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The acute phase reactant response to respiratory infection with *Chlamydia pneumoniae*: implications for the pathogenesis of atherosclerosis

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Abstract

The acute phase response to *C. pneumoniae* infection was analyzed over a 72 hr period post-infection in C57BL/6J mice. A single intranasal inoculation stimulated statistically significant increases in the plasma levels of IL-2, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN- γ , and serum amyloid A but not TNF- α , IL-1 β , IL-4 and serum amyloid P. There was also a decrease in the activity of the HDL protective enzyme paraoxonase as well as a reduced ability of HDL to prevent oxidation of palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphocholine by hydroperoxyoctadecadienoic acid at 48 and 72 hrs post-infection. To determine whether the *C. pneumoniae* induced acute phase response had any effect on atherosclerotic plaque stability, we measured the frequency of intra-plaque hemorrhage as a marker of plaque disruption in the innominate arteries of apolipoprotein E deficient mice at 29–30 weeks and 1.5–2.0 years of age. There was an increased frequency of intra-plaque hemorrhage only in the older mice infected with the live organism (8/14) as compared to mice treated with killed *C. pneumoniae* (2/11) or sham inoculated with PBS (2/12). These results suggest that acute phase reactant proteins produced in response to pulmonary infection with *C. pneumoniae* may contribute to the progression and destabilization of atherosclerotic lesions.

Keywords

Chlamydia pneumoniae; Inflammation; Cytokines; Mice

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1. Introduction

Chlamydia pneumoniae is an obligate intracellular bacterium that causes acute and chronic respiratory disease in humans including; sinusitis, pharyngitis, bronchitis, and pneumonia [1]. In addition, *C. pneumoniae* may be a risk factor for immuno-reactive disorders such as adult onset asthma, reactive airway disease in children, and reactive arthritis [1]. *C. pneumoniae* infection has also been associated with an increased risk of cardiovascular disease [2]. In younger apolipoprotein E deficient mice (apo E^{-/-}), which develop atherosclerosis spontaneously and in C57BL/6J mice fed an atherogenic diet, *C. pneumoniae* infection accelerates fatty streak formation [2].

Atherosclerosis is a disease of chronic inflammation. Pro-inflammatory cytokines such as TNF- α and IL-6 and other acute phase proteins, including C-reactive protein (CRP) and serum amyloid A (SAA) have been associated with progression of atherosclerosis and/or with increased risk of cardiovascular disease [3–5]. Pro-inflammatory cytokines induced by *C. pneumoniae* likely play an important role in the pathogenesis of both acute respiratory infection and chronic inflammatory diseases such as atherosclerosis [6–8]. CRP and serum amyloid P (SAP), the mouse equivalent of CRP, are acute phase proteins that are members of the pentraxin family [9]. These acute phase reactants as well as SAA are produced in the liver in response to inflammatory mediators such as IL-6 [9]. SAA proteins associate with HDL and may alter the atheroprotective functions of HDL [10]. For example, it has been proposed that SAA binding to HDL leads to the loss from HDL of protective enzymes such as paraoxonase (PON) and platelet activating factor acetylhydrolase (PAFH), which results in loss of the anti-inflammatory properties of HDL with respect to LDL oxidation [11]. Furthermore, during the acute phase response in humans after surgery, rabbits after corn oil injection, and mice following Influenza A infection, the loss of PON and PAFH occur concomitant with a marked increase in SAA levels [10]. *In vitro*, *C. pneumoniae* infection of human endothelial cells and monocytes results in increased secretion of cytokines including MCP-1, IL-8, IL-1 β , IL-6, and TNF- α [12–14]. However, previous studies have not provided plasma profiles of both pro- and anti-inflammatory cytokines and other acute phase reactants or the time course of secretion following acute infection *in vivo*. It is also currently unknown whether acute infection with *C. pneumoniae* alters the composition or stability of clinically relevant advanced atherosclerotic lesions. Over the past decade, we have studied the pathology of advanced lesions in the innominate artery of older chow-fed apoE^{-/-} mice. The innominate artery, also called the brachiocephalic trunk, is a small segment of artery at the base of the right carotid artery that has very irregular patterns of blood flow and a predilection for lesion development. We have shown that, comparable to the human disease, lesions with a large central necrotic core form at this site and with time become fibrotic, a-cellular and calcified plaques. We have also shown that there is plaque disruption leading to intra-plaque hemorrhage [15,16].

