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TLR/MyD88 and Liver X Receptor α Signaling Pathways Reciprocally Control Chlamydia pneumoniae-Induced Acceleration of Atherosclerosis


Experimental and clinical studies link Chlamydia pneumoniae infection to atherogenesis and atherothrombotic events, but the underlying mechanisms are unclear. We tested the hypothesis that C. pneumoniae-induced acceleration of atherosclerosis in apolipoprotein E (ApoE)−/− mice is reciprocally modulated by activation of TLR-mediated innate immune and liver X receptor α (LXRα) signaling pathways. We infected ApoE−/− mice and ApoE−/− mice that also lacked TLR2, TLR4, MyD88, or LXRα intranasally with C. pneumoniae followed by feeding of a high fat diet for 4 mo. Mock-infected littermates served as controls. Atherosclerosis was assessed in aortic sinuses and in en face preparation of whole aorta. The numbers of activated dendritic cells (DCs) within plaques and the serum levels of cholesterol and proinflammatory cytokines were also measured. C. pneumoniae infection markedly accelerated atherosclerosis in ApoE-deficient mice that was associated with increased numbers of activated DCs in aortic sinus plaques and higher circulating levels of MCP-1, IL-12p40, IL-6, and TNF-α. In contrast, C. pneumoniae infection had only a minimal effect on atherosclerosis, accumulation of activated DCs in the sinus plaques, or circulating cytokine increases in ApoE−/− mice that were also deficient in TLR2, TLR4, or MyD88. However, C. pneumoniae-induced acceleration of atherosclerosis in ApoE−/− mice was further enhanced in ApoE−/−/LXRα−/− double knockout mice and was accompanied by higher serum levels of IL-6 and TNF-α. We conclude that C. pneumoniae infection accelerates atherosclerosis in hypercholesterolemic mice predominantly through a TLR/MyD88-dependent mechanism and that LXRα appears to reciprocally modulate and reduce the proatherogenic effects of C. pneumoniae infection. The Journal of Immunology, 2008, 181: 7176–7185.

E xperimental and clinical studies demonstrate a link between Chlamydia pneumoniae infection and increased atherosclerosis and atherothrombotic events (1). C. pneumoniae has been isolated from the coronary arteries of patients with acute coronary syndrome (2) and from human aorta (3), carotid arteries (4), and peripheral arteries (5). Infection of cholesterol-fed C57BL/6 mice with C. pneumoniae produces aortic sinus lesions that are twice as large as those in uninfected control mice (1, 6). Most reports using murine models of atherosclerosis also indicate that C. pneumoniae infection exacerbates atherosclerosis compared with uninfected mice (7–10), although some studies have failed to find such a link (11, 12).

The precise mechanism(s) by which C. pneumoniae infection might accelerate atherosclerosis are unclear, but there are suggestions that mononuclear phagocytes and innate immune signaling may be involved. C. pneumoniae can infect and survive in circulating monocytes and tissue macrophages (13, 14) and can migrate from the lung to developing plaques via circulating monocytes and lymphocytes. Experimental studies have previously demonstrated that activation of innate immune signaling emanating from TLRs increases atherosclerosis in mice (15–18) and that C. pneumoniae can activate innate immunity via TLR-dependent signaling (19). Because mononuclear phagocytes express TLRs, it is conceivable that the ability of C. pneumoniae to promote atherogenesis could at least partly be explained by TLR-dependent effects of the pathogen (or molecules derived from the pathogen) on macrophages. Macrophages take up necrotic cellular debris that accumulate in lesions, lose migratory ability, and eventually can become transformed into plaque foam cells that can promote further plaque development and focal inflammation (20, 21). However, macrophages are also intimately involved in cholesterol homeostasis. Macrophage lipid uptake is counterbalanced by the activation of liver X receptors (LXR),3 that exist in two isoforms, LXRα and β.

3 Abbreviations used in this paper: LXR, liver X receptor; ApoE, apolipoprotein E; DC, dendritic cell; EC, endothelial cell; IFU, inclusion-forming unit; MOI, multiplicity of infection; ox-LDL, oxidized low-density lipoprotein.

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These nuclear receptors importantly regulate cholesterol absorption, transport, and efflux (22, 23). LXRα is activated by oxysterols and products of cholesterol, but they exert anti-inflammatory effects in macrophages by modulating the activation of NF-κB (24). Furthermore, LXR-dependent and TLR-dependent signaling pathways appear to reciprocally modulate one another (23, 25).

