# Immunomodulation of atherosclerosis

Hauer, A.D.

# Chapter 3

## Delivery of *Chlamydia pneumoniae* to the vessel wall aggravates atherosclerosis in LDLr$^{-/-}$ mice

A.D. Hauer[1], P. de Vos[1], N. Peterse[1], H. Ten Cate[2], Th.J.C. van Berkel[1], F.R.M. Stassen[3], J. Kuiper[1]

[1]Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, [2]Department of Internal Medicine and [3]Department of Medical Microbiology, Academic Hospital Maastricht, Cardiovascular Research Institute Maastricht, Maastricht, The Netherlands

*Cardiovascular Research 2006;69:280-8*

**Abstract**

The role of *Chlamydia pneumoniae* in atherosclerosis is still debated. In this study a novel mouse model was applied to determine the direct impact of *C. pneumoniae* on the arterial wall and the development of atherosclerosis. Direct effects of *C. pneumoniae* on collar-induced atherosclerosis were studied after local delivery of *C. pneumoniae* to carotid arteries of LDLr$^{-/-}$ mice. The presence of *C. pneumoniae* in the vessel wall was quantified by RT-PCR ($6.2 \times 10^4$ copies/artery) and resulted in a 2.0-fold increase in intima/media ratios ($p<0.05$) and a 1.7-fold increase in stenosis ($p<0.05$). Immunostaining revealed a 2.98-fold ($p<0.01$) increased macrophage content and a tendency towards lower numbers of smooth muscle cells and collagen in lesions of infected carotid arteries. Direct delivery of another respiratory pathogen, *M. pneumoniae*, to the carotids did not affect size or composition of the atherosclerotic lesions. Presence of *C. pneumoniae* in the carotid arteries resulted within 7 days in a marked upregulation of the expression of MCP-1 ($p<0.01$) and ICAM-1 as determined on mRNA and protein level. These *in vivo* data were in line with data obtained with *in vitro* infections of macrophages and endothelial cells with *C. pneumoniae*. We conclude that *C. pneumoniae* in carotid arteries leads to more pronounced atherosclerotic lesions with a more vulnerable morphology and that this model is suitable to monitor the direct effects of *C. pneumoniae* on atherosclerosis.

## Introduction

Atherosclerosis is considered to be an inflammatory disease[1] and possible involvement of infectious agents has been studied. Until now most evidence indicates that *Chlamydia pneumoniae* may promote atherogenesis. This gram-negative bacterium, a common human pathogen, causes mild upper respiratory tract infections. The majority of people are repeatedly infected with *C. pneumoniae* during lifetime. By the age of 65, anti-*C. pneumoniae* antibodies are found in 80% of the male and 70% of the female population.[2] Epidemiological studies show an association of serum antibodies against *C. pneumoniae* and cardiovascular disease.[3] Accumulating data revealed that, due to its obligate intracellular character, *C. pneumoniae* is able to disseminate via the circulation throughout the body within monocytes and can in this way enter the atherosclerotic lesion.[4] Its ability to infiltrate lesions is illustrated by the observation that in up to 50% of the human atherosclerotic lesions *C. pneumoniae* is detected.[5] Data from sero-epidemiological and pathology studies underline the possible relation between *C. pneumoniae* and atherosclerosis, but cannot provide definite proof for a causal relation between *C. pneumoniae* infection and atherosclerosis. Several clinical trials with antibiotics against these bacteria attempted to demonstrate a causal role in cardiovascular disease, but did not always show decreased pathology.[6] *In vitro*, *C. pneumoniae* has various pro-atherosclerotic effects on atheroma-associated cell types. Infection of endothelial cells (ECs) leads to increased expression of adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1),[7] chemokines, such as monocyte chemoattractant protein-1 (MCP-1),[8] and cytokines, such as interleukin (IL)-1, IL-8, and tumor necrosis factor-$\alpha$ (TNF-$\alpha$),[9] resulting in pro-atherosclerotic processes, such as enhanced adherence and migration of monocytes.[8] Infection of monocytes with *C. pneumoniae* increases adherence of infected monocytes to ECs[10,11] and promotes low density lipoprotein (LDL) oxidation,[12] resulting in accelerated uptake of cholesterol by macrophages and subsequent foam cell formation. Increased LDL oxidation was also observed upon infection of ECs with *C. pneumoniae.*[13] Smooth muscle cells (SMCs) respond to *C. pneumoniae* infection by elevated expression of matrix metalloproteinases (MMPs), which may contribute to plaque destabilization.[14] Although *in vitro* data point to pro-atherosclerotic effects of *C. pneumoniae*, most of these still have to be confirmed *in vivo*. Many groups have used animal models to chart the relation between *C. pneumoniae* infection and cardiovascular disease. However, until now these models did not result in a general consensus about the contribution of *C. pneumoniae* to atherosclerosis.[15-16] The differences in the observed effects of *C. pneumoniae* on atherosclerosis, may arise from the fact that *C. pneumoniae* could not always be detected at the site of atherosclerosis after initial lung infection.

Therefore, we developed a novel model in which the impact of *C. pneumoniae* on the arterial wall and subsequent atherogenesis can be studied. *C. pneumoniae* was delivered to the carotid artery of LDL receptor

deficient ($LDLr^{-/-}$) mice by means of local intra-vascular incubation. Subsequently, the influence of *C. pneumoniae*, present in the vessel wall, on atherosclerosis was studied by collar placement around the incubated carotid artery. In addition we determined the effect of *Mycoplasma pneumoniae* on lesion formation in the carotid artery. *M. pneumoniae* is, like *C. pneumoniae*, a mild respiratory pathogen that has directly been detected in atherosclerotic lesions.[17] The epidemiological behavior of *M. pneumoniae* and its ability to produce chronic sequelae following respiratory infection may point to a role for *M. pneumoniae* in atherosclerosis similar to *C. pneumoniae*.[18] Since *C. pneumoniae* may also affect cardiovascular disease by interfering with the thrombogenicity of the vessel wall or atherosclerotic plaque, we also determined the effect of *C. pneumoniae* presence in the vascular wall on acute arterial thrombus formation.