In this study, we utilized a multiplex cytokine array system to generate a more complete cytokine profile and determined the time course of secretion of cytokines and other acute phase proteins following acute pulmonary infection of C57BL/6 and apoE^{-/-} mice with *C. pneumoniae*. We also compared the effects of acute *C. pneumoniae* infection on the frequency of intra-plaque hemorrhage in atherosclerotic lesions in the innominate arteries of chow-fed apo E^{-/-} mice at 30 weeks of age with very old mice at 1.5–2 years of age.

2. Materials and Methods

2.1. Mouse Models

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME), and male and female apo E^{-/-} mice on a C57BL/6J background (bred in our animal facility) were housed in a specific pathogen free facility and fed normal chow and water *ad-libitum*. C57BL/6J mice were used

in this study to provide consistency with our previous studies of the effect of *C. pneumoniae* infection on atherosclerosis development [2]. *C. pneumoniae* strain AR-39, a respiratory isolate, was propagated in HL cells and purified by density gradient centrifugation with Hypaque®-76 (Nycomed Inc. Princeton, NJ) [17]. Purified organisms were resuspended in sucrose-phosphate-glutamic acid buffer (0.2 M sucrose, 3.8 mM KH₂PO₄, 6.7 mM Na₂HPO₄, and 5 mM L-glutamic acid, pH 7.4).

The mice were sedated by intra-peritoneal injection of a mixture of ketamine HCl (KETALECT® 100 mg/ml) and xylazine (Xyla-Ject® 20 mg/ml, Phoenix Pharmaceutical Inc, St. Joseph, MO). For determination of the plasma cytokine response, one group of mice (n=35) was infected via intra-nasal inoculation with 3×10^7 inclusion forming units (IFU) of *C. pneumoniae* strain AR-39 in 30 µl of PBS. A second group was sham infected with PBS (n=35), and a third group received no treatment in order to determine the baseline levels of plasma cytokines and other acute phase reactant proteins (n=5). For determination of plasma SAA and SAP, eight week old C57BL/6J mice were infected with *C. pneumoniae* or sham inoculated (n=20 mice per group). SAA and SAP were also measured in plasma from an additional group of older apoE^{-/-} mice, (24–52 weeks, male and female) that were infected with *C. pneumoniae* in PBS, heat inactivated *C. pneumoniae*, or PBS alone as part of a previous study. For determination of the effect of *C. pneumoniae* infection on the protective effects of HDL, eight week old C57BL/6J mice were infected as described above and blood was collected at 2, 3, 5, 7, and 9 days post-infection (n=6 per time point). For the studies of the effects of acute infection on the frequency of intra-plaque hemorrhage, 29 week old male mice (n = 15–23) were administered 1×10^7 IFU of live or heat killed *C. pneumoniae* (strain AR-39) or sham inoculated with PBS via intra-nasal inoculation twice and sacrificed one week post the second inoculation. Additional male and female mice that were between 75 and 102 weeks of age (n = 11–14) were inoculated once with 1×10^7 IFU of live or heat killed *C. pneumoniae* or sham inoculated with PBS and sacrificed at 4 or 7 days post inoculation. The differences in the number of inoculations and sacrifice schedule were necessitated by an unexpected increase in mortality of the older mice while infected with *C. pneumoniae*. Necropsies were not performed on the mice that died spontaneously, so it is not known whether their deaths were related to the disruption of the atherosclerotic plaques in the innominate arteries. Furthermore, the mice that survived the inoculation did not display any behavior consistent with cerebral ischemia. All animal protocols for this study were approved by the Institutional Animal Care and Use Committee of the University of Washington.

