (6)</td>
<td>– – 0.83±0.44 0.83±0.18 3.08±0.92** 2.00±0.69 1.80±0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mock (6)</td>
<td>– – 0.63±0.40 2.63±0.53** 1.88±0.32 2.13±0.24 1.86±0.11</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant shift from type 3 to type 4 (P=0.022).
**Statistically significant shift from type 4 to type 5a (P=0.002).
Total lesion area in *C. pneumoniae* - and mock-infected group at different time points pi.
*The mean number of lesions of each type is expressed as the mean frequency±S.E.M.
*Lesion type is not present.
Table 3

<table>
<thead>
<tr>
<th>Time post inoculation</th>
<th>Inoculation (No. of mice)</th>
<th>Lesion number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>C. pneumoniae (8)</td>
<td>4.38±0.88</td>
</tr>
<tr>
<td></td>
<td>Mock (8)</td>
<td>2.75±0.75</td>
</tr>
<tr>
<td>6 months</td>
<td>C. pneumoniae (7)</td>
<td>8.57±0.85</td>
</tr>
<tr>
<td></td>
<td>Mock (7)</td>
<td>8.00±0.88</td>
</tr>
<tr>
<td>9 months</td>
<td>C. pneumoniae (6)</td>
<td>6.67±1.01</td>
</tr>
<tr>
<td></td>
<td>Mock (8)</td>
<td>7.25±0.75</td>
</tr>
</tbody>
</table>

when the C. pneumoniae-infected and mock-infected groups were compared at the different time points.

The number of atherosclerotic lesions was analysed in all HE-stained sections. The number of lesions in the C. pneumoniae-infected and mock-infected groups was calculated at different time points pi. Data on lesion number are presented in Table 3. There were no significant differences in total lesion number between C. pneumoniae-infected and mock-infected mice at any time point pi.

3.3. C. pneumoniae infection results in an increased T-cell influx in atherosclerotic lesions

The effect of C. pneumoniae infection on the influx of T-cells was determined at every time point pi in atherosclerotic lesions of the C. pneumoniae-infected mice and the mock-infected mice. More specifically, at 1 month pi a significant increase in T-cell influx in the C. pneumoniae-infected atherosclerotic lesions was observed. Infection resulted in a significant increase in T-cells from 0.29 cells/μm² in the mock group to 2.63 cells/μm² in the C. pneumoniae group (P=0.0005, Fig. 4, 5). At 6 and 9 months pi, no effect of C. pneumoniae infection on the influx of T-cells was observed.

3.2. C. pneumoniae infection has no effect on lesion size and number of lesions

The HE-stained sections used for classification of atherosclerotic lesion types were also used for determination of lesion area and number of lesions. Data on the total lesion area are presented in Table 2. No statistically significant differences in lesion area could be detected when the C. pneumoniae-infected and mock-infected groups were compared at the different time points.

Fig. 2. Lesion types in the C. pneumoniae and mock groups 6 months post infection. The values are presented as the mean frequency ± S.E.M. at which that type of lesion was present. *P=0.022.

Fig. 3. Lesion types in the C. pneumoniae and mock groups 9 months post infection. The values are presented as the mean frequency ± S.E.M. at which that type of lesion was present. *P=0.002.

Fig. 4. Number of T-cells in atherosclerotic lesions at 1 month pi. Data are presented as number of T-cells divided by total plaque area. *P=0.0005.
Fig. 5. T-lymphocyte staining showing positive cells (arrows) in an atherosclerotic lesion of a *C. pneumoniae*-infected mouse 1 month pi.

3.4. Lipid profiles

At 1, 6 and 9 months pi, blood was collected from *C. pneumoniae*- and mock-infected mice for determination of plasma cholesterol and triglyceride levels (Table 4). When the *C. pneumoniae* and the mock groups were compared at one time point pi, no statistically significant difference was found in cholesterol and triglyceride plasma levels, suggesting that *C. pneumoniae* infection does not affect plasma lipid levels in the long term (data not shown).

3.5. *C. pneumoniae* infection

All *C. pneumoniae*-infected mice seroconverted after inoculations with *C. pneumoniae*, indicating that all our inoculated mice had indeed been successfully infected. Anti-*C. pneumoniae* antibodies were found in all these mice at plasma dilutions of 1:10 and 1:100. No anti-*C. pneumoniae* antibodies were detected in plasma of mock-infected mice.

*C. pneumoniae* antigen was detected by immunohistochemistry staining in about 30% of the aortic arches of the infected animals at the time points 1, 6 and 9 months pi. *C. pneumoniae* infection leads to an accelerated formation of complex atherosclerotic lesions. At 6 and 9 months pi, an acceleration in lesion type development was seen, whereas at 1 month pi, no effect of *C. pneumoniae* infection on early lesion formation was observed. This is an intriguing finding, as it contributes to the suggestion that *C. pneumoniae* infection may promote progression [11,14-16,29] of the atherosclerotic process rather than its initiation [33]. The effect of *C. pneumoniae* on lesion formation seems to be the result of a long-standing process, since its effect is

Table 4
Cholesterol and triglyceride levels in the *C. pneumoniae*- and mock-infected groups at different time points pi. Data are expressed as mean±S.E.M.