Collectively, these data led us to hypothesize that the effects of C. pneumoniae on atherosclerosis might be reciprocally modulated by activation of innate immunity through TLR/MyD88-dependent and LXRα-dependent signaling pathways. We report that infection with C. pneumoniae in hypercholesterolemic apolipoprotein E (ApoE)−/− mice increases atherosclerosis that is significantly inhibited in ApoE−/− mice lacking TLR2, TLR4, or MyD88, but in contrast is enhanced in ApoE/LXRα double knockout mice. Thus, in the context of hypercholesterolemia, C. pneumoniae infection directly promotes atherosclerotic plaque development via the TLR/MyD88-dependent signaling pathways, but this is counteracted by LXRα activation. These results provide novel insight into how C. pneumoniae infection impacts atherogenesis and suggest that therapeutic targeting of TLR2, TLR4, and LXRα signaling pathways might prove beneficial in treating atherosclerosis.

### Materials and Methods

**Generation of double knockout mice**

MyD88−/−, TLR2−/−, TLR4−/−, and LXRα−/− mice were crossed with ApoE−/− C57BL/6 mice as we previously described (16). Heterozygous mice were intercrossed to generate homozygous ApoE−/− mice bearing combinations of MyD88−/−, MyD88−/−, MyD88−/−, TLR4−/−, TLR4−/−, TLR4−/−, TLR2−/−, TLR2−/−, and TLR2−/− mice as we previously described (16). This process resulted in a backcross for a total of eight generations onto the C57BL/6 background. ApoE−/−/TLR2−/−, ApoE−/−/TLR4−/−, ApoE−/−/MyD88−/−, and ApoE−/−/LXRα−/− were used as littermate controls. Mice were fed with a high fat diet containing 0.15% cholesterol (Harlan Teklad) starting at 8 wk of age before infection and continuing until sacrifice. Mice were maintained under specific pathogen-free conditions and were used at 8 wk of age. Male and female ApoE−/− and double knockout mice were used, but the vast majority of the animals in each group were male (>80% in each group investigated). All experiments were approved by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee and performed according to institutional guidelines.

### C. pneumoniae infection

C. pneumoniae strain CM-1 (American Type Culture Collection) was propagated in Hep-2 cells as previously described. Hep-2 cells and the C. pneumoniae stocks were determined to be free of mycoplasma contamination by PCR. Mice were anesthetized with isoflurane before intranasal application of 5 × 10^4 inclusion-forming units (IFU) of C. pneumoniae suspended in sucrose-phosphate-glutamate buffer (40 μl...
per nostril) per mouse. The intranasal administration of the buffer alone was performed as a negative control (mock infection; data not shown). Mice were inoculated a total of three times 1 wk apart, high fat diet was continued for 4 mo, at which point mice were sacrificed and dependent variables were measured (see Fig. 1A).

**Assessment of atherosclerotic lesions in the aorta and aortic sinus**

Mice were anesthetized with isoflurane before the aorta and the heart were excised. Aortas were excised from the aortic arch to the iliac bifurcation. Adherent (advential) connective fat was removed, and cross-sections of the aortic sinus were stained with Oil Red O. Lesions areas were quantified with Image-Pro Plus (Media Cybernetics). Image analysis was performed by a trained observer blinded to the genotypes of mice as previously described (16). The lesion size in the aortic sinus was expressed as “aortic sinus lesion area” ((×10^5 μm^2/section), and the plaque composition in the aortic sinus was expressed as “lipid content aortic sinus” (percentage of plaque area staining with lipid). Five serial sections per animal were analyzed with the resulting data were averaged and presented as average aortic sinus lesion area.

The lesion area in the aorta en face preparations was expressed as “aortic lesion coverage” (percentage of total aortic surface staining with lipid) as previously reported (16).

**Immunohistochemical staining and quantification of dendritic cells (DCs) in the aortic sinus**

Frozen heart sections (aortic sinuses) were analyzed for infiltration of activated DCs using rat anti-mouse MIDC-8 Ab (Serotec) and a catalyzed signal amplification (CSA) kit from DakoCytomation as we described previously (26). Secondary biotinylated Ab was used followed by a streptavidin-biotin-peroxidase complex. An isotype control (IgG2a; Serotec) was used to demonstrate specificity of staining. The rat mAb MIDC-8, which binds a still unidentified Ag within intracellular granules of mature myeloid DCs (27, 28), has been used in multiple studies as a specific marker of mature (activated) myeloid DCs (27–29), including in murine atherosclerosis in ApoE-null mice (30). Pictures of the aortic sinuses were taken (330 TV lines resolution) with a charge-coupled device camera (Nikon). The stained DCs (MIDC8+) were determined in three representative aortic sinus regions (0.10 μm^2) as described (26). The number of activated DCs was quantified using Image-Pro Plus software (Media Cybernetics).