2.2. Sample collection and analysis of acute phase reactant proteins

Blood samples were obtained by cardiac puncture or via the retro-orbital sinus and were immediately centrifuged at 4°C. The plasma was stored at –70°C until assayed. The Beadlyte Multi-Cytokine Detection System (Upstate) was utilized to generate the plasma profiles for the cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), TNF-α, IFN-γ, GM-CSF. The assays were run in duplicate and averaged. SAA and SAP determinations were performed by ELISA as previously described [5]. Plasma total cholesterol levels were determined with a colorimetric kit (Diagnostic Chemicals Ltd) with cholesterol standards (Sigma). The mean ± standard deviation for each group at the different time points were determined and the mean of each group was compared by the two-sample, two-tailed Student's t-test with equal variance. A value of $P < 0.05$ was considered statistically significant.

2.3. Analysis of PON activity and the protective effects of HDL

2.3.1. PON activity—The PON assay tested neat plasma samples, or plasma HDL separated and pooled by size using fast phase liquid chromatography (FPLC) over two Superose 6 columns (Pharmacia) arranged in tandem. Samples were assayed for PON activity using paraoxon as substrate (1.0 mM paraoxon in 20 mM Tris/ HCl, pH 8.0), [18]. The reaction was

initiated by the addition of the plasma or lipoprotein sample, and the increase in the absorbance at 405 nm due to the liberation of 4-nitrophenol was recorded kinetically every 20 seconds for a 12 minute period. Blanks were included to correct for the spontaneous hydrolysis of paraoxon. Enzymatic activity was calculated from the molar extinction coefficient 1310 M.

2.3.2. Dichlorofluorescein (DCF) assay—Experiments investigating the effect of HDL in preventing the oxidation of palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphocholine (PAPC) by hydroperoxyoctadecadienoic acid (HPODE), were done as described by Navab et al. [19]. 2',7'-dichlorofluorescein diacetate (DCFH-DA) was dissolved in fresh methanol at 2.0 mg/ml and was incubated at room temperature and protected from light for 30 min. On interaction with lipid oxidation products DCFH forms the fluorescent DCF. Fluorescence intensity was determined with a Farrand (Valhalla, NY) system 3 scanning spectrofluorometer set at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. A sensitivity level of 0.1 and slit widths of 2.5 and 10 nm were used for excitation and emission, respectively. Ten μ l aliquots of DCFH in methanol were placed in polypropylene tubes and the methanol was evaporated under argon. Ten μ l of PAPC at 2.5 mg/ml in chloroform was placed in each tube, mixed, and evaporated under argon. Subsequently 10 μ l of HPODE at 0.1 mg/ml in ethanol was added and evaporated under argon. Twenty-five μ l of saline or HDL at 350 μ g/ml was added to each tube, the volume was adjusted to 1.0 ml with saline, and the mixture was vortexed for 1 min and incubated at room temperature in the dark. Fluorescence readings were obtained at the indicated time points.

2.4. Sacrifice and tissue preparation for the analysis of the frequency of intra-plaque hemorrhage

The mice were euthanized with Ketamine/Xylaject (I.P.) and perfused at physiological pressure with PBS followed by 10% buffered formalin through the left ventricle. The innominate artery was dissected out, embedded in paraffin, and serially sectioned (5 μ m). Five consecutive sections were placed on a glass microscope slide. A total of 30 to 40 slides were generated for each lesion and every fifth slide was stained with a modified Movat's pentachrome stain [20]. To determine the presence of calcification, adjacent slides were stained with the von Kossa stain that detects hydroxyapatite [21]. We chose to focus these studies solely on the innominate artery because in our experience, lesions with intra-plaque hemorrhage do not readily form in the aortic sinus or descending aorta in the chow-fed apo E^{-/-} mice.

