Induction of Macrophage Foam Cell Formation by *Chlamydia pneumoniae*

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Foam cell formation is the hallmark of early atherosclerosis. It was found that the intracellular bacterium *Chlamydia pneumoniae* induces foam cell formation by human monocyte-derived macrophages. Exposure of macrophages to *C. pneumoniae* followed by low-density lipoprotein (LDL) caused a marked increase in the number of foam cells and accumulation of cholesteryl esters. Foam cell formation was not inhibited by the antioxidant butylated hydroxytoluene nor fucoidan, suggesting that lipid accumulation did not involve scavenger receptors. In contrast, addition of heparin, which blocks binding of LDL to the LDL receptor, inhibited *C. pneumoniae*–induced foam cell formation, suggesting that the pathogen induced lipid accumulation by dysregulating native LDL uptake or metabolism (or both). These data demonstrate that an infectious agent can induce macrophage foam cell formation and implicate *C. pneumoniae* as a causative factor in atherosclerosis.

Atherosclerotic heart and vessel disease is the leading cause of morbidity and mortality in the Western Hemisphere [1]. High plasma low-density lipoprotein (LDL) concentration is a major risk factor for atherosclerosis [2], and early disease is characterized by arterial ''fatty-streak'' lesions that contain T cells and lipid-laden macrophages, or foam cells [3]. These cells have ingested great amounts of lipids and store the excess cholesterol as cholesteryl esters within the cytoplasm [4]. Because macrophage LDL receptors are tightly regulated [5], foam cell formation principally is thought to involve scavenger receptors that bind a modified form of LDL [4]. One such modification that may occur in vivo is LDL oxidation, and oxidized LDL has been shown to accumulate within macrophages following scavenger receptor binding [6].

The foam cell–rich fatty streaks may mature into intermediate lesions containing lipid-laden macrophages as well as smooth muscle cells, and continued endothelial damage and inflammation leads to complex, occlusive fibrous plaques [3]. Although atheroma development probably involves multiple factors, recent evidence suggests that infectious agents, such as *Chlamydia pneumoniae* and cytomegalovirus, contribute to atherosclerosis [7, 8]. Indeed, *C. pneumoniae* has been localized to atheromas by immunohistochemistry and polymerase chain reaction [9], demonstrated within atheroma foam cells by electron microscopy [10], isolated from atheromas [11], and associated with coronary artery disease by seroepidemiology [12]. Furthermore, a recent report suggests that taking a short course of azithromycin, the antibiotic most commonly used to treat respiratory tract *C. pneumoniae* infections, can lower adverse cardiovascular events in post–myocardial infarct patients [13]. However, direct evidence linking *C. pneumoniae* to atheroma development has not emerged.

To study one possible mechanism of *C. pneumoniae* pathogenesis in atherosclerosis, we examined the effect of *C. pneumoniae* infection on macrophage lipid uptake.

Materials and Methods

Reagents. Oxidized LDL was purchased from PerImmune (Rockville, MD). Anti-CD36 monoclonal antibody was purchased from Monosan (Uden, Netherlands). All other reagents were purchased from Sigma (St. Louis).

Isolation of LDL. LDL either was purchased from Sigma or isolated from normolipidemic donors by density gradient ultracentrifugation as previously described [14]. LDL was extensively dialyzed against 0.15 *M* NaCl and 0.05% EDTA and used within 2 weeks. Protein and total cholesterol contents were determined by use of a protein assay (Bio-Rad, Hercules, CA) and a cholesterol test kit (Sigma), respectively. Agarose gel electrophoresis showed no increase in relative electrophoretic mobility of LDL [15] within 2 weeks, indicating that isolated LDL did not become oxidized.