<table>
<thead>
<tr>
<th>Time post inoculation (No. of mice)</th>
<th>Cholesterol (mmol/l)</th>
<th>Triglycerides (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. pneumoniae</em> (8)</td>
<td>2.47±0.73</td>
<td>4.85±1.65</td>
</tr>
<tr>
<td>Mock (8)</td>
<td>2.93±0.63</td>
<td>5.15±1.41</td>
</tr>
<tr>
<td>6 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. pneumoniae</em> (7)</td>
<td>3.69±1.00</td>
<td>4.83±0.97</td>
</tr>
<tr>
<td>Mock (7)</td>
<td>6.87±3.30</td>
<td>2.90±0.80</td>
</tr>
<tr>
<td>9 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. pneumoniae</em> (6)</td>
<td>4.58±1.17</td>
<td>6.76±1.90</td>
</tr>
<tr>
<td>Mock (8)</td>
<td>6.83±2.73</td>
<td>4.92±1.44</td>
</tr>
</tbody>
</table>

4. Discussion

Infection with *C. pneumoniae* has been associated with the process of atherosclerosis [4,9], but whether infection influences the initiation or progression of atherosclerosis, or whether it plays a role in plaque (in)stability, has not been clarified. Most previously published experimental studies on *C. pneumoniae* infections have reported on the effect of infection on atherosclerotic lesion size [11,14,15,29]. No reports on the effect of *C. pneumoniae* infection on lesion type have been published. Since the type of atherosclerotic lesion may be a more important indicator for morbidity and mortality than lesion size or number, we evaluated the incidence of different atherosclerotic lesion types. In this study we demonstrate that *C. pneumoniae* infection leads to an accelerated formation of complex atherosclerotic lesions.
only found at later time points, 6 and 9 months pi. This suggests that an antigen-specific immune activation early after infection may contribute to the progression of atherosclerosis [1,6,9]. Since *C. pneumoniae* has been shown to infect macrophages and use them as a transport vehicle for dissemination [31] it may be speculated that *C. pneumoniae* enters the vascular wall in this way. Having entered the vascular wall, *C. pneumoniae*-infected macrophages may act as antigen-presenting cells, thereby attracting T-cells. Indeed, in the present study we demonstrated a markedly enhanced T-cell influx shortly after the first infection. Since activated T-cells are a powerful source of pro-atherogenic and pro-inflammatory cytokines, such as IFN-γ, IL-2 and TNF-α [34], this may explain the observed acceleration of plaque development in the long term. On the other hand, T-cells themselves may also function as a transport vehicle for *C. pneumoniae*. Recently, it has been shown that *C. pneumoniae* can infect and multiply in T-cells [26]. Furthermore, a significantly higher proportion of *C. pneumoniae*-positive T-cells were observed in patients with coronary artery disease compared with healthy blood donors [35]. Future co-localisation studies may shed some light on where *C. pneumoniae* is exactly located in the vascular wall of our infected mice.

Besides atherosclerotic lesion type, we also determined the effect of *C. pneumoniae* infection on lesion size and number and found no difference between the *C. pneumoniae*- and mock-infected mice. This corresponds to findings in previous studies that also found no effect of *C. pneumoniae* infection on lesion size [36,37]. In contrast, other studies reporting an increase in lesion size after infection with *C. pneumoniae* have also been published [11,14]. The difference between these other studies and ours may result from differences in the *C. pneumoniae* strain, the inoculation schedule or the type of animal model used. LDLR knockout [11] and Apo E knockout mice [14,36,37] were the mouse strains used by other groups. In the present study, Apo E3-Leiden mice were used [18]. It is known that different mouse strains vary in susceptibility to *C. pneumoniae* infection and the formation of atherosclerotic lesions [38]. Of the latter studies, only one reports elevated lipid levels after *C. pneumoniae* infection in LDLR knockout mice [11]. However, in the present study and in previous reports [14,36,37], no differences between infected and non-infected groups were observed in cholesterol or triglyceride levels, excluding this as a predominant factor.

In humans, the first association between *C. pneumoniae* infection, myocardial infarction and chronic heart disease was shown in patients with anti-*C. pneumoniae* antibodies by Saikku et al. [4]. Later, several other studies supported this finding, suggesting a correlation between *C. pneumoniae* infection and cardiovascular disease [8,9,39,40]. However, the correlation between the presence of anti-*C. pneumoniae* antibodies and *C. pneumoniae* antigen in atherosclerotic lesions is questionable [12,15,33,39,41]. A similar finding was also observed in our study, since all infected mice were positive for anti-*C. pneumoniae* antibodies, while *C. pneumoniae* antigen was detected in only one-third of the aortic arches. This discrepancy may be explained by the notion that immunohistochemical staining has a limited sensitivity for detecting *C. pneumoniae* in vascular tissue. A second explanation could be that anti-*C. pneumoniae* antibody in plasma and *C. pneumoniae* antigen in aortic tissue do not necessarily have to be present at the same time [36].

In summary, this study demonstrates that *C. pneumoniae* infections result in an acceleration of the formation of complex atherosclerotic lesions in Apo E3-Leiden mice, either by increasing the influx of activated T-cells to the vascular wall by infected and antigen-presenting macrophages, or by direct activation of T-cells.

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