## Methods

### *Bacteria*

*C. pneumoniae* strain AR39 (Washington Research Foundation, Washington, USA) was cultured in Buffalo Green Monkey cells. The titer was determined as previously described.[19] *M. pneumoniae* was kindly provided by dr Brugman and dr Thuis from the National Institute for Public Health and Environment (Bilthoven, The Netherlands).

### *Delivery of C. pneumoniae and induction of atherosclerosis*

Animal work was carried out in compliance with guidelines issued by the Dutch government, which are conform with NIH guidelines. Male $LDLr^{-/-}$ mice, 12-14 weeks old, were put on a "Western-type" diet, containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, UK). After two weeks mice were anesthetized as previously described,[20] and injected intravenously with 200 µl 20 U/ml heparin (Leo Pharma, Netherlands). Subsequently, 15 mice were inoculated with $5x10^5$ *C. pneumoniae* infection forming units (IFU) in 10 µl sucrose-phosphate-glutamic acid (SPG) medium by instillation into the right common carotid artery via the external carotid artery.[21] Carotids of 14 control mice were incubated with SPG medium. An additional group of mice (n=9) was incubated with $5x10^5$ *M. pneumoniae* bacteria. Suspensions were left inside the common carotid artery for 60 minutes and subsequently were aspirated from the common carotid artery. Thereafter, the external carotid artery was ligated to prevent blood loss.[21] Immediately after incubation atherosclerosis was induced by bilateral perivascular collar placement.[20] Furthermore, 83 male $LDLr^{-/-}$ mice were locally incubated (40 mice with *C. pneumoniae* and 43 mice with SPG medium) without induction of atherosclerosis by collar placement. Twelve mice per time point were sacrificed at day 1, 2, 4, and 7 post infection for gene expression studies. Another 16 mice were sacrificed 7 days post infection for DNA isolation or immunohistochemistry and in the remaining 19 animals acute thrombus formation was induced prior to their sacrifice at day 7

post infection. Acute thrombosis was induced in the right carotid artery by peri-arterial application of $FeCl_3$ (40%) resulting in the formation of platelet and fibrin-rich thrombi, as previously described.[22] A Doppler flow probe was used to measure blood flow. Time to occlusion was defined as the time after initiation of arterial injury with $FeCl_3$, required for blood flow to decline to <0.2 ml/min.

***Immunohistochemistry***

For immunohistochemistry carotid arteries were perfused and isolated as previously described.[20] Transverse 5 μm cryosections, prepared in a proximal direction from the carotid bifurcation, were routinely stained with hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). Each vessel was assessed ~0.5 mm proximal to the collar, and the site of maximal stenosis was used for morphometric assessment.[20] Corresponding sections were stained immunohistochemically with antibodies against a macrophage-specific antigen (MOMA-2, polyclonal rat $IgG_{2b}$, diluted 1:50; Research Diagnostics Inc., NJ), alpha smooth muscle cell actin (monoclonal mouse $IgG_{2a}$ (clone 1A4), dilution 1:500; Sigma Diagnostics), and ICAM-1 (monoclonal rat $IgG_{2a}$ (clone BSA2), dilution 1:200; R&D Systems, UK). Slides were incubated with primary antibodies for 2 hours at room temperature. As secondary antibodies goat anti mouse IgG peroxidase conjugate (dilution 1:500; Nordic, The Netherlands) and goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:50; Sigma Diagnostics) were used (one hour of incubation at room temperature), with 3,3'-diamino-benzidine (Sigma Diagnostics), nitro blue tetrazolium (Sigma Diagnostics) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Diagnostics) as enzyme substrates.[20] Sections were stained for collagen by a Picrosirius Red (Direct red 80) staining. Sections were analyzed using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK). Contens of macrophages, smooth muscle cells, and collagen were obtained by dividing the area of specific staining within the intima by the total intima area.

***Detection of C. pneumoniae***

DNA was isolated from incubated carotid arteries, after perfusion with PBS, using a QIAamp DNA MiniKit (Westburg, Netherlands). For detection of *C. pneumoniae* DNA quantitative PCRs were performed on an ABI PRISM 7700 machine (Applied Biosystems, CA) using primers for the outer membrane protein A (OMP A) gene (table 1), which is highly conserved within *C. pneumoniae* species,[23] and probe 5'-AAACTTAACTGCATGGACCCTTCTTT ACTAGG-3' (FAM), (reverse primers; 300 nM, forward primers; 900 nM, and probes; 200 nM). PCR conditions were the same as used in the gene expression assays. Amounts of *C. pneumoniae* copies were obtained after performing regression analysis on a standard curve, which was created by performing PCRs under the same conditions on dilutions of plasmids containing the OMP A gene in the presence of representative amounts of DNA isolated from murine carotids.