2.5. Morphometric analysis and analysis of lesion composition

The cross sectional area of the lumen and intima was measured in each Movat's stained section using computer assisted morphometric analysis (Image Pro, Media Cybernetics, Silver Spring, MD). The data was calculated as the average of all of the measured sections. Plaque composition and stability were evaluated by assessing the frequency, within each group of mice, of the following features: thin fibrous cap (a thin fibrous cap was defined as ≤ 3 cell layers), presence of a large necrotic core (defined as occupying $>50\%$ of the plaque volume), intra-plaque hemorrhage (defined as the presence of red blood cells), medial expansion (defined as the replacement of normal media with plaque components), chondrocyte like cells (defined on the basis of the morphology and verified previously using cell type specific markers [22]), the presence of lateral xanthomas/fatty streaks (defined as aggregates of macrophage-derived foam cells and lymphocytes situated on the lateral margins of the plaques) and the presence of calcification. All data collection was done with the operator blinded to the treatment groups.

2.6. Statistical Analysis

Comparisons of lesion and lumen areas were performed by two-tailed unpaired Student's T-test (Intercooled Stata 8). Frequency measures were compared using Chi-squared analysis and the Mann-Whitney test. Significance is denoted by $P < 0.05$.

3. Results

3.1. Cytokine response to infection

Inoculation with *C. pneumoniae* stimulated significant increases in the plasma levels of IL-2, IL-5, IL-6, IL-10, IL-12, GM-CSF and IFN- γ in comparison to the PBS sham inoculated mice (Table 1). In contrast, inoculation with *C. pneumoniae* did not significantly increase the plasma levels of TNF- α , IL-4 or IL-1 β (Table 1). Intranasal administration of PBS alone irritated the mice and stimulated a transient increase in all of the cytokines as compared to non-treated controls. The transient increases lasted for between 3 hrs and 6 hrs depending on the cytokine (Fig. 1 and data not shown). For this reason, an overall increase in cytokine levels in the infected mice was not calculated but increases were determined by comparison to the sham inoculated mice at each individual time point. The maximum statistically significant increase ranged from 5.6 fold to 77 fold for IFN- γ and IL-12 respectively. The time post-inoculation for the maximum fold increase varied with the different cytokines (Table 1, Fig. 1, and data not shown). Fig. 1 provides examples of the time course for two cytokines that showed statistically significant increases following infection (IL-10 and IL-12) and two which did not (TNF- α and IL-4).

3.2. SAA but not SAP levels increase following infection

Inoculation of normolipidemic C57BL/6J mice with *C. pneumoniae* significantly increased plasma SAA at 72 hrs post infection, but did not increase plasma SAP (data not shown), (Fig. 2A). The effect of acute infection of hyperlipidemic apo E $-/-$ mice was also investigated. As shown in Fig. 2B, statistically significant increases were observed in plasma SAA at 24, 48, and 72 hrs, post-infection in comparison to sham inoculated controls. The *C. pneumoniae* stimulated SAA production was abrogated in heat killed organism at 48 and 72 hr post-infection. SAP levels did not increase at any time analyzed post infection in the apo E $-/-$ mice (data not shown).

3.3 Infection results in loss of anti-inflammatory properties of HDL

Because SAA proteins associate with HDL and may change the protective functions of HDL, the activity of the HDL associated enzyme PON was determined. In addition, the ability of HDL to prevent oxidation of PAPC by HPODE was also measured. As shown in Fig. 3, there were statistically significant decreases in PON activity at 48 and 72 hrs post-infection. This was accompanied by a loss in the ability of HDL to prevent the formation of oxidized lipids at 72 hr (Fig. 4). These decreases in the protective activities of HDL following acute *C. pneumoniae* infection occurred concomitantly with the increase in plasma SAA.

