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# *Chlamydia pneumoniae*-Induced Foam Cell Formation Requires MyD88-Dependent and -Independent Signaling and Is Reciprocally Modulated by Liver X Receptor Activation<sup>1</sup>

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*Chlamydia pneumoniae* is detected by macrophages and other APCs via TLRs and can exacerbate developing atherosclerotic lesions, but how that occurs is not known. Liver X receptors (LXRs) centrally control reverse cholesterol transport, but also negatively modulate TLR-mediated inflammatory pathways. We isolated peritoneal macrophages from wild-type, TLR2, TLR3, TLR4, TLR2/4, MyD88, TRIF, MyD88/TRIF, and IFN regulatory factor 3 (IRF3) KO mice, treated them with live or UV-killed *C. pneumoniae* in the presence or absence of oxidized LDL, then measured foam cell formation. In some experiments, the synthetic LXR agonist GW3965 was added to macrophages infected with *C. pneumoniae* in the presence of oxidized LDL. Both live and UV-killed *C. pneumoniae* induced IRF3 activation and promoted foam cell formation in wild-type macrophages, whereas the genetic absence of TLR2, TLR4, MyD88, TRIF, or IRF3, but not TLR3, significantly reduced foam cell formation. *C. pneumoniae*-induced foam cell formation was significantly reduced by the LXR agonist GW3965, which in turn inhibited *C. pneumoniae*-induced IRF3 activation, suggesting a bidirectional cross-talk. We conclude that *C. pneumoniae* facilitates foam cell formation via activation of both MyD88-dependent and MyD88-independent (i.e., TRIF-dependent and IRF3-dependent) pathways downstream of TLR2 and TLR4 signaling and that TLR3 is not involved in this process. This mechanism could at least partly explain why infection with *C. pneumoniae* accelerates the development of atherosclerotic plaque and lends support to the proposal that LXR agonists might prove clinically useful in suppressing atherogenesis. *The Journal of Immunology*, 2008, 181: 7186–7193.

Numerous studies link *Chlamydia pneumoniae* infection with atherosclerosis in both humans (1) and experimental animal models (2–10), but much less is understood about the cellular and molecular mechanisms involved. *C. pneumoniae* can infect and survive in endothelial cells, smooth muscle cells (11–13), circulating monocytes, and tissue macrophages and can migrate from the lungs to developing plaque via circulating lymphocytes as well as macrophages (14–16). Macrophages are thought to be central to the pathobiology of atherosclerosis because they tend to be retained in developing atheroma, where they can express an array of proinflammatory chemokines and cytokines that promote plaque progression and plaque instability that often leads to clinical events such as myocardial infarction and stroke (17–19). In plaques, macrophages ingest necrotic cellular debris and modified lipids, which in turn triggers expression and secretion of proinflammatory molecules. Such lipid-laden macrophages are known as foam cells, owing to the foam-like appearance of their cytoplasm that is composed predominantly of accumulated lipid-filled vacuoles. Because macrophages can become infected with *C. pneumoniae*, one possibility is that ingested

pathogen or molecules derived from dead organisms or both might accelerate atherogenesis by somehow promoting formation of macrophage foam cells.

*C. pneumoniae* can be detected by innate immune pattern recognition receptors such as TLRs. Molecules derived from *C. pneumoniae* that are foreign to the host interact with several TLRs, including TLR2 and TLR4. Upon stimulation by ligands, these TLRs activate gene programs that involve expression and secretion of proinflammatory cytokines. Most TLRs (including TLR2 and TLR4) use the downstream adaptor MyD88 to transmit their signals. TLR3 relies exclusively upon Toll/IL-1R domain-containing adapter inducing IFN- $\beta$  (TRIF)<sup>3</sup> (20), which is a cytoplasmic adaptor that relays signals independently of MyD88 (21, 22). TLR4 is unique among TLRs because it is the only TLR that can signal via either the MyD88-dependent or the MyD88-independent pathway and that requires TRIF and TRAM (23). The MyD88-dependent pathway leads to activation of the NF- $\kappa$ B transcription factor, which directly controls proinflammatory genetic programs, but the primary target of the MyD88-independent pathway is IFN regulatory factor (IRF) 3 (IRF3), which controls genes that produce a response distinct from that of NF- $\kappa$ B-dependent programs.

To date, 13 murine and 11 human TLRs have been identified (24–26). Macrophages express an array of TLRs, including TLR2, TLR3, TLR4, and others (24–26). Reports from our laboratory (7, 27) and others (28–31) indicate that innate immune signaling via TLRs directly contributes to development of atherosclerotic

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<sup>3</sup> Abbreviations used in this paper: TRIF, Toll/IL-1R domain-containing adapter inducing IFN- $\beta$ ; ABCA1, ATP-binding cassette transporter 1; IRF, IFN regulatory factor; KO, knockout; LXR, liver X receptor; MOI, multiplicity of infection; ox-LDL, oxidized low-density lipoprotein.

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plaque. Moreover, molecules derived from *C. pneumoniae* are detected by TLR2 and TLR4, and signaling emanating from both these receptors triggers inflammation and promotes atherosclerosis (7, 27). Genetic loss-of-function approaches indicate that both TLR2 and TLR4 are involved in host responses to *C. pneumoniae* infection involving both MyD88-dependent and -independent signaling pathways (32, 33). Prior studies have shown that *C. pneumoniae* can induce foam cell formation in macrophages (3, 34) and that TLRs may play a role in this process (35). Collectively, these data suggest the possibility that *C. pneumoniae* infection might promote development of a proinflammatory phenotype that in turn would be expected to exacerbate atherosclerosis.

Metabolites of cholesterol bind to nuclear receptors called liver X receptors (LXRs) that play a central role in lipid metabolism and are master regulators of cholesterol metabolism (36). LXRs centrally control reverse cholesterol metabolism and also counterbalance the proinflammatory effects of TLRs and down-modulate TLR4-mediated NF- $\kappa$ B activation (36, 37). TLR-mediated innate immune responses and LXR-directed regulation of cholesterol metabolic pathways influence one another bidirectionally. Stimulation of macrophages with LPS and LXR agonists reduce proinflammatory responses by a mechanism that is MyD88 dependent (37). Hence, LXRs suppress TLR signaling; conversely, TLR signaling reciprocally inhibits LXR activation (38). Indeed, stimulation of TLR3 or TLR4 by pathogen-derived ligands inhibits expression of LXR-dependent gene targets and macrophage cholesterol efflux. This effect proceeds via a MyD88-independent mechanism and involves IRF3 (38). Thus, LXR-TLR cross-talk provides a potential mechanism to explain how microbial infections might interfere with cholesterol metabolism and contribute to cardiovascular disease. These results underscore the intimate association of lipid metabolic pathways with innate immune host defenses (36, 38–40), both of which are central to the pathobiology of atherosclerotic plaque development (17).