| Gene | Forward primer (5'- '3) | Reverse primer (5'- '3) |
|---|---|---|
| E-selectin | CCCTGCCCACGGTATCAG | CCCTTCCACACAGTCAAACGT |
| HPRT | TTGCTCGAGATGTCATGAAGGA | AGCAGGTCAGCAAAGAACTTATAG |
| ICAM-1 | GGACCACGGAGCCAATTTC | CTCGGAGACATTAGAGAACAATGC |
| IL-1β | TGGTGTGTGACGTTCCCATTA | AGGTGGAGAGCTTTCAGCTCATAT |
| IL-6 | GAAGAATTTCTAAAAGTCACTTT GAGATCTAC | CACAGTGAGGAATGTCCACAAAC |
| IL-12 | AGTGAAAATGAAGCTCTGCATCC | GATAGCCCATCACCCTGTTGA |
| MCP-1 | GCATCTGCCCTAAGGTCTTCA | GCATCTGCCCTAAGGTCTTCA |
| OMP A | TCCGCATTGCTCAGCC | AAACAATTTGCATGAAGTCTGAGAA |
| TF | TCCTGGCCACCATCTTTATCAT | GCAGGCAGTGAGCGTTAG |
| TNFα | GCCAGCCGATGGGTTGTA | AGGTTGACTTTCTCCTGGTATGAGA |
| VCAM-1 | ACAAAACGATCGCTCAAATCG | CGCGTTTAGTGGGCTGTCTATC |

**Table 1.** Primer sequences used in quantitative PCR.

***Cell Culture***

Murine macrophages (RAWs) and endothelial cells (H5Vs) were cultured in DMEM containing 10% Fetal Bovine Serum (FBS), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker Europe) in a humidified atmosphere (5% $CO_2$) at 37°C. Prior to infection cells were transferred into 24-well plates ($2.5x10^5$ RAWs/well or $1.0x10^5$ H5Vs/well) and incubated overnight.

***In vitro infection***

Cells were washed twice with DMEM containing 10% FBS and 2 mmol/L L-glutamine and subsequently inoculated with *C. pneumoniae* with a m.o.i of 1. Mock infection was performed with SPG medium. Immediately after inoculation, plates were centrifuged for 60 minutes with 800xg at 37°C. After another 60 minutes of inoculation with *C. pneumoniae*, infected cells were washed twice and incubated for 0, 2, 4, 6, and 8 hours prior to total RNA isolation.

***Analysis of gene expression by real time quantitative PCR***

Total RNA was isolated from RAWs, H5Vs and carotid arteries using TRIzol reagent (Invitrogen, Netherlands) according to manufacturer's instructions. Two arteries were pooled resulting in n=3 per treatment per time point. Generation of cDNA, quantitative RT-PCR, calculations of ratios of target genes and the house hold gene, Hypoxanthine-guanine phosphoribosyl-transferase (HPRT), and the design of primers (table 1) were performed as previously described.[24]

***Data analysis***

Values are expressed as mean±sem. A 2-tailed Student's t-test was used to compare individual groups of mice or cells. Levels of $p<0.05$ were considered significant.

## Results

Local incubation of the right common carotid artery with *C. pneumoniae* did not affect total serum cholesterol levels (Fig.1C). Successful infection of the arterial wall with *C. pneumoniae* was confirmed by quantitative RT-PCR on the OMP A gene of *C. pneumoniae*. Every artery incubated with *C. pneumoniae* was highly positive for the OMP A gene and on average, $6.2x10^4$ *C. pneumoniae* copies were detected in bacteria incubated carotid arteries 7 days post local incubation. *C. pneumoniae* DNA was absent in lungs, livers and spleens of *C. pneumoniae* incubated mice and in right carotid arteries of control incubated mice.

***Effect of C. pneumoniae presence in the vessel wall on atherosclerosis***
After positive identification of the presence of *C. pneumoniae* we determined the effect of *C. pneumoniae* on *de novo* atherosclerosis in carotid arteries of $LDLr^{-/-}$ mice. Five weeks after local incubation of carotid arteries and simultaneous collar placement we determined the degree of atherosclerosis. Incubation of the carotid artery with *C. pneumoniae* resulted in 1.87-fold increased neointima area (Fig.1A,B,D; 57,711±10,943 vs 30,891±9,997 $\mu m^2$), which translated into markedly 2.03-fold increased intima/media ratios (Fig.1E; 1.27±0.23 vs 0.62±0.15, $p<0.05$) and a 1.74-fold increased degree of lumen stenosis (Fig.1F; 0.53±0.08 vs 0.30±0.07, $p<0.05$), as compared to SPG medium incubated arteries.

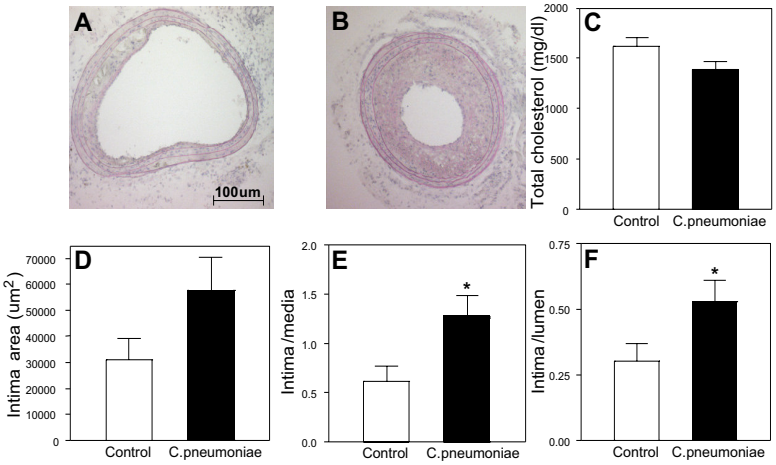


**Figure 1.** Five weeks after collar placement and simultaneous *C. pneumoniae* infection mice were sacrificed and carotid arteries were isolated for quantification of lesion formation. Representative cross-sections of collar induced plaques in carotid arteries of $LDLr^{-/-}$ mice are shown after incubation with *C. pneumoniae* (B) or SPG medium (A). During the experiment total serum cholesterol levels were monitored (C). Using computer assisted morphometric analysis we determined intima area (D), intima/media ratio (E), and intima/lumen ratio (F) after incubation with *C. pneumoniae* or SPG medium (*=$p<0.05$).