Propagation of chlamydiae. *C. pneumoniae* (TW-183) was purchased from American Type Culture Collection (ATCC, Rockville, MA) and propagated in HEp-2 cells (ATCC); infectious elementary bodies were purified by Renografin gradient centrifugation as described elsewhere [16]. Propagated organisms were suspended in sucrose phosphate buffer (0.22 *M* sucrose–10 m*M* NaH_2PO_4–3.8 m*M* KH_2PO_4–5 m*M* glutamic acid, pH 7.4), aliquoted, and frozen at −70°C. *Chlamydia* titers were determined by infecting HEp-2 cells with 10-fold dilutions of thawed stock, incubating for 36 h, fixing cells with methanol, and fluorescent staining chlamydial inclusions with fluorescein isothiocyanate–conjugated anti–major outer membrane protein antibody (Syva, San Jose, CA). Infectious elementary bodies were determined by counting chlamydial inclusions in several confluent HEp-2 cell monolayers and are reported here as infection-forming units (ifu). In some experiments, chlamydiae were inactivated by incubating

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organisms at 65°C for 30 min. This treatment eliminated viable organisms completely, as determined by standard infectivity assays for ifu.

Isolation, culture, and infection of human monocytes/macrophages. Human peripheral blood mononuclear cells were isolated from healthy donor blood by ficoll-hypaque centrifugation and suspended in serum-free RPMI 1640 medium. Cells were plated in 96-well microtiter plates at 8×10^5 cells/well for 45 min at 37°C and washed four times with medium. Adherent cells were >95% monocytes as confirmed by surface CD14 expression. Some wells were trypsinized and gently scraped for cell counting and yielded $\sim 1-2 \times 10^5$ monocytes/well. Adherent monocytes were incubated for 7–10 days in 200 μL of RPMI 1640 supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), 25 m*M* HEPES buffer, 50 μg/mL vancomycin, and 10 μg/mL gentamicin before use.

C. pneumoniae infection of monocyte-derived macrophages was done by exposing macrophages to 2×10^5 ifu for 2 h in 50 μL of RPMI 1640 + 10% FBS, followed by addition of 150 μL of RPMI 1640 + 10% FBS with or without 100 μg/mL LDL. In separate experiments, these titers were shown to infect all macrophages with <5% loss in viability. FBS was used in all experiments, since pooled human serum has been reported to induce lipid accumulation [17], and delipidized calf serum and lipoprotein-deficient human serum were found toxic to macrophages. In experiments in which heparin was used to block LDL receptor function, 100 U/mL heparin was added to cultured cells only after completion of the 2-h inoculum adsorption period to be certain that treatment did not interfere with *C. pneumoniae* entry into macrophages.

Quantitation of foam cell formation. To visualize cellular neutral lipid accumulation, macrophages were fixed in 2% paraformaldehyde for 15 min, stained with 1% Oil-Red O (in 60% isopropanol) for 30 min, washed, and examined at ×200 magnification on an inverted microscope (Diaphot 200; Nikon, Garden City, NY). Lipid-laden macrophages were scored by previously established criteria [18, 19].

Quantitation of macrophage cholesterol and cholesteryl ester content. Total cellular cholesterol and cholesteryl ester content was quantitated by use of a slightly modified method from Gamble et al. [20]. The assay is based on an enzymatic reaction that breaks down cholesterol to produce hydrogen peroxide, which then reacts with *p*-hydroxyphenylacetic acid to produce a fluorescent molecule. Briefly, macrophages were fixed in 2% paraformaldehyde, washed three times with PBS, and incubated for 30 min with 200 μL of absolute ethanol at 4°C to extract cellular lipids. To determine total cholesterol content, 95 μL of ethanol-lipid extract was incubated with 800 μL of assay solution [20] for 1 h at 37°C, and fluorescence was measured by a fluorometer (Aminco-Bowman Series 2; SLM-Aminco, Urbana, IL) set to 325 nm excitation, 415 nm emission. To determine free cholesterol content, 95 μL of ethanol-lipid extract was incubated with 800 μL of a modified assay solution prepared without cholesterol esterase [20]. Cholesteryl ester content was calculated by subtracting free cholesterol from total cholesterol for each sample.

After lipid extraction, protein content of each sample was determined. Briefly, lipid-extracted cells were washed three times with distilled water and resuspended in 160 μL of water. Next, 40 μL of protein assay dye (Bradford; Bio-Rad) was added to each well, and the optical density (570 nm) was read after 5 min. Cellular cholesterol and protein contents were calculated from a standard curve and are expressed as nanograms of cholesterol per microgram of protein.