**Lipid profiles**

Total cholesterol concentrations were determined in duplicate by using a colorimetric assay (infinity cholesterol reagent’ Sigma Diagnostics). Triglyceride concentrations were determined by using the L-type triglyceride H assay according to the manufacturer’s instructions (Wako Chemicals). These assays were performed on serum obtained from blood withdrawn at the time of sacrifice from mice that had undergone an overnight fast.
Serum levels of cytokines and chemokines

Serum concentrations of TNF-α, IL-6, IL-12p40, and MCP-1 (BD Biosciences and R&D Systems for MCP-1) were detected by means of ELISA according to the manufacturer’s instructions.

Assessment of foam cells

Peritoneal macrophages were isolated from C57BL/6 wild-type mice after the instillation of PBS buffer with 2 mM EDTA (Sigma-Aldrich). Cells were plated on coverslips (Fisher Scientific) previously coated with gelatin (0.1% Sigma-Aldrich) in a 24-well plate. Oxidized low-density lipoprotein (ox-LDL) (100 μg/ml; Sigma-Aldrich), live C. pneumoniae (5 × 10^7 IFU/ml), and GW3965 (1 μM), a specific LXR agonist, were added, and 24 h later Oil Red O staining was performed. Quantification of foam cells was calculated using Image-Pro Plus software (Media Cybernetics) by counting the Oil Red O-positive cells and comparing that number to the total amount of macrophages (expressed as percentage of foam cells/total macrophages).

GM-CSF induction by C. pneumoniae in murine endothelial cells (ECs)

Primary murine aortic ECs were isolated and purified to 95% purity from wild-type, MyD88−/−, TLR2−/−, and TLR4−/− mice as we previously described (16, 31) ECs were grown to 80% confluency and stimulated overnight with live C. pneumoniae (multiplicity of infection (MOI) = 5 and 10), GM-CSF release into the cell-free supernatant was determined after 24 h of treatment using ELISA (eBioscience).

GM-CSF expression by atherosclerosis gene array

Total RNA from aortas of infected and uninfected ApoE−/− mice were isolated using RNeasy mini kit (Qiagen) and subjected to DNase treatment (Invitrogen) to eliminate possible DNA contaminations. cDNA synthesis was performed using an Omniscript kit (Qiagen). Quantitative real-time PCR assays were performed using an iCycler thermal cycler (Bio-Rad Laboratories) in 96-well plates using the SYBR Green method (Applied Biosystems). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and imaged using a florescent microscope. Omission of the primary Ab and isotype control Ab were included as controls and to determine staining specificity. Quantitative staining for GM-CSF was performed with Image-Pro Plus (Media Cybernetics). Image analysis was performed by a trained observer blinded to the genotypes of mice as previously described (16). The lesion area and GM-CSF-stained areas in the aortic sinus were measured. GM-CSF staining in aortic root plaques was expressed as the percentage of positive stained area divided by the total plaque area.

Statistical analysis

Data are presented as mean values ± SEM. Statistically significant differences were defined as p < 0.05 by Student’s t test and one-way ANOVA for multiple comparisons.

C. pneumoniae infection and serum cholesterol and triglyceride levels

We administered C. pneumoniae or an equivalent volume of buffer intranasally to ApoE−/−, ApoE−/−TLR4−/−, ApoE−/−TLR2−/−, ApoE−/−MyD88−/−, and ApoE−/−LXRα−/− mice and control littersmates (ApoE−/−TLR4+/+, ApoE−/−TLR2+/+, ApoE−/−MyD88+/+, and ApoE−/−LXRα+/+) mice were started on a high fat diet (at age 8 wk) and then infected once a week for 3 consecutive weeks, and the high fat diet was maintained until the time of sacrifice 4 mo later (Fig. 1A). The dose of C. pneumoniae was chosen to ensure that infection was sublethal and was based on estimates of mortality after infection in wild-type and ApoE−/− mice (33). Serum cholesterol and plasma triglyceride concentration were similar in C. pneumoniae-infected or uninfected mice (data not shown). There were also no significant differences in lipoprotein profiles between various genotypes (data not shown).