In comparison we performed similar experiments and incubated the carotid artery with *M. pneumoniae.* Incubation with *M. pneumoniae* did not affect the extent of atherosclerosis as compared to SPG medium incubated arteries, which was reflected by similar intima area, intima/media and intima/lumen ratios.

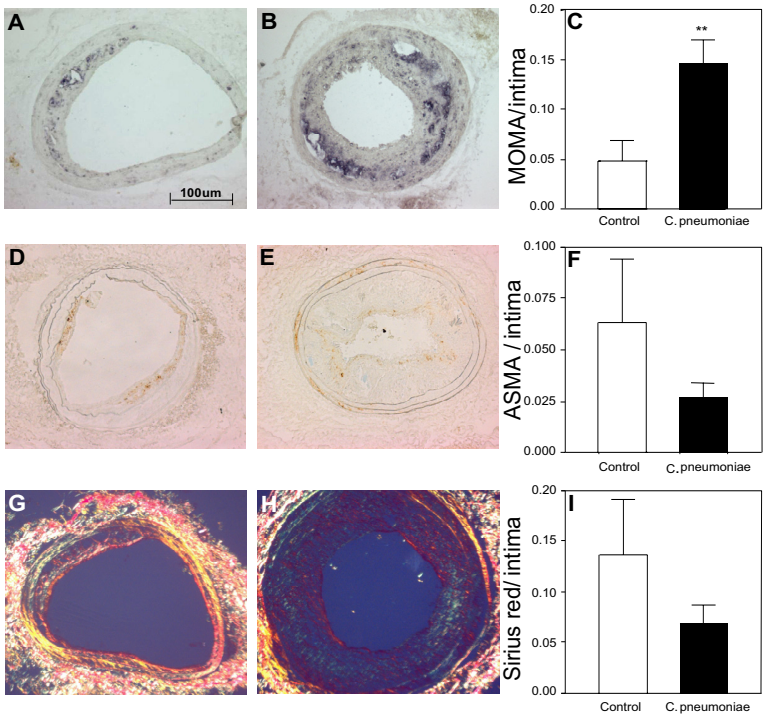


**Figure 2.** Immunohistochemical analysis of plaques developed upon *C. pneumoniae* incubation (B,E,H) or control incubation (A,D,G). Increased MOMA-2 staining per intima area was observed in bacteria incubated arteries (A,B,C). Both ASMA (D,E) and Picrosirius Red staining (G,H) per intima area were diminished in bacteria incubated arteries as compared to controls (F,I, respectively) (**= p<0.01)

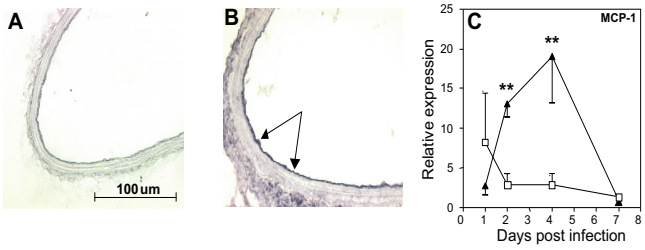


**Figure 3.** Analysis of inflammatory effects of *C. pneumoniae* on the arterial wall. Seven days post incubation antibody staining showed that *C. pneumoniae* in the arterial wall increased ICAM-1 expression (B, arrows) as compared to controls (A). Expression of MCP-1 in the arterial wall on mRNA level was increased markedly at day 2 and 4 after local incubation with *C. pneumoniae* (▲) as compared with control incubation (□). (C, **= p<0.01).

***Plaque morphology***

The composition of collar induced lesions was determined by histological and immunohistochemical staining techniques. The increased lesion size in *C. pneumoniae* infected arteries was found to be largely attributable to a significant 2.98-fold increased macrophage content of the lesion, as determined by MOMA-2 staining (Fig.2C; 0.145±0.025 vs 0.048±0.020, $p<0.01$). The higher macrophage content was accompanied by a decrease in smooth muscle cells and collagen. In *C. pneumoniae* infected carotid arteries a tendency towards less staining for alpha-smooth muscle cell actin per intima area was observed as compared with control incubated carotid arteries (Fig.2F; 0.027±0.007 vs 0.064±0.031), which was in parallel with the lower collagen content after *C. pneumoniae* incubation (Fig.2I; 0.136±0.055 vs 0.069±0.018). Again incubation with *M. pneumoniae* did not change the composition of the atherosclerotic plaques as compared to SPG medium incubated carotid arteries.

***Inflammatory effect on the vessel wall***

Since *M. pneumoniae* in no way affected the size or composition of the plaque we subsequently focused on the effect of the presence of *C. pneumoniae* on the expression of inflammatory markers in the vessel wall both at mRNA and protein level in $LDLr^{-/-}$ mice without induction of atherosclerosis. The presence of *C. pneumoniae* in the vessel wall after local incubation resulted seven days later in a marked increase in ICAM-1 expression at protein level. Increased ICAM-1 staining was mainly located at the endothelial cell layer (Fig.3A,B). Furthermore, at day 2 and 4 after local incubation with *C. pneumoniae* mRNA levels of MCP-1 in *C. pneumoniae* incubated carotids appeared to be significantly elevated 4.64- ($p<0.01$) and 6.79-fold ($p<0.01$), respectively (Fig.3C), whereas no changes in mRNA levels for ICAM-1, VCAM-1, E-selectin, IL-6, IL-12, and Tissue Factor (TF) were observed.