These considerations led us to hypothesize that *C. pneumoniae* might promote development of foam cells by triggering proinflammatory TLR-dependent signaling, but that this would be inhibited by activation of LXRs. Using a genetic loss-of-function approach, we report that in the presence of oxidized low-density lipoprotein (ox-LDL), *C. pneumoniae* promotes foam cell formation by interacting not only with TLR2 but also with TLR4 to trigger activation of NF- $\kappa$ B and IRF3 and can therefore use both the MyD88-dependent and the MyD88-independent pathways. This was confirmed using macrophages derived from TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, TLR2<sup>-/-</sup>:TLR4<sup>-/-</sup> double knockout (KO), MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, IRF3<sup>-/-</sup>, and MyD88<sup>-/-</sup>:TRIF<sup>-/-</sup> double KO mice. However, signaling emanating from TLR3 was not involved. We also found that *C. pneumoniae*-induced foam cell formation was blocked by pharmacologic LXR agonist treatment. These results therefore elucidate molecular mechanisms that link host defenses with lipid metabolic pathways and implicate both in a potential explanation as to how *C. pneumoniae* might accelerate atherosclerosis on a cellular and molecular level.

## Materials and Methods

### Generation of KO mice

C57BL/6 wild-type, TLR2<sup>-/-</sup>, and TRIF<sup>Lps2/Lps2</sup> mice were purchased from The Jackson Laboratory. MyD88<sup>-/-</sup> and TLR4<sup>-/-</sup> mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan), TLR2<sup>-/-</sup>:TLR4<sup>-/-</sup> double KO mice were obtained from Dr. C. Wilson (University of Washington, Seattle, WA), and IRF3<sup>-/-</sup> and TLR3<sup>-/-</sup> mice were provided from Dr. G. Cheng (University of California, Los Angeles, CA). A homogenous population of these mice was established by backcrossing onto the C57BL/6 background for at least eight generations as previously described (27). The MyD88<sup>-/-</sup>:TRIF<sup>-/-</sup> double KO mice were estab-

lished in our laboratory by crossing the MyD88<sup>-/-</sup> with TRIF<sup>Lps2/Lps2</sup> mice. Mice were fed a standard chow diet and housed under pathogen-free conditions at Cedars-Sinai Medical Center (Los Angeles, CA). All animal experiments were performed under protocols that had been approved by the Institutional Animal Care and Use Committee at our facility.

### Preparation of peritoneal macrophages

Native peritoneal macrophages were isolated by injecting HBSS (Invitrogen) into the peritoneal cavity of mice immediately after euthanization. Resident peritoneal cells from three or four mice were pooled and washed, seeded in 24-well plates ( $2.5 \times 10^5$  cells/well) in RPMI 1640 medium (Cellgro) with 10% FBS, incubated at 37°C in 5% CO<sub>2</sub> for 3 h, washed twice with HBSS to remove nonadherent cells, and cultured for 24 h before treatment.

### Infection of macrophages

*C. pneumoniae* CM-1 strain (American Type Culture Collection) was propagated in Hep-2 cells as previously described (41). *Mycoplasma* contamination was assessed by PCR (42). Inocula of *C. pneumoniae* were expressed as multiplicities of infection (MOI). UV inactivation of *C. pneumoniae* was performed by exposure of 150  $\mu$ l in a sterile tissue culture dish in a laminar flow hood to shortwave UV light at a distance of 7 cm for 25 min, with mixing after 15 min. The absence of live *C. pneumoniae* was confirmed by infecting Hep-2 cells with the bacteria. Murine peritoneal macrophages were infected with live or UV-killed *C. pneumoniae* (MOI = 5) overnight. Cells were washed and incubated with human ox-LDL (100  $\mu$ g/ml); thiobarbituric acid-reactive substances, 18.4 nmol of MDA per mg of protein; Biomedical Technologies, MA) for an additional 24 h in the presence or absence of an LXR agonist, GW3965 (2  $\mu$ M) (35). Trypan blue staining showed that none of the treatment affected macrophage viability at 24 h (>95% of the macrophages were viable).

### Assessment of foam cell formation

Foam cells were identified using Oil Red O staining. Peritoneal macrophages were washed twice with PBS and then fixed and stained with 1% of Oil Red O solution (60% isopropanol; Sigma-Aldrich). Cells were washed three times with PBS and examined by light microscopy (original magnification:  $\times 40$ ). Designation of a macrophage as a foam cell required positive Oil Red O staining. Foam cells were quantified by light microscopy by the same investigator who was blinded to the genotypes of the cells with Image-Pro Plus 5.1 software program. The foam cells were defined as cells with intracellular Oil Red O-positive droplets and were expressed as the percentage of positive Oil Red O cells to total cells (area of stained cells vs the total cytoplasm area: >20%). Each treatment condition was performed in triplicate.

### NF- $\kappa$ B activity assay

RAW 264.7 cells were stably transfected with an NF- $\kappa$ B reporter plasmid (ELAM-luciferase, where ELAM is endothelial leukocyte adhesion molecule) (43). Cells were seeded onto 96-well plates ( $1 \times 10^5$  cells/well) and treated 24 h later for 4 h before any measurement of luciferase activity. Cells were stimulated with live or UV-killed *C. pneumoniae* (MOI = 5), or LPS (0.01  $\mu$ g/ml) with or without ox-LDL (100  $\mu$ g/ml) in the presence or absence of the LXR agonist GW3965 (2  $\mu$ M). After 4 h, cells were washed with PBS and lysed using a lysis buffer (Promega). Luciferase activity was measured by means of the Dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. Data were expressed as mean values of three independent experiments, with each treatment performed in triplicate.

### IRF3 activity assay

The DNA-binding capacity of IRF3 was determined by using the TransAM transcription factor assay kit (Active Motif). The nuclear extract was subjected to the binding of IRF3 to an immobilized consensus sequence in a 96-well plate. Primary and secondary Abs were added as suggested by the manufacturer's instructions. After the colorimetric reaction, the samples were measured in a spectrophotometer.

### Statistical analysis

Data are reported as mean  $\pm$  SEM. Statistical differences were assessed by Student's *t* test between two groups and one-way ANOVA for three or more groups, and values of *p* < 0.05 were considered significant.