Results

C. pneumoniae induces foam cell formation. Compared with uninfected, LDL-treated human macrophages (figure 1A), most macrophages infected with *C. pneumoniae* and treated with LDL developed into lipid-laden foam cells (figure 1B). Untreated macrophages and infected macrophages cultured in serum-free medium showed little lipid accumulation (<10% foam cells). In a separate experiment, foam cells were counted and found to be increased from 28.3% to 70.6% after infection with *C. pneumoniae* (figure 2). Although lipid accumulation varied between uninfected macrophages from different donors, *C. pneumoniae* consistently induced an increase of 30%–50% in foam cell numbers. Viability was monitored by trypan blue staining and remained >95% in both infected and uninfected cultures.

Since the excess cholesterol that accumulates within foam cells is stored as cytoplasmic cholesteryl esters [4], total cholesterol and cholesteryl ester levels between infected and uninfected macrophages were determined. As shown in figure 3, *C. pneumoniae* infection resulted in a significant increase in cholesteryl ester content and a small increase in total cholesterol content.

C. pneumoniae–induced foam cell formation is reversible with heparin. Since macrophages are known to become foam cells by ingesting modified LDL that bind to scavenger receptors [4], *C. pneumoniae*–induced foam cell formation was monitored in the presence of the antioxidant butylated hydroxytoluene or fucoidan, which blocks scavenger receptors. Incubation of cultures with butylated hydroxytoluene (up to 200 μ*M*) or fucoidan (50 μg/mL) did not inhibit *C. pneumoniae*–induced foam cell formation (not shown), suggesting that lipid accumulation did not result from uptake of modified LDL. Interestingly, fluorescent microscopy showed that *C. pneumoniae* slightly inhibited surface levels of a scavenger receptor (CD36) (not shown). In contrast, when *C. pneumoniae*–exposed macrophages were cultured in the presence of heparin, which competes with the LDL receptor in binding LDL, a marked reduction in foam cells was observed (table 1). As expected, addition of heparin to oxidized LDL-treated cultures did not inhibit foam cell formation, indicating that the molecule did not interfere with scavenger-receptor uptake of modified LDL. Addition of lactoferrin, which binds the LDL receptor–related protein, also did not inhibit *C. pneumoniae*–induced foam cell formation. Taken together, these data suggest that *C. pneumoniae*–induced foam cell formation results from dysregulation of native LDL uptake and/or metabolism.

Discussion

Clinical data linking *C. pneumoniae* to atherosclerosis [9–12] are impressive yet indirect, because a possible mechanism