***Effect of C. pneumoniae on acute arterial thrombosis***

The effect of *C. pneumoniae* in the vessel wall on the thrombogenicity of the carotid artery was assessed using a $FeCl_3$-induced acute arterial thrombosis model. Seven days after infection of the carotid artery with *C. pneumoniae* no significant differences in time to occlusion after thrombus induction were observed between SPG medium and bacteria incubated arteries (1001±138 vs 1147±164 sec, respectively).

***In vitro effect of C. pneumoniae on macrophages and endothelial cells***

To further elucidate by which mechanisms *C. pneumoniae* is capable of stimulating atherogenesis, effects of *C. pneumoniae* on different cell types, present in the vessel wall, such as ECs and macrophages were evaluated at mRNA level. The initial level of expression was measured at t=0 hrs post infection, which represents the time point immediately after 1 hour of centrifugation plus 1 hour of incubation with *C. pneumoniae.* Infection of H5Vs with *C. pneumoniae* showed an inflammatory response, reflected in

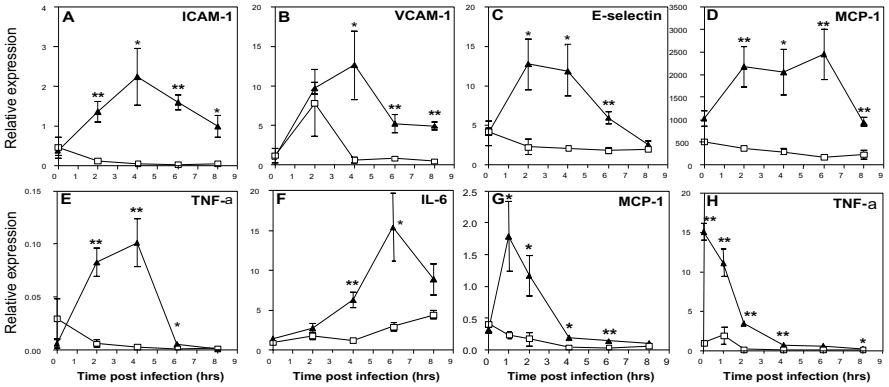


**Figure 4.** Effects of *C. pneumoniae* on H5Vs (A-F) or RAWs (G,H). Cells were incubated with *C. pneumoniae* (▲) or SPG medium (□) for 2 hours, including 1 hour of centrifugation at 800xg. Subsequently, cells were washed and incubated with medium prior to RNA isolation at indicated timepoints. Quantitative RT-PCR revealed that expression levels of several adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin were markedly elevated upon infection of H5Vs (A-C). Infection of H5Vs also caused an upregulation of MCP-1, TNF-α and IL-6 (D-F). Expression levels of MCP-1 and TNF-α were also elevated in RAWs upon infection with *C. pneumoniae* (*= $p<0.05$, **= $p<0.01$).

increased mRNA levels for MCP-1, for a number of adhesion molecules (E-selectin, ICAM-1, and VCAM-1) and for the cytokines TNF-α and IL-6. Peak values were observed at 2-6 hrs post infection (Fig.4A-F). At 8 hrs post infection differences in expression levels between control and infected cells were smaller, while cell viability did not change. In RAWs, TNF-α and MCP-1 expression levels were elevated upon infection (Fig.4G,H) and reached a maximum within the first hour, which was earlier than in H5Vs.

## Discussion

In the present study, we investigated how direct infection of the vessel wall with *C. pneumoniae* affects atherosclerosis and thrombogenicity of the carotid artery. Although many groups have studied effects of lung infection with *C. pneumoniae* on atherosclerosis, little is known about the direct effect of *C. pneumoniae* in the vessel wall on this inflammatory process. Local administration of *C. pneumoniae* in coronary arteries of pigs or rabbits has been reported,[25,26] but was never performed in atherosclerosis prone mice. Therefore, we established a novel model in which *C. pneumoniae* is delivered from the luminal side of the carotid artery to the vessel wall. This model mimics the delivery of *C. pneumoniae* to the arterial wall by infected monocytes and subsequent infection of arterial wall cells by *C. pneumoniae* after proliferation within macrophages. Using this model we can be sure that

the arterial wall is infected. Therefore, this model may be preferred to study the *in vivo* effect of *C. pneumoniae* on the arterial wall, since in previous publications effects of *C. pneumoniae* on atherosclerosis via airway infection were not always accompanied by the positive identification of *C. pneumoniae* in the arterial wall. The fact that infection via the respiratory tract does not per se lead to positive infection of the arterial wall with *C. pneumoniae* may explain some of the conflicting results on the effects of *C. pneumoniae* in atherosclerosis prone mice. Furthermore, this local incubation model offers the opportunity to study the role of *C. pneumoniae* in the arterial wall without interference of indirect effects, such as effects on lipid metabolism or on systemic inflammation.