## Results

### *C. pneumoniae* promotes the formation of foam cells in the presence of ox-LDL

Peritoneal macrophages from wild-type (C57BL/6) mice were pre-infected with live *C. pneumoniae* or UV-killed *C. pneumoniae* (MOI = 5) and then treated with ox-LDL (100  $\mu\text{g/ml}$ ). Foam cell formation was characterized by the engulfment of positive Oil Red O lipid droplets in the macrophages. Foam cell formation was significantly induced by the presence of live *C. pneumoniae* (Fig. 1, A and B) or UV-killed *C. pneumoniae* (Fig. 1B) and ox-LDL compared with the bacteria or ox-LDL alone. To evaluate the effect of LXRs on foam cell formation induced by the addition of *C. pneumoniae* and ox-LDL, we coadministered the LXR agonist GW3965 (2  $\mu\text{M}$ ). GW3965 treatment significantly decreased foam cell formation induced by live or UV-killed *C. pneumoniae* (Fig. 1C).

### *C. pneumoniae* induces foam cell formation via activation of either TLR2 or TLR4

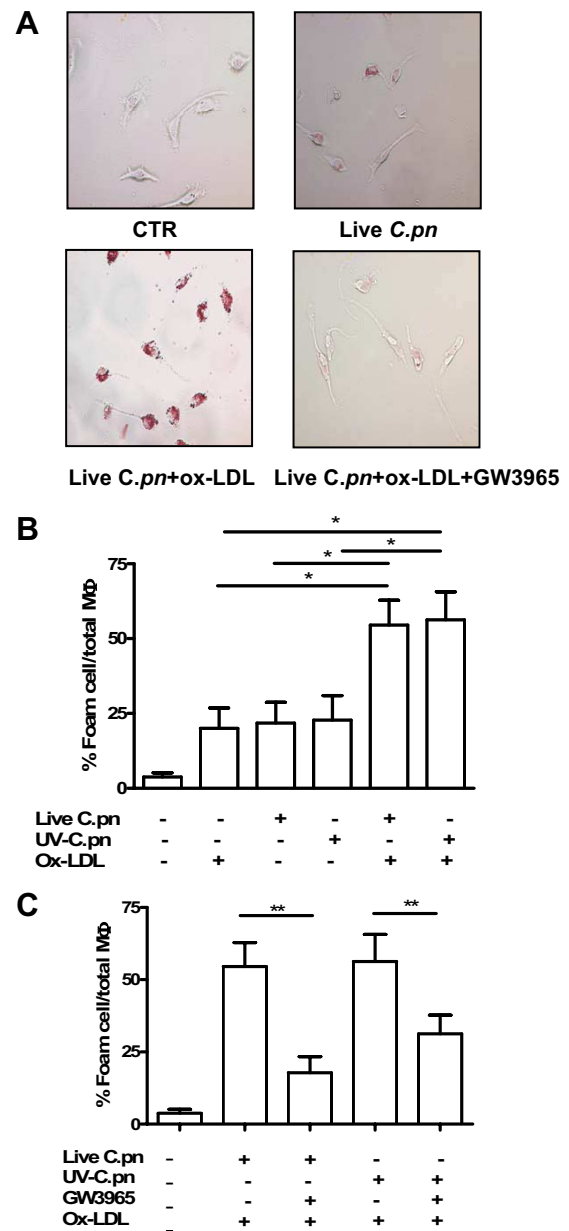
*C. pneumoniae* infection can trigger both TLR2- and TLR4-dependent host responses (33, 44). We therefore isolated peritoneal macrophages from TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, and TLR2<sup>-/-</sup>:TLR4<sup>-/-</sup> mice. Genetic deficiency in either TLR2 or TLR4 significantly reduced foam cell formation from macrophages treated with either live (Fig. 2A) or UV-killed *C. pneumoniae* (Fig. 2B) in the presence of ox-LDL. Although the double deficiency of TLR2 and TLR4 further reduced foam cell formation from macrophages treated with UV-killed *C. pneumoniae* (Fig. 2B), this did not reach significance. These results indicate that molecules derived from *C. pneumoniae* can promote foam cell formation by triggering signaling through both TLR2 and TLR4. There were no significant differences between live and UV-killed *C. pneumoniae*-induced foam cell formation.

### *C. pneumoniae* promotes foam cell formation by activating both MyD88-dependent and MyD88-independent signaling pathways

MyD88, an adaptor molecule used by both the TLR2 and TLR4 signaling pathways, plays an essential role in *C. pneumoniae*-induced infection (32). Although TLR2 relies exclusively upon MyD88, TLR4 can signal through both MyD88-dependent and MyD88-independent (TRIF-IRF3) pathways. To understand whether *C. pneumoniae*-mediated foam cell formation was mediated by a MyD88-dependent or MyD88-independent pathway, we isolated peritoneal macrophages from MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, and MyD88<sup>-/-</sup>:TRIF<sup>-/-</sup> double KO mice and compared them with wild type-derived peritoneal macrophages. The loss of either MyD88 or TRIF resulted in a significant reduction in the formation of foam cells induced by either live (Fig. 2C) or UV-killed (Fig. 2D) *C. pneumoniae* in the presence of ox-LDL. These results are most consistent with the interpretation that in the presence of ox-LDL, *C. pneumoniae* promotes formation of foam cells by activating both the MyD88-dependent and MyD88-independent and TRIF/IRF3-dependent signaling pathways. Although there was a tendency for lower baseline foam cell formation in TRIF<sup>-/-</sup> or MyD88<sup>-/-</sup> cells in response to ox-LDL alone, this difference did not reach statistical significance.