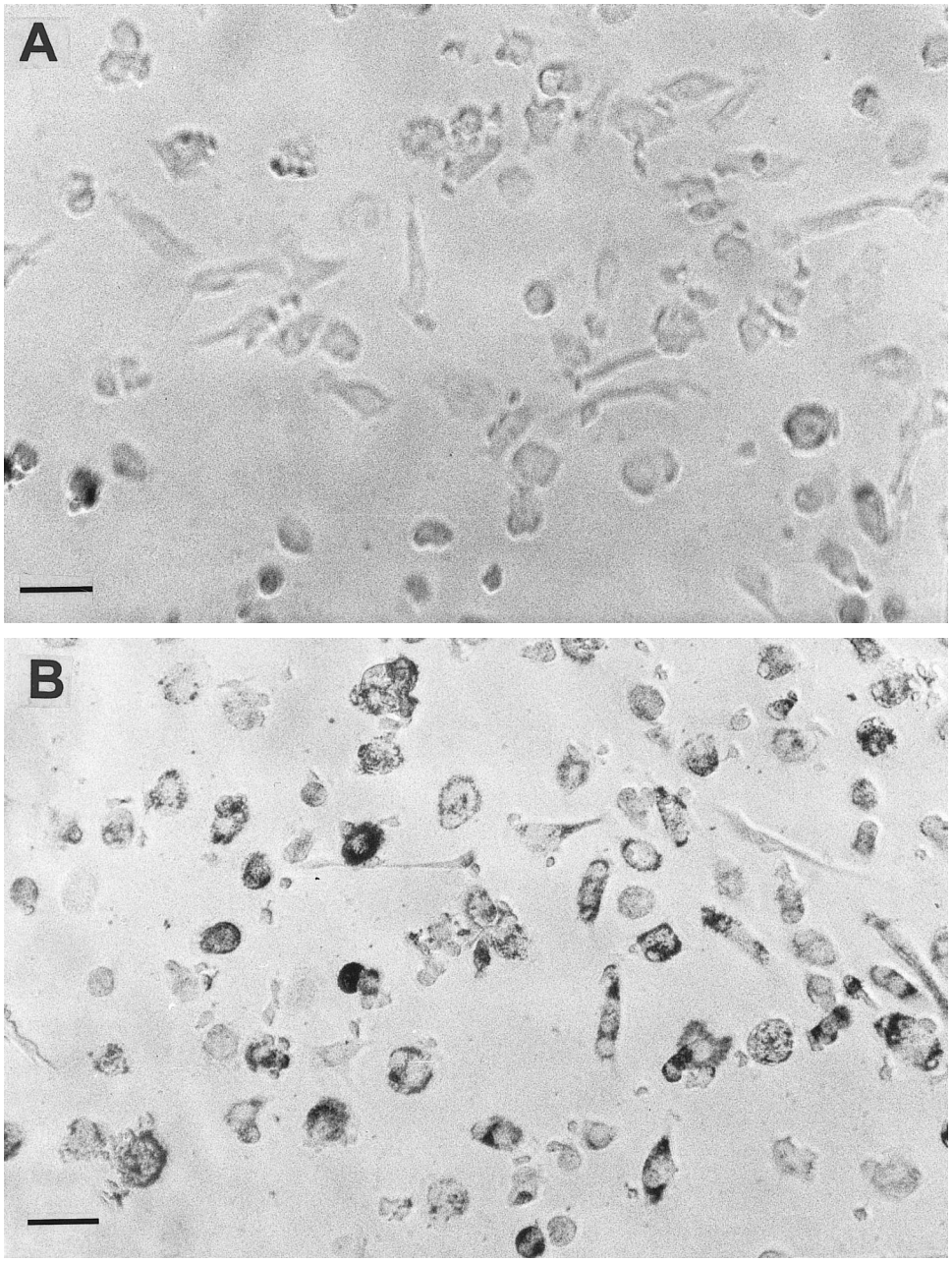

Figure 1. Foam cell formation by uninfected (**A**) and infected (**B**) human monocyte-derived macrophages. Cells were infected or not infected for 2 h and incubated with 100 μg/mL low-density lipoprotein for 24 h. Micrographs show Oil-Red O–stained macrophages. Bar = 50 μm.

of disease has not been demonstrated. Since lipid-laden macrophages are the hallmark of early atherosclerosis and contribute to initial fatty streaks as well as developing atheromas [3, 18, 21], data presented here show that *C. pneumoniae* may initiate or promote atheroma development by inducing foam cell formation.

Although increased plasma LDL levels remain a major risk factor for development of atherosclerosis, the role of native LDL in foam cell formation has been questioned because of stringent cellular control of LDL receptor expression [5]. In contrast, acetylated and oxidized LDL bypass the LDL-receptor pathway and become ingested by scavenger receptors, resulting in macrophage foam cell formation in vitro [4, 21]. Data presented herein suggest that high levels of native LDL can be ingested by macrophages following exposure to *C. pneumoniae,* since results showed that *C. pneumoniae*–exposed macrophages cultured under nonoxidizing conditions ingested LDL to become foam cells and that foam cell formation was inhibited by heparin, which blocks LDL binding to the LDL receptor. Lopes-Virella et al. [22] induced foam cell formation through a similar mechanism by incubating monocyte-derived macrophages with high levels of *Escherichia coli* lipopolysaccharide (LPS). Interestingly, like *E. coli* LPS, the chlamydial antigen causing lipid accumulation was heat-resistant, since organisms

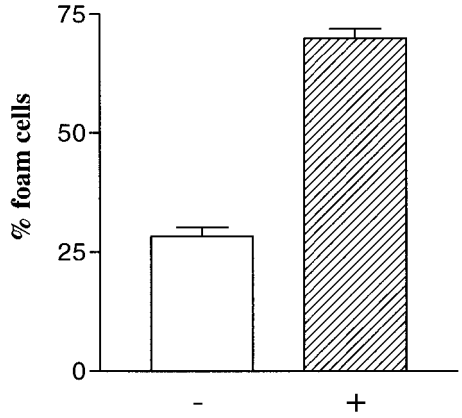

Figure 2. Foam cell formation by uninfected (−) and infected (+) human monocyte-derived macrophages. Cells were infected or left undisturbed for 24 h, incubated with 100 μg/mL low-density lipoprotein for 24 h, and stained with Oil-Red O. About 500 cells were counted from each well. Data are means of triplicates with SE.