By means of local intra-vascular incubation with *C. pneumoniae* in the common carotid artery it is possible to introduce these bacteria in the vessel wall, which was confirmed by detecting the *C. pneumoniae* specific Omp A gene in the vessel wall 7 days after incubation in all *C. pneumoniae* incubated arteries. This detection method circumvents the problems that are met when using immunohistochemistry, in which antigens may be missed while cutting sections or when cross-reactivity with non-chlamydial plaque constituents may occur.[27] In addition, by detecting *C. pneumoniae* specific DNA it is possible to quantify the number of bacteria that infected the arterial wall. While *C. pneumoniae* was detected in high amounts in the arterial wall of incubated arteries, these bacteria were not detected in the lungs, liver and spleen of the mice that were locally incubated with *C. pneumoniae*, indicating the lack of systemic infection upon local incubation. Since ECs form the first barrier that has to be past and *C. pneumoniae* is able to infect these cells, both *in vivo* and *in vitro*, and replicate inside ECs,[28,29] entrance of the elementary bodies seems to occur via infection of ECs. Although this model not entirely mimics the physiological situation, in which bacteria are presumed to enter the vessel wall primarily while residing in monocytes and in which relatively less *C. pneumoniae* is found in the atherosclerotic plaque, it offers the opportunity to study effects of the actual presence of *C. pneumoniae* in the vessel wall.

With this local incubation model we demonstrate that *C. pneumoniae* in the vessel wall enhances collar-induced atherosclerosis in carotid arteries of $LDLr^{-/-}$ mice. Larger lesions were observed in bacteria incubated arteries, which translated into significant larger intima/media and intima/lumen ratios. These results indicate that presence of *C. pneumoniae* in the arterial wall creates inflammatory stimuli that enhance atherosclerosis and that systemic inflammation as induced by airway infection with *C. pneumoniae* is not a necessary stimulation for atherosclerosis development. In contrast, infection of the carotid artery with *M. pneumoniae,* a mild respiratory pathogen that has also been implicated in atherosclerosis, did not affect the size and composition of the atherosclerotic lesions in $LDLr^{-/-}$ mice.

In order to further evaluate the role of *C. pneumoniae* in different processes involved in atherosclerosis, lesion composition was elucidated by immunohistochemistry. Although larger lesions usually contain relatively less macrophages than smaller lesions, in this experiment the larger lesions in the

bacteria incubated group contained relatively more macrophages. Presence of mononuclear infiltrates in vascular tissue upon infection with *C. pneumoniae* was studied previously. However, the outcome of these studies was not univocal.[30-33] The higher macrophage content observed after *C. pneumoniae* infection is in line with our *in vitro* and *in vivo* data, which showed elevated expression levels of ICAM-1 and MCP-1 upon infection, which can result in increased adherence of circulating monocytes to the endothelium and attraction of these cells into the intima. Furthermore, the finding that *C. pneumoniae* infection of monocytes can rescue these cells from apoptosis can also contribute to the increased macrophage content.[34]
Lesions developed after *C. pneumoniae* incubation showed a tendency towards decreased $\alpha$-actin and Picrosirius Red staining, which is indicative for reduced numbers of VSMCs and reduced collagen content, respectively. *In vitro* studies have shown that proliferation of SMCs can be stimulated by *C. pneumoniae* both directly and indirectly.[35,36] However, conditioned medium from *C. pneumoniae* infected SMCs inhibits proliferation of SMCs through induction of prostaglandin $E_2$.[37] The sum of the *in vitro C. pneumoniae* induced effects is not well documented *in vivo.* Distinct observations have been reported in which both stimulatory and inhibiting actions of *C. pneumoniae* on SMCs proliferation were suggested.[38,39,25] Our study makes a net stimulatory effect of *C. pneumoniae* on SMCs *in vivo* less likely. Since collagen is mainly produced by the VSMCs in atherosclerotic lesions, the observed trend in the amount of collagen in the *C. pneumoniae* incubated carotids fits with the decreased amount of SMCs observed in the atherosclerotic lesions. Several groups have reported that upon infection with *C. pneumoniae* both macrophages and SMCs produce MMPs,[40,14] which are able to degrade collagen. Previously, enhanced MMP-2 and MMP-9 production was observed in atherosclerotic lesions upon infection with *C. pneumoniae* in LDLr/ApoE double knock out mice.[39] This lesion morphology with more macrophages and the lack of compensatory elevated amounts of VSMCs and collagen points to a net destabilizing effect of *C. pneumoniae*.
Presence of *C. pneumoniae* in the vessel wall resulted in enhanced expression of MCP-1 at mRNA level. It remained unclear from these experiments to which cell types this increased expression can be contributed. However, since both ECs, macrophages and VSMCs[41] *in vitro* show elevated expression of MCP-1 upon infection with *C. pneumoniae* they can be all involved in this response. Immunohistochemistry revealed increased ICAM-1 protein expression mainly on ECs, which, combined with the found elevated MCP-1 expression level, could contribute to the increased macrophage content, as observed in infected lesions. The lack of increased expression of ICAM-1 at mRNA level could be explained by the fact that expression levels at mRNA level were not specifically measured in the endothelium, but in the total vessel wall, which may dilute the ICAM-1 signal.
*In vitro* studies have shown that *C. pneumoniae* increases the expression of the pro-coagulant proteins TF and plasminogen activator inhibitor-1 in ECs, SMCs and macrophages,[42,43] and accelerates clotting and phosphatidylserine exposure in several cell types.[44] However, in our *in vivo* model, in which