### LXR agonist (GW3965) reduces *C. pneumoniae*-induced NF- $\kappa$ B activation

Stimulation of the LXR pathway inhibits NF- $\kappa$ B activation and TLR-dependent signaling and also activates cholesterol efflux by inducing the ATP-binding cassette transporter 1 (ABCA1) gene (38). We therefore predicted that treatment with the LXR agonist

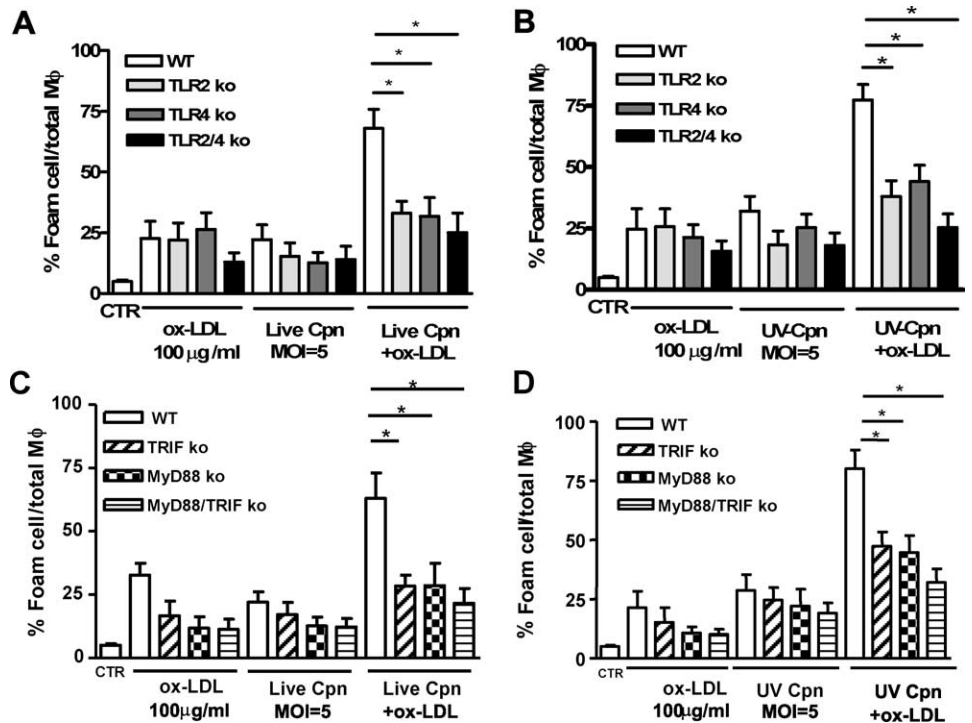


**FIGURE 1.** *C. pneumoniae* (C.pn) promotes the formation of foam cells in the presence of ox-LDL from wild type-derived macrophages. **A**, Representative formation of foam cells following *C. pneumoniae* (MOI = 5) or ox-LDL (100  $\mu\text{g/ml}$ ) alone or in combination. CTR, Control. **B**, The amount of foam cells was significantly increased following live ( $p < 0.05$ ) or UV-killed ( $p < 0.05$ ) *C. pneumoniae* and ox-LDL administration. **C**, The treatment with GW3965 (2  $\mu\text{M}$ ) significantly reduced foam cell formation induced by the addition of ox-LDL and live ( $p < 0.01$ ) or UV-killed ( $p < 0.01$ ) *C. pneumoniae*. Foam cells are expressed as percentage of positive Oil Red O cells compared with total macrophages (M $\phi$ ). Data represent mean  $\pm$  SEM, experiments were repeated in four times in triplicate. Statistical difference was evaluated by means of one-way ANOVA followed by Tukey's posttest (**B**) and by Student's *t* test (**C**); \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

GW3965 would attenuate foam cell formation induced by ox-LDL and *C. pneumoniae*. We stably transfected the RAW264.7 murine macrophage cell line with an NF- $\kappa$ B luciferase reporter plasmid (RAW-NF- $\kappa$ B). Cells were stimulated with live or UV-killed *C. pneumoniae* and ox-LDL. LPS (0.01  $\mu\text{g/ml}$ ), TLR4 ligand, and Pam<sub>3</sub>CSK<sub>4</sub> (1  $\mu\text{g/ml}$ ; a TLR2 ligand) were used as positive controls (data not shown). The activation of the NF- $\kappa$ B reporter



**FIGURE 2.** Live or UV-killed *C. pneumoniae* (Cpn)-induced foam cell formation is TLR2, TLR4, MyD88, and TRIF dependent. *A* and *B*, Peritoneal macrophages of wild type (WT), TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup> and TLR2<sup>-/-</sup>:TLR4<sup>-/-</sup> mice were treated with live (*A*) or UV killed (*B*) *C. pneumoniae* (MOI = 5) with or without ox-LDL (100 μg/ml). *C* and *D*, The same experimental protocol was applied to macrophages derived from MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, and MyD88<sup>-/-</sup>:TRIF<sup>-/-</sup> mice (*C*, live *C. pneumoniae*; *D*, UV-killed *C. pneumoniae*). Foam cells are expressed as percentage of positive Oil Red O cells compared with total macrophages (Mφ). Data represent mean ± SEM; experiments were repeated three times in triplicate. Statistical difference, denoted as \* for *p* < 0.05, was evaluated by means of Student's *t* test. CTR, Control.

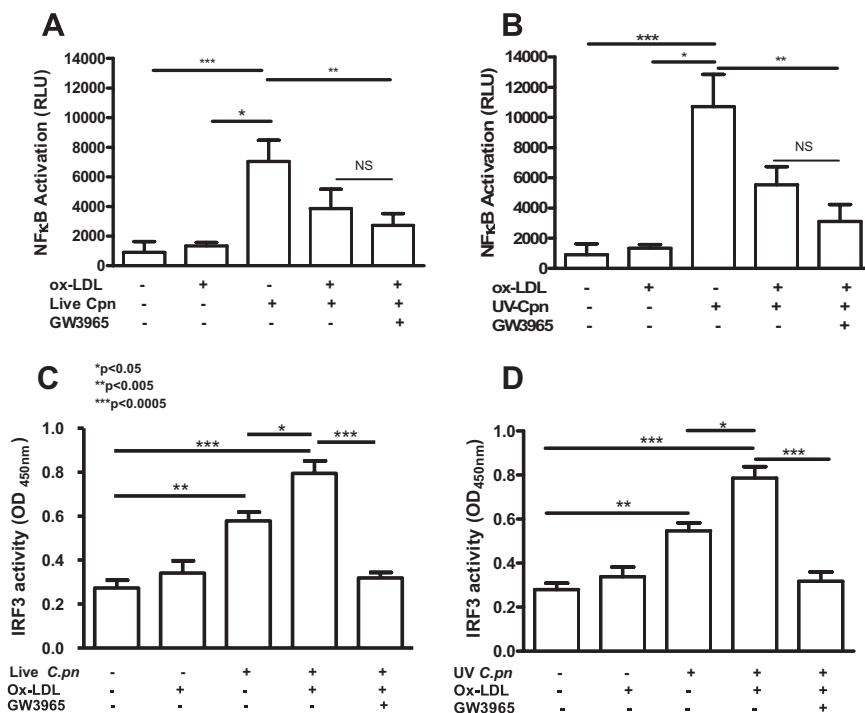


ELAM-luciferase was measured. Compared with untreated cells, there was no change in NF-κB activation after treatment with ox-LDL alone (Fig. 3, *A* and *B*). However, live or UV-killed *C. pneumoniae* infection significantly increased NF-κB activation (Fig. 3, *A* and *B*). Interestingly, treatment with ox-LDL together with infection with either live or UV-killed *C. pneumoniae* decreased NF-κB activation (Fig. 3, *A* and *B*), as was reported by others as well (45). Indeed, Robbesyn et al. reported that ox-LDL may exert a biphasic effect on NF-κB activation, where low concentrations for a short period of time may be stimulatory while higher concentrations may be inhibitory (46). As expected, the addition of the LXR agonist GW3965 de-

creased significantly NF-κB activation between *C. pneumoniae* alone vs *C. pneumoniae* plus ox-LDL and GW3965 (Fig. 3, *A* and *B*).