inactivated at 65°C for 30 min also induced foam cell formation (unpublished data). In addition, the finding that macrophage scavenger receptor levels were inhibited by *C. pneumoniae* also has been observed with *E. coli* LPS [23], further suggesting that *C. pneumoniae* modulates these macrophage functions through its LPS antigen. Studies are underway to define the chlamydial antigen(s) responsible for induction of macrophage foam cell formation by *C. pneumoniae.*

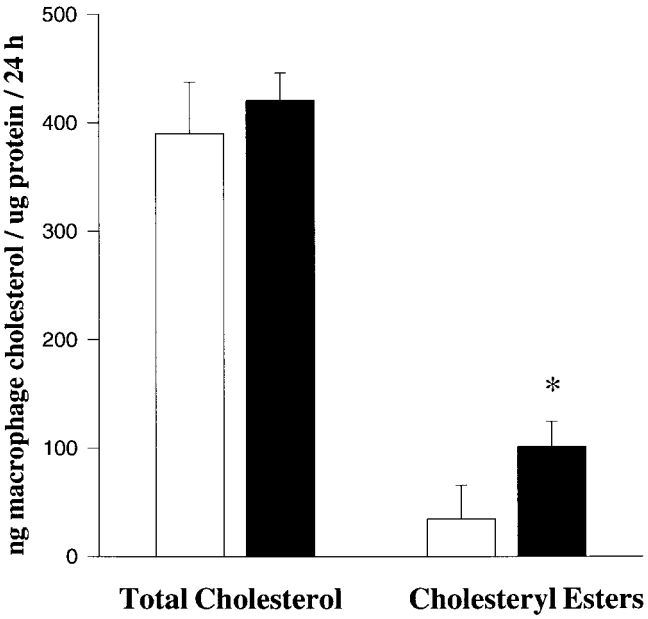

Figure 3. Total cholesterol and cholesteryl ester levels by uninfected (open bars) and infected (solid bars) human monocyte-derived macrophages. Cells were infected or not infected for 2 h and incubated with 100 μg/mL low-density lipoprotein for 24 h. Cholesterol levels were normalized to protein content and are expressed as ng of cholesterol/μg of protein. Data are means of triplicates with SE. * Statistically significant difference compared with uninfected samples, $P < .05$ (t test).

Table 1. Heparin inhibits *C. pneumoniae*–induced foam cell formation by human monocyte-derived macrophages.

	Untreated	With heparin
No treatment	85/500 (17%)	95/508 (18.7%)
+ *C. pneumoniae*	276/500 (55.2%)	135/500 (27%)

NOTE. Macrophages were exposed to 2×10^5 ifu for 2 h and incubated in presence of 100 μg/mL low-density lipoprotein with or without 100 U/mL heparin. Cells then were fixed and stained with Oil-Red O. Cell counts are expressed as no. foam cells/no. total cells (%).

The mechanism of *C. pneumoniae*–induced foam cell formation currently is not known, although data presented here suggest that the pathogen disrupts native LDL uptake or metabolism (or both). One possibility is that *C. pneumoniae* may dysregulate macrophage LDL receptor expression. *C. pneumoniae*–exposed macrophages secrete high levels of tumor necrosis factor-α and interleukin-1β [24], cytokines shown to up-regulate hepatocyte LDL receptor gene expression [25], and these cytokines also may up-regulate macrophage LDL receptors to contribute to foam cell formation. Alternatively, since chlamydiae are intracellular bacteria capable of disrupting various host cell functions [26], *C. pneumoniae* may induce foam cell formation by dysregulating intracellular lipoprotein metabolism or cholesterol storage. Future work will identify the mechanism of foam cell formation, partly by using fluorescently labeled LDL to quantitate lipoprotein binding, uptake, and degradation after infection with *C. pneumoniae.*