C. pneumoniae infection accelerates atherosclerosis in ApoE−/− mice

Quantification of the lesion area of aortic sinus plaques revealed a significant increase in lesion size in infected ApoE−/− mice compared with mock-infected littermate controls (p < 0.01; Fig. 1B). Infection with C. pneumoniae also significantly increased the extent of lipid accumulation in both the aortic sinus plaque lesions (p < 0.01; 153% average increase; Fig. 1, B and C) and the total lesion area in the en face aorta (p < 0.01; 122% average increase; Fig. 1, D and E). All infected mice developed similar levels of...
specific Ab titers against *C. pneumoniae*, whereas all uninfected controls were Ab negative (data not shown).

**TLR2, TLR4, and MyD88 are required for *C. pneumoniae*-induced acceleration of atherosclerosis**

We generated double knockout mice by crossing ApoE−/− mice with either TLR2−/−, TLR4−/−, or MyD88−/− mice (16). Mice from all genotypes were backcrossed against the C57BL/6 strain for at least eight generations to enhance congenicity and reduce secondary sources of variance. Littermates that were ApoE−/− but wild type for TLR2, TLR4, or MyD88 served as controls. Consistent with previous reports by us and others (16, 17), uninfected ApoE−/−/TLR2−/−, ApoE−/−/TLR4−/−, and ApoE−/−/MyD88−/− mice demonstrated a reduction in the cross-sectional area of the lesion, less lipid content in aortic sinus plaques, and a reduction in the size of atherosclerotic lesions in the aorta compared with littermate controls (Fig. 2, A–C). However, following *C. pneumoniae* infection, cross-sectional lesion area and plaque lipid content in aortic sinuses (expressed as percentage of plaque area; Fig. 2, A and B) and aortic lesion size (Fig. 2C) were significantly reduced in ApoE−/−/TLR2−/−, ApoE−/−/TLR4−/−, and ApoE−/−/MyD88−/− mice compared with infected ApoE−/− littermate controls (Fig. 2, A–C). These

**FIGURE 4. *C. pneumoniae* (*C.pn*)-induced acceleration of atherosclerosis in ApoE−/− and in ApoE−/−/LXRα−/− mice is associated with increased circulating levels of cytokines.** Infected ApoE-null mice had significantly increased cytokine levels compared with uninfected littermate controls. A and B, In contrast, the genetic absence of TLR2, TLR4, and MyD88 significantly reduced serum IL-12p40 (A) and IL-6 (B) in both uninfected and infected ApoE−/− mice. Cytokine concentrations were measured using ELISA. Data are presented as mean values ± SEM, n = 8–10.

**FIGURE 5. *C. pneumoniae* (*C.pn*) infection leads to increased numbers of active DCs in the aortic sinus of ApoE−/− mice.** A, Quantification of active DCs in the aortic sinus of infected and noninfected ApoE−/−, ApoE−/−/TLR2−/−, ApoE−/−/TLR4−/−, and ApoE−/−/MyD88−/− mice. B, Representative MIDC-8-positive staining in infected vs uninfected ApoE−/− and ApoE−/−/MyD88−/− mice. Infection with *C. pneumoniae* (*5 × 10^9 IFU/mouse*) led to greater accumulation of MIDC-8-positive DCs in the aortic sinus from ApoE−/− mice, but not in ApoE−/−/TLR2−/−, ApoE−/−/TLR4−/− or ApoE−/−/MyD88−/− mice. Data are presented as mean values ± SEM; n = 10–12. CTR, Control.
reductions in lesion areas in the aortic sinus plaques averaged 60, 70, and 70% in ApoE\(^{-/-}\) TLR2\(^{-/-}\), TLR4\(^{-/-}\), and MyD88\(^{-/-}\) infected mice, respectively (Fig. 2B). Aorta lesion sizes (en face) were reduced by 70, 45, and 65% in ApoE\(^{-/-}\) TLR2\(^{-/-}\), TLR4\(^{-/-}\), and MyD88\(^{-/-}\) infected mice, respectively (Fig. 2, C and D). Hence, while C. pneumoniae infection induced an average of 153% increase in aortic plaque lipid content and 122% increase in aortic lesion size in ApoE\(^{-/-}\) mice, it failed to induce a significant acceleration of atherosclerosis in the ApoE\(^{-/-}\) TLR2\(^{-/-}\), ApoE\(^{-/-}\) TLR4\(^{-/-}\), and ApoE\(^{-/-}\) MyD88\(^{-/-}\) double knockout mice despite a modest stimulatory effect (Fig. 2, A–C). Furthermore, the significantly reduced acceleration of C. pneumoniae-induced atherosclerotic lesions in ApoE\(^{-/-}\) MyD88\(^{-/-}\) mice was not due to reduced bacterial infectivity in MyD88-deficient macrophages. Indeed, if anything, MyD88\(^{-/-}\) mice have diminished bacterial lung clearance of C. pneumoniae (C.pn).
**FIGURE 7.** LXRα deficiency enhances *C. pneumoniae* (C.p.)-mediated acceleration of atherosclerosis in ApoE−/− mice. A–C, Quantification of lesion size shown as area (A), plaque composition as lipid content in the aortic sinus (B), and lipid content in aorta lesions en face (C) from ApoE−/− LXRα−/− and ApoE−/− LXRα−/− with and without *C. pneumoniae* infection (5 × 10^4 IFU/mouse). D, Representative Oil Red O staining of aortic sinus plaques from ApoE−/− LXRα−/− and ApoE−/− LXRα−/− mice with and without infection. Lesions from ApoE−/− LXRα−/− mice showed increased lipid content after *C. pneumoniae* infection. Data are presented as mean values ± SEM; n = 8–10. Statistically significant differences are denoted by *, **, and ***, which indicate p < 0.05, p < 0.01 and p < 0.001, respectively.