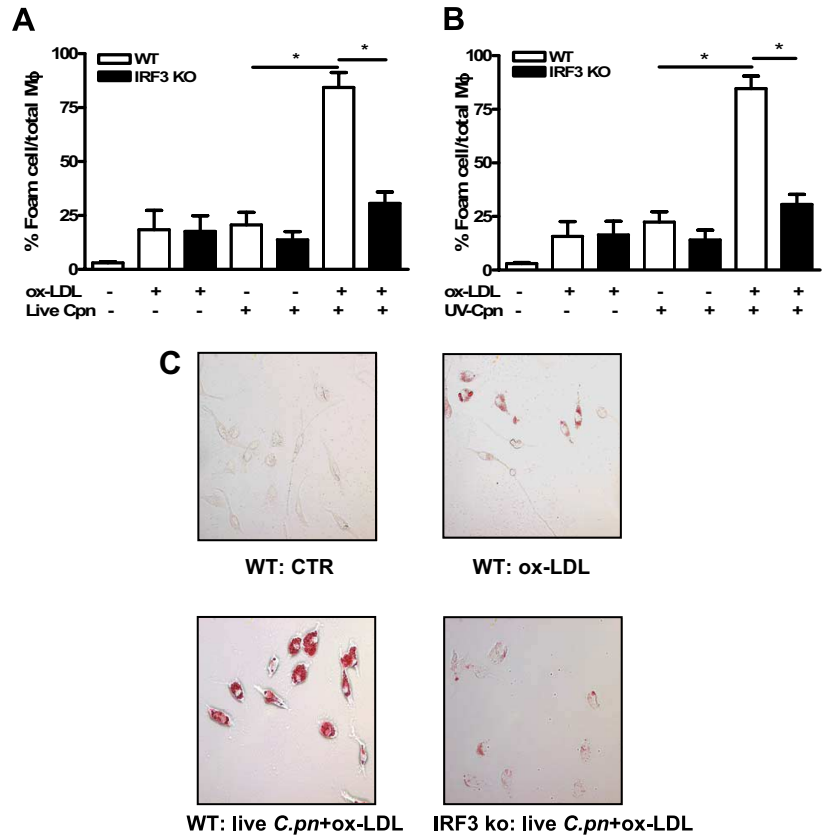
*C. pneumoniae*-induced foam cell formation uses the TRIF/IRF3 pathway in macrophages; modulation of IRF3 activity by the LXR agonist GW3965

Previous reports suggest that *Chlamydia trachomatis* activates the IRF3 pathway in a MyD88-independent manner (i.e., TRIF-dependent pathway) (47), as well as in a MyD88-dependent manner (48). However, the role of IRF3 in *C. pneumoniae*-induced responses and in particular foam cell formation has not been studied. First,



**FIGURE 3.** *C. pneumoniae* (Cpn) induced NF-κB and IRF3 activation and modulation by the LXR agonist GW3965. *A* and *B*, NF-κB activation with live (*A*) or UV-killed (*B*) *C. pneumoniae* in the presence of ox-LDL. The treatment with GW3965 further reduced NF-κB activation. RLU, Relative light unit. *C* and *D*, Detection of IRF3 activation following live (*C*) or UV-killed (*D*) *C. pneumoniae* and ox-LDL. GW 3965 significantly reduced the IRF3 activation (*C* and *D*, *p* < 0.001 and *p* < 0.001, respectively). Data represent mean ± SEM; experiments were repeated three times in triplicate. Statistical difference, denoted as \*, *p* < 0.05, \*\*, *p* < 0.005, and \*\*\*, *p* < 0.0005 as evaluated by means of one-way ANOVA followed by Tukey's *posttest*.

**FIGURE 4.** *C. pneumoniae* (Cpn)-mediated foam cell formation requires IRF3 activation as IRF3<sup>-/-</sup> macrophages (MΦ) are resistant to infection-mediated foam cell production. *A* and *B*, Peritoneal macrophages from IRF3<sup>-/-</sup> mice and wild-type (WT) mice were stimulated with live (*A*) or UV-killed (*B*) *C. pneumoniae* and ox-LDL. *C*, Representative picture of foam cells induced in peritoneal macrophages obtained in wild-type mice compared with those obtained from IRF3<sup>-/-</sup> mice in the presence of live *C. pneumoniae* and ox-LDL. Data represent mean ± SEM; experiments were repeated three times in triplicate. Statistical difference, denoted as \* for  $p < 0.05$ , was evaluated by means of one-way ANOVA followed by Tukey's posttest.