3.4 Infection is associated with an increased frequency of intra-plaque hemorrhage in the older mice

There were no significant differences between infected and control groups of mice with regard to plasma cholesterol levels (average of all infected mice = 380 ± 80 mg/dl versus sham infected = 335 ± 43 mg/dl). There was no effect of infection on lesion area (data not shown). The frequency of features of advanced plaque composition was also tabulated in the step sections from each mouse and recorded as binomial measures. These included the frequency of thin fibrous caps, large central necrotic cores, presence of red blood cells, calcification, chondrocytes, medial expansion, and presence of inflammatory cells in lateral xanthomas/fatty streaks. With the exception of the frequency of intra-plaque hemorrhage, there were no

differences between the infected and control mice with regard to the frequency of any of the listed features (data not shown). As shown in table 2, there was an increase in the frequency of intra-plaque hemorrhage in the very old mice that were inoculated once and sacrificed at either 4 or 7 days post-infection. Figure 5 shows a representative example of the presence of red blood cells within the necrotic core of an advanced lesion from one of the much older mice (panel A) and the lack of hemorrhage in a lesion from one of the younger mice (panel B).

4. Discussion

This is the first report on the complex profile of acute phase reactant proteins induced by acute respiratory infection of C57BL/6J mice with *C. pneumoniae*. It is also the first report that the anti-inflammatory effects of HDL are decreased with *C. pneumoniae* infection and that there is an increased frequency of intra-plaque hemorrhage in older apo E^{-/-} mice. The plasma cytokine profile measured in this study, in part, parallels *in vitro* studies that have reported increased expression of cytokines such as IL-6 and IL-10 [23,24] but are in contrast to studies showing increases in IL-1 β or TNF- α [8]. In keeping with the present study, infection of a human lung epithelial cell line with *C. pneumoniae* did not increase secretion of TNF- α and IL-1 β [25]. However, human patients infected with *C. pneumoniae*, *Mycoplasma pneumoniae* or Influenza A had increased plasma levels of IFN- γ , IL6 and TNF- α [26]. This does not appear to be the case with mice. In agreement with our studies, at 2 days post-infection IFN- γ levels were increased significantly while no increase in TNF- α levels were observed in BALB/c mice [27]. Furthermore, in a study with C3H/HeJ mice infected with low inoculum levels (10⁶ EBs) of *C. pneumoniae*, no increase in IL-6, IL-10, TNF- α and SAA were detected in lung supernatants, bronchoalveolar lavage or serum at 2, 4, 11, 18, and 33 days post-inoculation [28]. Collectively, these studies indicate that species and strain differences as well as differences in inoculum concentration, the route of infection, the time point of sampling, cell types and *in vitro* versus *in vivo* approaches account for the differences observed in individual studies of the acute phase reactant response to *C. pneumoniae* infection.

It is currently unclear how increased secretion of a complex combination of both pro- and anti-inflammatory cytokines contributes to acute and chronic inflammatory processes. In the present study, the plasma concentration of the anti-inflammatory cytokine IL-10 was increased by 6 hrs post *C. pneumoniae* inoculation. This rapid increase in IL-10 may have contributed to the suppression of the secretion of TNF- α and IL-1 β . There were also significant increases in IL-2, IL-5, IL-6, IL-12, and IFN- γ . IL-5 plays a pivotal role in the maturation, activation and survival of eosinophils [29]. IL-6 enhances the proliferation of multipotential haematopoietic progenitor cells and is a regulator of the acute phase response including activation of SAA [30]. IL-12 is produced mainly by monocytes, macrophages, and dendritic cells and plays a role in activation of antigen presenting cells and sustaining the antigen-specific T-cell response [31–33]. IFN- γ activates macrophages with increased production of reactive oxygen intermediates and proteases [34]. IFN- γ also induces the expression of indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS), [35]. Both IDO and iNOS have been positively correlated with inhibition of chlamydial growth *in vitro* [35–38]. Interestingly, the pro-inflammatory T cell cytokines, IFN- γ and IL-2 are found in a large proportion of advanced atherosclerotic plaques [39].