*C. pneumoniae* (33) and ApoE−/− MyD88−/− macrophages have a higher level of infectivity compared with ApoE−/− macrophages in vitro (data not shown).

**Infection-induced acceleration of atherosclerosis in ApoE−/− mice is associated with increased circulating levels of inflammatory cytokines**

*C. pneumoniae* infection in ApoE−/− mice was associated with significant increases in serum concentrations of MCP-1 (Fig. 3A), IL-12p40 (Fig. 3B), TNF-α (Fig. 3C), and IL-6 (Fig. 3D). In contrast, *C. pneumoniae* infection did not increase circulating concentrations of these cytokines in ApoE−/− mice lacking either TLR2, TLR4, or MyD88 (Fig. 4, A and B, and data not shown). These results appear most consistent with the interpretation that at least part of the acceleration of atherosclerosis observed in *C. pneumoniae*-infected ApoE−/− mice may be mediated by a general increase in circulating levels of proatherogenic inflammatory cytokines. Circulating IL-12p40 was significantly reduced in uninfected ApoE−/− MyD88−/− mice compared with ApoE−/− mice (Fig. 4A), as we have previously reported (16).

**Infection-induced acceleration of atherosclerosis is associated with increased numbers of activated DCs in aortic sinus plaques**

DCs directly control immune responses that occur during inflammatory diseases such as atherosclerosis (34–36). DCs are present in normal arteries, but the numbers of activated DCs increase as atherosclerosis develops (26, 37–39). We reasoned that after infection of atherosclerosis-prone mice with *C. pneumoniae*, activated DC numbers in the aortic sinus plaques should increase, but this response should be blunted in mice genetically deficient in TLR2, TLR4, or MyD88. To test this, we performed immunohistochemical staining using MIDC-8 Ab to quantitatively measure numbers of mature, activated myeloid DCs as reported by several investigators (27–30). As anticipated, infection of ApoE−/− mice with *C. pneumoniae* led to a significant increase in the numbers of activated, mature DCs in the aortic sinus plaques (p < 0.0005; Fig. 5, A and B). In contrast, *C. pneumoniae*-infected ApoE−/− TLR2−/−, ApoE−/− TLR4−/−, and ApoE−/− MyD88−/− mice showed no such increase following infection and also had significantly lower numbers of activated DCs in the aortic sinus plaques compared with infected ApoE−/− mice (p < 0.0001, Fig. 5, A and B). MIDC-8-positive DCs were also CD11c+ by immunohistochemistry in sequentially obtained slides (data not shown). The striking differences in DC numbers seen between genotypes were not due to an overall reduction in cellularity in the double knockout mice, as the cellularity was comparable between genotypes (data not shown). These data suggest that acceleration of atherosclerosis induced by *C. pneumoniae* infection is accompanied by increased numbers of activated DCs in plaques but, as was the case with lesion size and circulating cytokine levels, this was significantly blocked in ApoE−/− mice with additional genetic deficiencies in TLR2, TLR4, or MyD88.