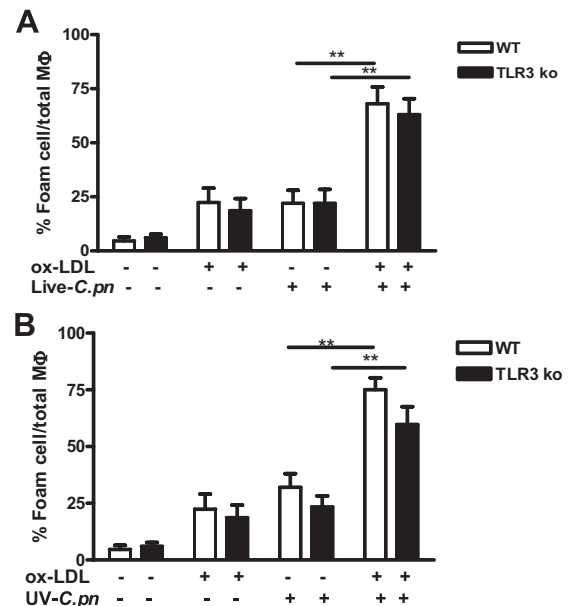


we investigated whether *C. pneumoniae* induces IRF3 activity in macrophages by using an in vitro IRF3 activity assay. Infection with live *C. pneumoniae* or stimulation with UV-killed *C. pneumoniae* increased significantly IRF3 activity in wild-type macrophages, which was further increased in the presence of ox-LDL (Fig. 3, *C* and *D*). Surprisingly, the addition of the LXR agonist GW3965 to live or UV-killed *C. pneumoniae* significantly inhibited IRF3 activation (Fig. 3, *C* and *D*), suggesting a bidirectional interaction between the IRF3 and LXR pathways. To investigate the role of the IRF3 activation pathway in *C. pneumoniae*-induced foam cell formation, we isolated IRF3<sup>-/-</sup> peritoneal macrophages and treated them in the same manner as in experiments described above. IRF3 deficiency completely inhibited the formation of foam cells in response to either live (Fig. 4*A*) or UV-killed (Fig. 4*B*) *C. pneumoniae* in the presence of ox-LDL. Peritoneal macrophages obtained from IRF3<sup>-/-</sup> mice were resistant to *C. pneumoniae*-induced foam cell formation as compared with wild-type macrophages as seen in a representative picture (Fig. 4*C*).

#### *C. pneumoniae*-induced foam cell formation does not involve TLR3

Results in the previous section demonstrate that *C. pneumoniae* uses TRIF to activate IRF3. TLR4 can signal through either MyD88 or TRIF; however, TLR3 signaling is completely dependent upon TRIF (21, 49). Results summarized above indicate that TLR4 signaling was involved, but it is also possible that molecules derived from *C. pneumoniae* stimulated foam cell formation by triggering signaling via both TLR4 and TLR3. To clarify this issue, we isolated peritoneal macrophages from TLR3<sup>-/-</sup> mice and treated them with live or UV-killed *C. pneumoniae* in the presence or absence of ox-LDL. Formation of foam cells was not affected by the genetic absence of TLR3 as compared with macrophages derived from wild-type mice when either live (Fig. 5*A*) or UV-killed

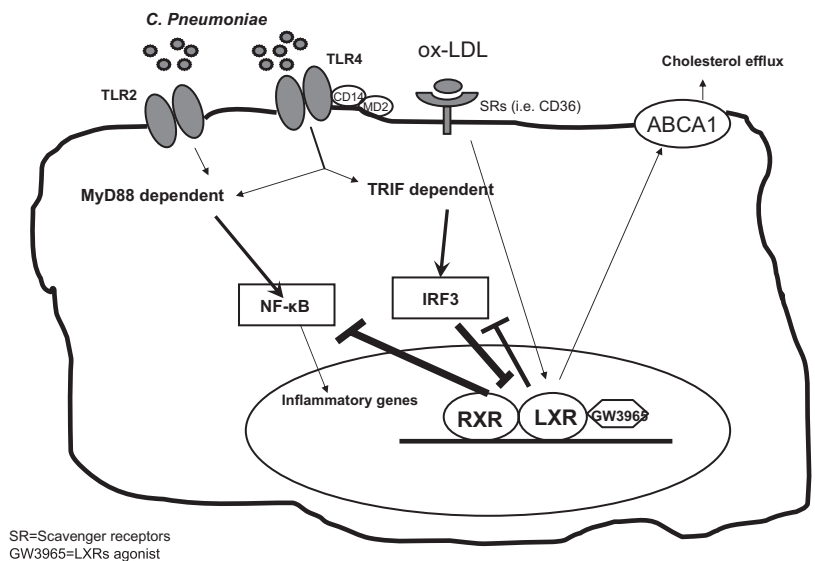
(Fig. 5*B*) *C. pneumoniae* was added to ox-LDL. Furthermore, TLR3 was not essential for ox-LDL or *C. pneumoniae* alone or together to promote formation of Oil Red O-positive foam cells.



**FIGURE 5.** *C. pneumoniae* (Cpn)-mediated foam cell production does not involve TLR3 signaling. Peritoneal macrophages (MΦ) derived from wild-type (WT) or TLR3<sup>-/-</sup> mice were stimulated with live (*A*) or UV-killed (*B*) *C. pneumoniae*, and the percentages of Oil Red O-positive stained cells were calculated to measure foam cell formation. Data represent mean ± SEM; experiments were repeated three times in triplicate. Statistical difference, denoted as \*\* for  $p < 0.01$ , was evaluated by means of one-way ANOVA followed by Tukey's posttest.

### **Chlamydia pneumoniae-Induced Foam Cell Formation**

**FIGURE 6.** Schematic representation of *C. pneumoniae*-mediated foam cell formation. *C. pneumoniae* infection mediates foam cell formation through at least two signaling pathways: TLR2- and TLR4-dependent signaling through MyD88, and TLR4-TRIF-dependent signaling. The MyD88 pathway leads to activation of NF- $\kappa$ B, and the TRIF pathway leads to activation of IRF3. The addition of ox-LDL increases *C. pneumoniae*-promoted foam cell formation and activates NF- $\kappa$ B and IRF3 signaling pathways. LXR agonists like GW3965 reverse *C. pneumoniae*-induced foam cell formation by increasing cholesterol efflux via ABCA1 expression. Others have shown that TLRs that can activate TRIF and IRF3 inhibit LXR responses (38), and we show that the LXR agonist GW3965, in turn, can inhibit *C. pneumoniae*-induced IRF3 activation, suggesting a bidirectional cross-talk. Thus, LXR activation can negatively modulate both TLR/MyD88- and TLR/TRIF-dependent innate immune signaling networks.



Hence, these results exclude the possibility that TLR3 is involved in the formation of foam cells induced by *C. pneumoniae*.

## Discussion

Numerous studies indicate that the retention of lipids in developing plaques and the formation of lipid-laden macrophages (i.e., foam cells) are hallmarks of the atherosclerotic process (17, 18). Clinical and experimental studies suggest that infection with *C. pneumoniae* exacerbates atherosclerosis (50), but the cellular and molecular signaling mechanisms involved are unclear. In this study we report that accumulation of lipids in macrophages and the consequent formation of foam cells is induced by infection of macrophages with *C. pneumoniae* or even simply treatment of macrophages with UV-killed organisms in the presence of ox-LDL. This process was mediated by signaling through either TLR2, TLR4, or both, used both the MyD88-dependent and MyD88-independent (TRIF/IRF3) pathways, and activated both NF- $\kappa$ B and IRF3, but did not involve TLR3 signaling. Foam cell formation promoted by *C. pneumoniae* was inhibited through activation of the LXR pathway by a synthetic agonist. Hence, our results provide important insights into the specific innate immune signaling mechanisms linking infection by *C. pneumoniae* with facilitation of plaque development and, in addition, underscore the close interconnections of host immune defenses with cholesterol metabolic pathways (36, 51) in the pathobiology of atherosclerosis.