This study also established that acute infection of mice with *C. pneumoniae* leads to a reduction in the anti-inflammatory properties of HDL. This observation is in keeping with a previous study showing that infection of mice with Influenza A has the same effect on HDL [10]. It has been proposed that SAA binding to HDL may be most responsible for these changes in HDL [40]. In this study, plasma SAA levels were increased at 24 to 72 hours post-infection in both the C57BL/6J and apoE^{-/-} mice while there was no increase in plasma SAP. In a previous study with C57BL/6J mice fed an atherogenic diet and inoculated intravenously with *C.*

pneumoniae, there were also no increases in serum SAP as compared to sham-inoculated mice [41]. It has also been shown that administration of an atherogenic diet independent of infection has no effect on SAP. In contrast, feeding the atherogenic diet increases SAA levels that are correlated with increases in atherosclerotic lesion size [5].

Acute infection did not stimulate the rate of progression or alter the composition of the atherosclerotic lesions in the innominate arteries in either the 30 week old mice or the very old mice. This is in contrast to previous studies in younger mice showing that chronic infection accelerates formation of fatty streaks in the aortic sinus or descending aorta [42–44]. It is also in contrast to a previous study showing *C. pneumoniae* induced thinning of the fibrous cap and increased matrix metalloproteinase expression in apo E^{-/-}, LDL-R^{-/-} double knockout mice [45]. The failure to see any acute effects of infection on lesion progression and composition is not surprising as measurable increases in lesion area are unlikely to occur within 7 days post-inoculation.

It is unclear how *C. pneumoniae* infection and the resulting acute phase response increased the frequency of intra-plaque hemorrhage. Intra-plaque hemorrhage can be the result of either plaque disruption along the luminal surface or due to fragile neovessels within the body of the plaque [46]. We and others have previously shown that the advanced plaques in the innominate artery of the apo E^{-/-} mice develop disruptions through the lateral fatty streaks that lead to pooling of red blood cells at these sites [16]. These disruptions are not analogous to the rupture of the fibrous cap that occurs in human plaques and that lead to formation of occlusive thrombi. We have to date, not observed any thrombosis associated with plaque disruptions in the chow-fed apo E^{-/-} mice. Although neovessels have been reported to form in the aortic arch of the apo E^{-/-} mice [47], we have also not observed any neovessels within the advanced lesions of the innominate arteries. Thus, it can be presumed that infection in some way increased disruption of the very advanced lesions. It is not known what causes plaque disruption in either humans or mice. It is possible that vasoconstriction in response to increased acute phase proteins or endothelin mechanically precipitates plaque disruption. It has recently been reported that *C. pneumoniae* induces expression of endothelin in infected U937 cells [48]. Other mechanisms such as activation of matrix metalloproteinases (MMPs) must also be considered as others have reported that *C. pneumoniae* infection induces expression of MMPs and MMPs and *C. pneumoniae* have been co-localized in human and mouse plaques [6,45]. However, in the present study we have no evidence for the presence of *C. pneumoniae* at the sites of plaque disruption.

Older people are more susceptible to *C. pneumoniae* infection and have the highest frequency of myocardial infarction and stroke [1]. Thus, it is highly significant that *C. pneumoniae* infection of the very old apo E^{-/-} mice led to an increased frequency of intra-plaque hemorrhage. This result may help explain the association between *C. pneumoniae* infection and morbidity from CVD in older people. It is also consistent with reports of increased antibody titers in people with acute coronary syndrome [6,49] and with reports of a higher frequency of *C. pneumoniae* in thrombosed ruptured plaques from people who died of acute myocardial infarction [50].

In conclusion, we have shown that acute infection of mice with *C. pneumoniae* stimulates secretion of a complex combination of both pro- and anti-inflammatory cytokines and increases plasma levels of SAA. The increase in SAA corresponds with a reduction in the anti-inflammatory properties of HDL. We have also shown that acute infection is associated with an increase in the frequency of intra-plaque hemorrhage. Taken together these observations could help explain how *C. pneumoniae* infection contributes to the progression of atherosclerosis and to the clinically significant disruption of advanced lesions.