Shaposhnik et al. (32) recently reported that lack of GM-CSF in hypercholesterolemic mice resulted in smaller lesions and a dramatic decrease in the numbers of DCs in plaques. We therefore reasoned that *C. pneumoniae* infection could stimulate expression of GM-CSF, which in turn might lead to increased recruitment of DCs to plaques and/or increased retention within plaques. To address this possibility, we isolated primary mouse aortic ECs from wild-type or TLR2, TLR4, or MyD88 knockout mice, treated the cells with *C. pneumoniae* (or buffer), and assessed GM-CSF secretion. As anticipated, infection of mouse aortic ECs with *C. pneumoniae* (MOI of 5 and 10) significantly increased GM-CSF release, whereas the genetic absence of TLR2, TLR4, and MyD88 completely abolished the release of the growth factor (Fig. 6A). These results suggest that the differential induction of *C. pneumoniae*-induced GM-CSF production may play a role in the significantly increased accumulation of activated DCs in the aortic sinus plaques that we observed following infection in ApoE−/− mice but not in ApoE−/− TLR2−/−, ApoE−/− TLR4−/−, and ApoE−/− MyD88−/− mice (Fig. 5). Consistent with this interpretation, we also observed significantly increased GM-CSF gene expression in the aortas of ApoE−/− mice infected with *C. pneumoniae* when compared with uninfected littermate
FIGURE 8. GW3965, an LXR agonist, reduces ox-LDL- and C. pneumoniae (Cpn)-induced foam cell formation by peritoneal macrophages. A, Live C. pneumoniae (5 × 10^5 IFU/ml) induced foam cell formation in the presence of ox-LDL (100 μg/ml) in wild-type primary peritoneal macrophages. Representative Oil Red O staining of peritoneal macrophage-derived foam cells. B, Treatment of macrophages with GW3965 (1 μM), an LXR agonist, reduced foam cells produced after stimulation with C. pneumoniae plus ox-LDL. Oil Red O-positive cells were compared with the total amount of macrophages and expressed as the percentage of foam cells from total macrophages. Data are presented as mean values ± SEM; n = 4.

Control 100 μg/ml ox-LDL Live Cpn (MOI=5) +ox-LDL

LXRα deficiency is associated with further acceleration of C. pneumoniae-mediated increase of atherosclerotic lesions in ApoE−/− mice

LXRs orchestrate whole body cholesterol homeostasis, especially macrophage cholesterol metabolism and apoptosis (25). However, LXR activation is negatively influenced by TLR-dependent signaling, and in turn, LXRα suppresses expression of downstream targets of TLR signaling (23). These cross-talk mechanisms can importantly modulate atherosclerosis; hence, mice with genetic deficiency in both LXRα and ApoE develop accelerated atherosclerosis (40), and this can be reversed by the administration of LXR agonists (41). We therefore hypothesized that LXRα and TLR/MyD88 signaling pathways would show reciprocal modulation of C. pneumoniae-induced acceleration of atherosclerosis in ApoE−/− mice. If so, then ApoE−/−LXRα−/− double knockout mice should be particularly susceptible to C. pneumoniae-induced acceleration of atherosclerosis. As expected, uninfected ApoE−/−LXRα−/− compound mutant mice developed increased atherosclerotic lesions in both en face aorta and aortic sinus lesions and increased lipid accumulation in the aortic sinus plaques compared with uninfected ApoE−/−LXRα+/- littermates (Fig. 7, A–C). Infection with C. pneumoniae markedly enhanced aortic sinus lesion area (80% increase; Fig. 7A) and also resulted in a significantly greater accumulation of lipid in lesions in the aortic sinus and in the aorta en face (Fig. 7, B and C). Infection with C. pneumoniae significantly increased serum levels of IL-6 and TNF-α as well (Fig. 4, C and D). This could not be explained by differing levels of serum cholesterol, because serum cholesterol levels in ApoE−/−LXRα−/− and ApoE−/−LXRα+/- were similar regardless of whether mice were infected or not (data not shown). Furthermore, C. pneumoniae-induced acceleration of atherosclerotic plaques in ApoE−/−LXRα−/− mice was not due to increased bacterial infectivity in macrophages or degree of lung infectivity or delayed bacterial lung clearance in ApoE−/−LXRα−/− compared with ApoE−/− mice (data not shown).