Our results confirm and significantly extend previous work by Byrne and coworkers (3, 34, 35, 52). These investigators first reported that *C. pneumoniae* infection of human monocyte-derived macrophages (34) or murine RAW264.7 cells (3) in the presence of LDL causes accumulation of cholesteryl esters by macrophages, thus leading to foam cell formation, and that uptake of cholesterol occurred independently of the LDL receptor (52). Conversely, Blessing and colleagues reported that, once formed, foam cells poorly support the growth of *C. pneumoniae*, but when foam cells are exposed to *C. pneumoniae*, the secretion of proinflammatory cytokines is similar to that of macrophages that have not yet become foam cells (45). Very recent electron microscopic data indicate that oxidized lipids and *C. pneumoniae* colocalize in foam cells obtained from human carotid arteries, and these data document the amalgamation of *C. pneumoniae* inclusions with lipid droplets (53). Similarly, our results also show that infection of

peritoneal macrophages with either the live or UV-killed pathogen induces foam cell formation. Clearly then, data reported thus far in both human and murine cell culture studies indicate that *C. pneumoniae* promotes foam cell formation, at least in the context of a lipid-rich environment, and our data now provide the molecular signaling pathways involved in this process that may play a role in the acceleration of atherosclerosis.

More recent studies have begun to shed light on the participation of innate immune signaling networks in this proatherogenic process. Cao et al. infected murine RAW264.7 macrophages with *C. pneumoniae* and also treated cells with known TLR2 or TLR4 ligands (Pam<sub>3</sub>CSK<sub>4</sub> and LPS, respectively) in the presence of LDL (35). They reported that either treatment induced foam cell formation, and experiments with TLR2-deficient cells appeared to implicate TLR2 as the primary signaling pathway that instigates *C. pneumoniae*-mediated foam cell formation (35). Our results with TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> primary peritoneal macrophages confirmed the involvement of TLR2, but also showed that TLR4 is involved as well. Furthermore, based on results obtained with MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, IRF3<sup>-/-</sup>, and MyD88<sup>-/-</sup>:TRIF<sup>-/-</sup> macrophages, *C. pneumoniae* in the presence of ox-LDL triggered signals to activate both the MyD88-NF- $\kappa$ B pathway and the TRIF/IRF3 downstream effectors. Although Cao et al. did not find evidence for TLR4 involvement (35), it might be pertinent to note that their study used the GG2EE macrophage cell line derived from C3H/HeJ mice that express a mutant TLR4 containing a proline-to-histidine mutation at amino acid residue 714. Hence, C3H/HeJ mice and cells isolated from these mice do not respond normally to TLR4 ligands. However, our approach was more direct and instead used macrophages derived from homozygous TLR4 KO mice. Of interest Kalayoglu et al. reported that *C. pneumoniae*-derived components that induce foam cell formation include the chlamydial LPS and chlamydial HSP60 both of which are TLR4 ligands (3, 54, 55) (reviewed in Ref. 33). The fact that TRIF<sup>-/-</sup> and IRF3<sup>-/-</sup> macrophages produced significantly diminished foam cell formation similar to that seen in TLR4<sup>-/-</sup> macrophages and that foam cell production was not altered in TLR3<sup>-/-</sup> macrophages and was similar to that in wild-type macrophages further corroborates the interpretation that TLR4 is directly involved in foam cell formation induced by *C. pneumoniae*. However, our



studies cannot rule out any potential contribution of the intracellular TLRs (TLR7, TLR8, and TLR9) to *C. pneumoniae*-mediated foam cell formation.

Previous data suggest that *C. trachomatis* activates the IRF3 pathway in a MyD88-independent manner (i.e., TRIF-dependent) that results in up-regulation of type I IFNs, at least in oviduct epithelial cells, (47), as well as in a MyD88-dependent manner (48). In the current study we show that *C. pneumoniae* is also able to induce IRF3 activation in macrophages. Macrophages express both TLR4 and TLR3. However, our data using TLR3<sup>-/-</sup> cells exclude the possibility that *C. pneumoniae* activates TLR3 and then TRIF/IRF3. TLR3 responds primarily to dsRNA and other viral ligands (56) and, to date, no *Chlamydia* ligands for TLR3 have been identified (47). Because there is no direct evidence that *C. pneumoniae* stimulates TLR3, the most likely interpretation of our results is that activation of the TRIF/IRF3 pathway by the bacteria requires intact TLR4 and does not involve TLR3. However, another study reported that *C. pneumoniae*-induced activation of NF- $\kappa$ B and expression of proinflammatory cytokines was TRAF6 dependent and that IRF3 and IRAK4 were redundant, and the authors did not observe increased IRF3 phosphorylation in *C. pneumoniae*-infected bone marrow-derived macrophages (57). In contrast, our results emphasize that *C. pneumoniae* directly stimulates IRF3 activation in peritoneal macrophages, which in turn promotes foam cell formation in the presence of ox-LDL. These differing results could be due to innate differences between the structural components of the distinct *C. pneumoniae* strains used between the two studies, CM-1 vs Kajaani 6, or to the use of bone marrow-derived macrophages in the previous study vs peritoneal macrophages used in this study. Indeed, significant variations in responses to different chlamydial strains have been reported and recently discussed by Nagarajan et al. (48). Besides IRF3, another key transcription factor that plays a role in type I IFN induction is IRF7, and future studies will need to investigate whether IRF7 also contributes to *C. pneumoniae*-induced foam cell formation.