acute thrombus formation was induced by peri-arterial application of $FeCl_3$, no pro-thrombotic effect was observed 7 days after delivery *of C. pneumoniae* to the arterial wall. In addition, no upregulation of mRNA levels for TF were detected in the vessel wall during the first week after delivery of *C. pneumoniae*. These data suggest that previously shown *in vitro* indications for increased thrombogenicity upon infection with *C. pneumoniae* may be compensated by antithrombotic actions of *C. pneumoniae in vivo,* such as induction of the anti-thrombotic cytokine IL-10,[34,45] resulting in the absence of a significant effect of *C. pneumoniae* in the vessel wall on thrombogenicity.
*In vitro* infections of mouse ECs and macrophages with *C. pneumoniae* were performed to support the results observed *in vivo*. As demonstrated previously, infection of ECs induced an inflammatory response marked by elevated mRNA levels of IL-6, TNF-$\alpha$, E-selectin, ICAM-1, and VCAM-1. Combined with the elevated MCP-1 expression in both macrophages and ECs, these data suggest that *C. pneumoniae* promotes monocyte adhesion and migration to the inner layers of the vessel wall, which is in accordance with the *in vivo* results.
In conclusion, this study demonstrates that direct infection of the carotid artery with *C. pneumoniae* enhances atherosclerosis in a novel model. *C. pneumoniae* affects the composition of the developing lesions towards a more vulnerable morphology via an increase in macrophage content, which was not observed after infection of the carotid artery with *M. pneumoniae*. Therefore, this study supports the specific contribution of *C. pneumoniae* to the progression of atherosclerosis. Enhanced macrophage content caused the major increase in lesion formation, and chemokines contributed to this increase. This local delivery model offers the opportunity to further elucidate the site-specific influences of *C. pneumoniae* on atherosclerosis.

## References

1. Ross R. Atherosclerosis-an inflammatory disease. N Engl J Med. 1999;340:115-26.
2. Grayston JT. Infections caused by Chlamydia pneumoniae strain TWAR. Clin Infect Dis. 1992;15:757-61.
3. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, et al. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet. 1988;2:983-6.
4. Moazed TC, Kuo CC, Grayston JT, Campbell LA. Evidence of systemic dissemination of Chlamydia pneumoniae via macrophages in the mouse. J Infect Dis. 1998;177:1322-5.
5. Kuo CC, Shor A, Campbell LA, Fukushi H, Patton DL, Grayston JT. Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. J Infect Dis. 1993;167:841-9.
6. Muhlestein JB. Antibiotic treatment of atherosclerosis. Curr Opin Lipidol. 2003;14:605-14.
7. Kaukoranta-Tolvanen SS, Ronni T, Leinonen M, Saikku P, Laitinen K. Expression of adhesion molecules on endothelial cells stimulated by Chlamydia pneumoniae. Microb Pathog. 1996;21:407-11.

8. Molestina RE, Miller RD, Ramirez JA, Summersgill JT. Infection of human endothelial cells with Chlamydia pneumoniae stimulates transendothelial migration of neutrophils and monocytes. Infect Immun. 1999;67:1323-30.
9. Coombes BK, Mahony JB. cDNA array analysis of altered gene expression in human endothelial cells in response to Chlamydia pneumoniae infection. Infect Immun. 2001;69:1420-7.
10. Kalayoglu MV, Perkins BN, Byrne GI. Chlamydia pneumoniae-infected monocytes exhibit increased adherence to human aortic endothelial cells. Microbes Infect. 2001;3:963-9.
11. May AE, Redecke V, Gruner S, Schmidt R, Massberg S, Miethke T, et al. Recruitment of Chlamydia pneumoniae-infected macrophages to the carotid artery wall in noninfected, nonatherosclerotic mice. Arterioscler Thromb Vasc Biol. 2003;23:789-94.
12. Kalayoglu MV, Hoerneman B, LaVerda D, Morrison SG, Morrison RP, Byrne GI. Cellular oxidation of low-density lipoprotein by Chlamydia pneumoniae. J Infect Dis. 1999;180:780-90.
13. Dittrich R, Dragonas C, Mueller A, Maltaris T, Rupp J, Beckmann MW, et al. Endothelial Chlamydia pneumoniae infection promotes oxidation of LDL. Biochem Biophys Res Commun. 2004;319:501-5.
14. Rodel J, Prochnau D, Prager K, Pentcheva E, Hartmann M, Straube E. Increased production of matrix metalloproteinases 1 and 3 by smooth muscle cells upon infection with Chlamydia pneumoniae. FEMS Immunol Med Microbiol. 2003;38:159-64.
15. Campbell LA, Rosenfeld M, Kuo CC. The role of Chlamydia pneumoniae in atherosclerosis--recent evidence from animal models. Trends Microbiol. 2000;8:255-7.
16. Caligiuri G, Rottenberg M, Nicoletti A, Wigzell H, Hansson GK. Chlamydia pneumoniae infection does not induce or modify atherosclerosis in mice. Circulation. 2001;103:2834-8.
17. Higuchi ML, Sambiase N, Palomino S, Gutierrez P, Demarchi LM, Aiello VD, et al. Detection of Mycoplasma pneumoniae and Chlamydia pneumoniae in ruptured atherosclerotic plaques. Braz J Med Biol Res. 2000;33:1023-6.
18. Taylor-Robinson D. Chlamydia pneumoniae infection and coronary heart disease. Trials should assess whether antibiotics eliminate organism from atherosclerotic lesions. BMJ. 1997;315:1538.
19. Verkooyen RP, Harreveld S, Ahmad Mousavi Joulandan S, Diepersloot RJ, Verbrugh HA. Survival of Chlamydia pneumoniae following contact with various surfaces. Clin Microbiol Infect. 1995;1:114-118.
20. von der Thusen JH, van Berkel TJ, Biessen EA. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. Circulation. 2001;103:1164-70.
21. von der Thusen JH, van Vlijmen BJ, Hoeben RC, Kockx MM, Havekes LM, van Berkel TJ, et al. Induction of atherosclerotic plaque rupture in apolipoprotein E-/- mice after adenovirus-mediated transfer of p53. Circulation. 2002;105:2064-70.
22. Weiler H, Lindner V, Kerlin B, Isermann BH, Hendrickson SB, Cooley BC, et al. Characterization of a mouse model for thrombomodulin deficiency. Arterioscler Thromb Vasc Biol. 2001;21:1531-7.
23. Jantos CA, Heck S, Roggendorf R, Sen-Gupta M, Hegemann JH. Antigenic and molecular analyses of different Chlamydia pneumoniae strains. J Clin Microbiol. 1997;35:620-3.
24. Hoekstra M, Kruijt JK, Van Eck M, Van Berkel TJ. Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. J Biol Chem. 2003;278:25448-53.