LXR signaling significantly reduces C. pneumoniae-induced foam cell formation in vitro

The results in the previous section are consistent with the notion that lack of LXRα facilitated acceleration of atherosclerosis by removing inhibitory constraints against TLR-mediated signaling instigated by C. pneumoniae. However, LXR-dependent signaling also induces reverse cholesterol transport by macrophages. We therefore reasoned that LXR agonists might suppress C. pneumoniae-induced foam cell formation in macrophages in vitro. To further evaluate this possibility, we isolated peritoneal-derived macrophages from wild-type C57BL/6 mice and stimulated them with ox-LDL and live C. pneumoniae. As anticipated, treatment of macrophages with ox-LDL (100 μg/ml) and live C. pneumoniae (5 × 10^5 IFU/ml) induced a significant increase in the formation of foam cells (p < 0.0001; Fig. 8, A and B). However, cotreatment with the LXR agonist GW3965 (1 μM) together with ox-LDL and live C. pneumoniae significantly decreased foam cell formation (Fig. 8, A and B). Similar results were obtained when cells were cotreated with ox-LDL, GW3965, and UV-killed C. pneumoniae (data not shown). These findings suggest that LXR signaling may counter acceleration of atherogenesis and foam cell formation both by promoting cholesterol efflux and by blocking TLR-dependent stimulation of proinflammatory signaling.

Discussion

Although numerous epidemiologic (42–44) and experimental (7, 9, 10) studies link infection with development of atherosclerosis and cardiovascular outcomes, the precise mechanisms contributing to this link have remained elusive. In this study we report data that provide new insight into molecular pathways linking C. pneumoniae infection to accelerated atherosclerosis in murine models. Consistent with previous studies (7, 9, 10), we found that infection...
of atherosclerosis-prone hypercholesterolemic ApoE−/− mice with C. pneumonia resulted in accelerated atherosclerosis associated with increased plaque lipid content, numbers of activated DCs in lesions, and serum levels of proinflammatory cytokines (IL-12p40, MCP-1, TNF-α and IL-6) compared with mock-infected littermate controls. Conversely, in ApoE−/− mice with additional genetic deficiency in TLR2, TLR4, or the common adaptor MyD88, atherosclerotic plaque development was significantly inhibited, and this was accompanied by significant reductions in lesion lipid content, numbers of activated DCs in lesions, and serum levels of proinflammatory cytokines.

It is important to note that not all pathogens seem to be capable of promoting atherogenesis. Atherosclerotic lesions in mice did not develop after infection with Chlamydia trachomatis (45). Furthermore, the atypical bacterial pathogen Mycoplasma pneumoniae, which causes lung pathology similar to that of C. pneumoniae, failed to cause inflammatory changes or induce atherosclerotic lesions in rabbits (46). In contrast, C. pneumoniae-induced acceleration of atherosclerosis requires viable organism replication (47). Other studies using alternative modes of delivery such as local carotid artery application or i.v. injection also indicate that infection with the live bacteria is necessary to promote plaque development (8). Collectively, these data suggest that either pre-existing heat labile factors, organism replication, or perhaps more likely, continued chronic infection and inflammation were essential for C. pneumoniae-induced acceleration of atherosclerosis.

Several investigators proposed that the extent of infectious burden from all pathogenic sources is the key determinant of the impact of pathogens on the atherosclerotic disease process (48–51). Our data suggest a reason why this may be so. Molecular motifs derived from C. pneumoniae are detected by multiple TLRs (52–55). In our study, we clearly show that either TLR2 or TLR4 can mediate the proatherogenic effects of C. pneumoniae infection. Although the ApoE−/−TLR2−/−, ApoE−/−TLR4−/−, and ApoE−/−MyD88−/− double knockout mice displayed a substantially reduced proatherogenic response to infection, the modest 17–30% increases in lesion size we noted are consistent with the interpretation that TLR-independent signaling and/or MyD88-independent signaling may also contribute to some extent to infection-mediated progression of atherosclerosis. Together, these data strongly suggest that C. pneumoniae can exacerbate atherosclerosis in multiple ways that involve innate immune signaling networks.