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Fig. 1. Kinetics of plasma cytokines IL-10, IL-12, TNF- α and IL-4 levels during 72 hrs following acute *Chlamydia pneumoniae* lung infection. Solid line: *C. pneumoniae* inoculated group. Dotted line: Sham inoculated group. *, $P < 0.05$ in comparison to sham infected group. Error bars indicate one standard deviation. N=5 at each time point.

Fig. 2.

C. pneumoniae infection induces SAA in C57BL/6J and ApoE^{-/-} mice. Mice were sham infected with PBS, *C. pneumoniae* or heat-inactivated organisms (ApoE^{-/-} only). SAA levels were measured by ELISA at 24, 48 and 72 hrs post-inoculation. Panel A – C57BL/6J mice; Panel B – ApoE^{-/-} mice. *, $P < 0.05$ in comparison to sham infected group.

Fig. 3.

C. pneumoniae infection decreases paraoxynase activity in C57BL/6J mice. Eight week old mice were infected with *C. pneumoniae* and sera collected at various times post-infection. Neat plasma samples or HDL proteins separated and pooled from plasma samples by fast phase liquid chromatography were assayed for PON activity using paraoxon as substrate.

Fig. 4.

C. pneumoniae infection decreases the effect of HDL in preventing the oxidation of oxidized palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphocholine (PAPC) by HPODE. Eight week old mice were infected with *C. pneumoniae* and blood collected at various times post-infection and HDL separated and pooled. Ten μ l aliquots of DCFH in methanol were placed in the polypropylene tubes and the methanol was evaporated under argon. Ten microliters of PAPC at 2.5 mg/ml in chloroform was placed in each tube, mixed, and evaporated. Subsequently, 10 μ l of HPODE (0.1 mg/ml in ethanol) was added and evaporated. 25 μ l of saline or HDL at 350 μ g/ml was added to each tube, the volume was adjusted to 1.0 ml with saline, and the mixture was vortexed for 1 min and incubated at room temperature in the dark. Fluorescence readings were obtained at the indicated time points.

Fig. 5. Disrupted and stable atherosclerotic plaques in the innominate arteries of *C. pneumoniae* infected apolipoprotein E deficient mice.
 Panel A. Representative example of a disrupted atherosclerotic plaque from a *C. pneumoniae* infected, 75 week old, chow-fed, female apo E^{-/-} mouse. Red blood cells can be seen within the necrotic core region of the plaque (arrow), Movat's pentachrome stain, 200X final magnification. Panel B. Representative example of an intact plaque from a *C. pneumoniae* infected, 30 week old, chow-fed, male apo E^{-/-} mouse. Movat's pentachrome stain, 100X final magnification.

Table 1Plasma Cytokines Induced by Acute Lung Infection with *Chlamydia pneumoniae*.

Cytokine	Peak Levels (hours post inoculation)*	Degree of Increase**	P<0.05***
IL-2	12, 48	++++	12, 48 hrs
IL-5	48, 72	++++	72 hrs
IL-6	3,6,48,72	+++	72 hrs
IL-10	6, 24	++++	12–72 hrs
IL-12	3–72	++++	12–72 hrs
GM-CSF	12–72	++++	6, 72 hrs
IFN- γ	24–72	+++	24–72 hrs
IL-1 β	48	+++	None
IL-4	6–12	++	None

* Plasma samples were obtained at 1,3,6,12,24,48, and 72 hours post inoculation.

** Increase in comparison to sham infected group.

*** Time points with statistically significant increases.

+ = ~ 1–2 fold increase, ++ = ~ 2 fold, +++ = ~ 3 fold, ++++ = > 4 fold

Table 2

Effects of *C. pneumoniae* infection on the frequency of intra-plaque hemorrhage in the innominate artery of older apolipoprotein E deficient mice.

Age at Sacrifice		Frequency of Intra-plaque Hemorrhage	
	Control	Live CP	Dead CP
31 Weeks	5/23 (22%)	2/15 (13%)	3/17 (18%)
>75 Weeks	2/12 (17%)	8/14 (57%)*	2/11 (18%)

* P<0.05 compared to control and dead CP.