25. Pislaru SV, Van Ranst M, Pislaru C, Szelid Z, Theilmeier G, Ossewaarde JM, et al. Chlamydia pneumoniae induces neointima formation in coronary arteries of normal pigs. Cardiovasc Res. 2003;57:834-42.
26. Muhlestein JB. Chlamydia pneumoniae-induced atherosclerosis in a rabbit model. J Infect Dis. 2000;181 Suppl 3:S505-7.
27. Hoymans VY, Bosmans JM, Ursi D, Martinet W, Wuyts FL, Van Marck E, et al. Immunohistostaining assays for detection of Chlamydia pneumoniae in atherosclerotic arteries indicate cross-reactions with nonchlamydial plaque constituents. J Clin Microbiol. 2004;42:3219-24.
28. Laitinen K, Laurila A, Pyhala L, Leinonen M, Saikku P. Chlamydia pneumoniae infection induces inflammatory changes in the aortas of rabbits. Infect Immun. 1997;65:4832-5.
29. 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;64:1614-20.
30. Neureiter D, Heuschmann P, Stintzing S, Kolominsky-Rabas P, Barbera L, Jung A, Ocker M, et al. Detection of Chlamydia pneumoniae but not of Helicobacter pylori in symptomatic atherosclerotic carotids associated with enhanced serum antibodies, inflammation and apoptosis rate. Atherosclerosis. 2003;168:153-62.
31. Aalto-Setala K, Laitinen K, Erkkila L, Leinonen M, Jauhiainen M, Ehnholm C, et al. Chlamydia pneumoniae does not increase atherosclerosis in the aortic root of apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2001;21:578-84.
32. Campbell LA, Blessing E, Rosenfeld M, Lin T, Kuo C. Mouse models of C. pneumoniae infection and atherosclerosis. J Infect Dis. 2000;181 Suppl 3:S508-13.
33. Davidson M, Kuo CC, Middaugh JP, Campbell LA, Wang SP, Newman WP 3rd, et al. Confirmed previous infection with Chlamydia pneumoniae (TWAR) and its presence in early coronary atherosclerosis. Circulation. 1998;98:628-33.
34. Geng Y, Shane RB, Berencsi K, Gonczol E, Zaki MH, Margolis DJ, et al. Chlamydia pneumoniae inhibits apoptosis in human peripheral blood mononuclear cells through induction of IL-10. J Immunol. 2000;164:5522-9.
35. Coombes BK, Mahony JB. Chlamydia pneumoniae infection of human endothelial cells induces proliferation of smooth muscle cells via an endothelial cell-derived soluble factor(s). Infect Immun. 1999;67:2909-15.
36. Sasu S, LaVerda D, Qureshi N, Golenbock DT, Beasley D. Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. Circ Res. 2001;89:244-50.
37. Rodel J, Prochnau D, Prager K, Baumert J, Schmidt KH, Straube E. Chlamydia pneumoniae decreases smooth muscle cell proliferation through induction of prostaglandin E2 synthesis. Infect Immun. 2004;72:4900-4.
38. Coombes BK, Chiu B, Fong IW, Mahony JB. Chlamydia pneumoniae infection of endothelial cells induces transcriptional activation of platelet-derived growth factor-B: a potential link to intimal thickening in a rabbit model of atherosclerosis. J Infect Dis. 2002;185:1621-30.
39. Ezzahiri R, Stassen FR, Kurvers HA, van Pul MM, Kitslaar PJ, Bruggeman CA. Chlamydia pneumoniae infection induces an unstable atherosclerotic plaque phenotype in LDL-receptor, ApoE double knockout mice. Eur J Vasc Endovasc Surg. 2003;26:88-95.
40. Kol A, Sukhova GK, Lichtman AH, Libby P. Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression. Circulation. 1998;98:300-7.

41. Dechend R, Gieffers J, Dietz R, Joerres A, Rupp J, Luft FC, et al. Hydroxymethylglutaryl coenzyme A reductase inhibition reduces Chlamydia pneumoniae-induced cell interaction and activation. Circulation. 2003;108:261-5.
42. Dechend R, Maass M, Gieffers J, Dietz R, Scheidereit C, Leutz A, et al. Chlamydia pneumoniae infection of vascular smooth muscle and endothelial cells activates NF-kappaB and induces tissue factor and PAI-1 expression: a potential link to accelerated arteriosclerosis. Circulation. 1999;100:1369-73.
43. Bea F, Puolakkainen MH, McMillen T, Hudson FN, Mackman N, Kuo CC, et al. Chlamydia pneumoniae induces tissue factor expression in mouse macrophages via activation of Egr-1 and the MEK-ERK1/2 pathway. Circ Res. 2003;92:394-401.
44. Goth SR, Stephens RS. Rapid, transient phosphatidylserine externalization induced in host cells by infection with Chlamydia spp. Infect Immun. 2001;69:1109-19.
45. Caligiuri G, Rudling M, Ollivier V, Jacob MP, Michel JB, Hansson GK, et al. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. Mol Med. 2003;9:10-7.