Recent studies indicate an important role of DCs in atherosclerosis (38). We observed that C. pneumoniae-induced acceleration of atherosclerosis was similarly accompanied by significantly increased numbers of activated DCs accumulating in plaques from infected ApoE-null mice when compared with uninfected littermate controls. No such increase in DC numbers was observed in infected ApoE−/−TLR2−/−, ApoE−/−TLR4−/−, and ApoE−/−MyD88−/− mice. Of interest, Shaposhnik et al. showed that GM-CSF plays a key role in DC migration into lesions and that LDLR−/−GM-CSF−/− mice exhibit both diminished lesion size and decreased DC accumulation in the plaques (32). Accordingly, we observed that C. pneumoniae induces a dose-dependent release of GM-CSF in primary aortic ECs in a TLR2−/−, TLR4−/−, and MyD88-dependent manner. We also observed that GM-CSF gene expression was significantly higher in the aortic tissue as well as aortic sinus lesions obtained from infected ApoE−/− mice compared with uninfected littermate controls. Therefore, it is tempting to propose that the differential GM-CSF induction by C. pneumoniae observed in wild-type ECs vs TLR2−/−, TLR4−/−, or MyD88−/− ECs may play a role in recruiting and/or retaining DCs in developing plaques and may provide an additional explanation for how C. pneumoniae accelerates plaque formation. Although we used an EC infection model for these studies to show that C. pneumoniae can induce GM-CSF in vitro, the mechanism of GM-CSF induction during in vivo infection may be different and may be indirectly modulated by the induced cytokines.

Our data also provide important new evidence that suggests that LXR activation integrates cholesterol homeostasis with innate immune host defenses in a manner that can critically modulate atherogenesis. When mice with genetic deficiency of both ApoE and LXRα were challenged with C. pneumoniae, markedly accelerated atherosclerosis occurred that was significantly greater than that observed in either ApoE−/−LXRα−/− littermate controls or uninfected ApoE−/−LXRα−/− controls. In vitro studies with peritoneal-derived cells obtained from wild-type mice showed that treatment with GW3965, an LXR agonist, inhibited foam cell formation induced by ox-LDL and live C. pneumoniae. Collectively then, our data indicate the following: 1) C. pneumoniae infection does indeed accelerate atherogenesis in hypercholesterolemic mice in a TLR/MyD88-dependent manner, perhaps in part through a significant increase in serum cytokine levels and accumulation of activated DCs in the aortic sinus lesions; and 2) proatherogenic effects of C. pneumoniae infection are substantially enhanced with genetic deficiency of LXRα.

Previous reports indicate that signaling triggered by TLR3 or TLR4 can inhibit expression of LXR target genes (23). In contrast, C. pneumoniae promotes foam cell formation in a TLR2-dependent manner, but this can be counteracted by treatment with LXR agonists (52). Gain-of-function and loss-of-function studies both indicate that LXRα activation retards atherogenesis by promoting cholesterol efflux (40). The model that emerges suggests that TLR-dependent signaling and LXR activity reciprocally modulate one another. Our results extend this theme and show that when this balance is tipped by C. pneumoniae-induced innate immune signaling, acceleration of atherosclerosis occurs. Acceleration of atherosclerosis in LXR-deficient mice cannot be attributed to compromised host immune defenses, because in vitro infectivity of ApoE−/−LXRα−/− and ApoE−/−LXRα−/− macrophages with C. pneumoniae was similar (data not shown). Instead, acceleration of atherosclerosis in ApoE−/−LXRα−/− mice was probably due at least in part to defective cholesterol efflux by macrophages. Hence, our data suggest that infection in the setting of hypercholesterolemia and lack of LXRα signaling promotes atherosclerosis most likely by two mechanisms: defective reverse cholesterol transport and inflammation instigated by C. pneumoniae that in turn triggers TLR-dependent signaling.

We demonstrate here that C. pneumoniae can directly influence atherosclerotic plaque development in a TLR2−/−, TLR4−/−, and MyD88-dependent manner by promoting foam cell formation and enhancing recruitment and/or retention of activated DCs in plaques. In contrast, LXR signaling may counteract C. pneumoniae-induced atherosclerosis in at least two ways: by decreasing foam cell formation via promotion of cholesterol efflux and by blocking TLR-dependent stimulation of proinflammatory signaling. Our data thus suggest a close interaction between cholesterol homeostatic pathways orchestrated by LXRs and innate immune host defenses such asTLRs. This reciprocal relationship may become skewed in favor of inflammation (and hence accelerated atherosclerosis) when infection with C. pneumoniae occurs, at least in the setting of hypercholesterolemia. Whether other pathogens that have been linked to atherosclerosis might similarly alter this balance will be an intriguing question to be addressed by future studies.

Disclosures
The authors have no financial conflict of interest.