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References

1. McMillan G. Historical review of research on atherosclerosis. Adv Exp Med Biol **1995**;369:1–6.
2. Gofman J, Lindgren F, Elliott H. The role of lipids and lipoproteins in atherosclerosis. Science **1950**;111:166–71.
3. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature **1993**;362:801–9.
4. Brown M, Goldstein J. Lipoprotein metabolism in the macrophage. Annu Rev Biochem **1983**;52:223–61.
5. Goldstein J, Brown M. The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem **1977**;46:897–930.
6. Steinberg D, Parthasarathy S, Carew T, Khoo J, Witztum J. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med **1989**;320:915–24.
7. Ridker P, Cushman M, Stampfer M, Tracy R, Hennekens C. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med **1997**;336:973–9.

8. Mendall M, Patel P, Ballam L, Strachan D, Northfield T. C-reactive protein and its relation to cardiovascular risk factors—a population based cross sectional study. Br Med J **1996**;312:1061–5.
9. Campbell L, O'Brien E, Cappuccio A, et al. Detection of *Chlamydia pneumoniae* TWAR in human coronary atherectomy tissues. J Infect Dis **1995**;172:585–8.
10. Kuo C, Shor A, Campbell L, Fukushi H, Patton D, Grayston T. Demonstration of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries. J Infect Dis **1993**;167:841–9.
11. Ramirez J, Ahkee S, Summersgill J, et al. Isolation of *Chlamydia pneumoniae* from the coronary artery of a patient with coronary atherosclerosis. Ann Intern Med **1996**;125:979–82.
12. Saikku P, Leinonen M, Tenkanen L, et al. Chronic *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki Heart Study. Ann Intern Med **1992**;116:273–8.
13. Gupta S, Leatham E, Carrington D, Mendall M, Kaski J, Camm J. Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. Circulation **1997**;96:404–7.
14. Redgrave T, Roberts D, West C. Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal Biochem **1975**;65:42–9.
15. Esterbauer H, Gebicki J, Puhl H, Jurgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Radic Biol Med **1992**;13:341–90.
16. Caldwell H, Kromhout J, Schachter J. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis.* Infect Immun **1981**;31:1161–76.
17. Garner B, Baoutina A, Dean R, Jessup W. Regulation of serum-induced lipid accumulation in human monocyte-derived macrophages by interferon-gamma. Correlations with apolipoprotein E production, lipoprotein lipase activity, and LDL receptor-related protein expression. Atherosclerosis **1997**;128:47–58.
18. Schaffner T, Taylor K, Bartucci E, et al. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. Am J Pathol **1980**;100:57–80.
19. Huh H, Pearce S, Yesner L, Schindler J, Silverstein R. Regulated expression of CD36 during monocyte-to-macrophage differentiation: potential role of CD36 in foam cell formation. Blood **1996**;87:2020–8.
20. Gamble W, Vaughan M, Kruth H, Avigan J. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. J Lipid Res **1978**;19:1068–70.
21. Aviram M. Interaction of oxidized low density lipoprotein with macrophages in atherosclerosis, and the antiatherogenicity of antioxidants. Eur J Clin Chem Clin Biochem **1996**;34:599–608.
22. Lopes-Virella M, Klein R, Stevenson H. Low density lipoprotein metabolism in human macrophages stimulated with microbial or microbial-related products. Arteriosclerosis **1987**;7:176–84.
23. Van Lenten B, Fogelman A, Seager J, Ribi E, Haberland M, Edwards P. Bacterial endotoxin selectively prevents the expression of scavenger-receptor activity on human monocyte-macrophages. J Immunol **1985**;134:3718–21.
24. Kaukoranta-Tolvanen S, Teppo A, Kaitinen K, Saikku P, Linnavuori K, Leinonen M. Growth of *Chlamydia pneumoniae* in cultured human peripheral blood mononuclear cells and induction of a cytokine response. Microb Pathog **1996**;21:215–21.
25. Stopeck A, Nicholson A, Mancini F, Hajjar D. Cytokine regulation of low density lipoprotein receptor gene transcription in HepG2 cells. J Biol Chem **1993**;268:17489–94.
26. Moulder J. Interaction of chlamydiae and host cells in vitro. Microbiol Rev **1991**;55:143–90.