LXR activation promotes cholesterol efflux from lipid-loaded macrophages, and the LXR agonist GW3965 was shown to decrease atherosclerotic lesions by 50% in hypercholesterolemic mouse models (58, 59). Cross-talk between TLRs and LXR pathways converge on IRF3 signaling, because TLR3-TRIF- or TLR4-TRIF-mediated IRF3 signaling down-regulates LXR functions as evidenced by a reduction of ABCA1 transcription and reduced cholesterol efflux from macrophages (38). Therefore, microbial agents associated with acceleration of atherosclerosis, i.e., *C. pneumoniae*, can trigger TLR4/TRIF/IRF3 activation, down-regulate LXRs, and shift cholesterol transport toward pro-foam cell production and therefore accelerate atherogenesis. In direct support of the notion that *C. pneumoniae* alters the processing of ingested lipids are our results demonstrating that treatment with the LXR agonist GW3965 blocked foam cell formation in the presence of the bacteria. These results were consistent with previous data (35) and underscore the intimate interconnections between cholesterol metabolism and innate immune host defenses. Activation of LXRs by LXR ligands in macrophages leads to induction of the ABCA1 transporter and stimulation of lipid efflux to extracellular acceptors (58). Based on this established mode of action, the most likely mechanism of LXR agonist-mediated inhibition of *C. pneumoniae*-induced foam cell formation is increased lipid efflux, but because we did not directly measure effects on transporters such as ABCA1, our results cannot rule out any other mechanism, including reduced lipid uptake. Although LXR activation was reported to reduce TLR4-mediated NF- $\kappa$ B activity (36, 37) and TLR-IRF3 pathways were shown to inhibit LXR activation (38), to our knowledge our data represent the first demonstration that an

LXR agonist can in turn down-modulate *C. pneumoniae*-mediated IRF3 activation in macrophages (Fig. 6; schematic).

Collectively then, our data (Fig. 6) indicate the following: 1) *C. pneumoniae* infection promotes foam cell formation in a TLR2- and TLR4-dependent manner through both MyD88- and TRIF-dependent signaling pathways, and this process does not require TLR3 signaling; 2) LXR activation by a synthetic agonist (GW3965) inhibits *C. pneumoniae*-mediated foam cell formation; 3) *C. pneumoniae* infection induces IRF3 activation in peritoneal macrophages and IRF3 in turn contributes to foam cell formation; 4) The LXR agonist GW3965 in turn reduces *C. pneumoniae*-induced IRF3 activation in macrophages. We therefore provide important new insights into the molecular signaling mechanisms involved in the formation of foam cells promoted by infection with *C. pneumoniae*, and hence we provide potential explanations as to how *C. pneumoniae* infection might accelerate the development of atherosclerosis and so contribute to adverse clinical events such as myocardial infarction and stroke.

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## Disclosures

The authors have no financial conflict of interest.

## References

- Saikku, P., M. Leinonen, K. Mattila, M. R. Ekman, M. S. Nieminen, P. H. Makela, J. K. Huttunen, and V. Valtonen. 1988. Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* 2: 983–986.
- Kaukoranta-Tolvanen, S. S., T. Ronni, M. Leinonen, P. Saikku, and K. Laitinen. 1996. Expression of adhesion molecules on endothelial cells stimulated by *Chlamydia pneumoniae*. *Microb. Pathog.* 21: 407–411.
- Kalayoglu, M. V., and G. I. Byrne. 1998. A *Chlamydia pneumoniae* component that induces macrophage foam cell formation is chlamydial lipopolysaccharide. *Infect. Immun.* 66: 5067–5072.
- Kiechl, S., E. Lorenz, M. Reindl, C. J. Wiedermann, F. Oberhollenzer, E. Bonora, J. Willeit, and D. A. Schwartz. 2002. Toll-like receptor 4 polymorphisms and atherogenesis. *N. Engl. J. Med.* 347: 185–192.
- Molestina, R. E., R. D. Miller, J. A. Ramirez, and J. T. Summersgill. 1999. Infection of human endothelial cells with *Chlamydia pneumoniae* stimulates transendothelial migration of neutrophils and monocytes. *Infect. Immun.* 67: 1323–1330.
- Coombes, B. K., and J. B. Mahony. 1999. *Chlamydia pneumoniae* infection of human endothelial cells induces proliferation of smooth muscle cells via an endothelial cell-derived soluble factor(s). *Infect. Immun.* 67: 2909–2915.
- Michelsen, K. S., T. M. Doherty, P. K. Shah, and M. Arditi. 2004. TLR signaling: an emerging bridge from innate immunity to atherogenesis. *J. Immunol.* 173: 5901–5907.
- Belland, R. J., S. P. Ouellette, J. Gieffers, and G. I. Byrne. 2004. *Chlamydia pneumoniae* and atherosclerosis. *Cell. Microbiol.* 6: 117–127.
- Rosenfeld, M. E., E. Blessing, T. M. Lin, T. C. Moazed, L. A. Campbell, and C. Kuo. 2000. *Chlamydia*, inflammation, and atherogenesis. *J. Infect. Dis.* 181 (Suppl 3): S492–S497.
- Campbell, L. A., and C. C. Kuo. 2004. *Chlamydia pneumoniae*: an infectious risk factor for atherosclerosis? *Nat. Rev. Microbiol.* 2: 23–32.
- Gaydos, C. A., J. T. Summersgill, N. N. Sahney, J. A. Ramirez, and T. C. Quinn. 1996. Replication of *Chlamydia pneumoniae* in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect. Immun.* 64: 1614–1620.
- Jahn, H. U., M. Krull, F. N. Wuppermann, A. C. Klucken, S. Rosseau, J. Seybold, J. H. Hegemann, C. A. Jantos, and N. Suttrop. 2000. Infection and activation of airway epithelial cells by *Chlamydia pneumoniae*. *J. Infect. Dis.* 182: 1678–1687.
- Shemer-Avni, Y., and D. Lieberman. 1995. *Chlamydia pneumoniae*-induced ciliostasis in ciliated bronchial epithelial cells. *J. Infect. Dis.* 171: 1274–1278.
- Haranaga, S., H. Yamaguchi, H. Friedman, S. Izumi, and Y. Yamamoto. 2001. *Chlamydia pneumoniae* infects and multiplies in lymphocytes in vitro. *Infect. Immun.* 69: 7753–7759.
- Kaul, R., J. Uphoff, J. Wiedeman, S. Yadlapalli, and W. M. Wenman. 2000. Detection of *Chlamydia pneumoniae* DNA in CD3<sup>+</sup> lymphocytes from healthy blood donors and patients with coronary artery disease. *Circulation* 102: 2341–2346.
- Moazed, T. C., C. C. Kuo, J. T. Grayston, and L. A. Campbell. 1998. Evidence of systemic dissemination of *Chlamydia pneumoniae* via macrophages in the mouse. *J. Infect. Dis.* 177: 1322–1325.



17. Hansson, G. K. 2005. Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* 352: 1685–1695.
18. Libby, P. 2002. Inflammation in atherosclerosis. *Nature* 420: 868–874.
19. Lusis, A. J. 2000. Atherosclerosis. *Nature* 407: 233–241.
20. Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon- $\beta$  induction. *Nat. Immunol.* 4: 161–167.
21. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301: 640–643.
22. Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda, and S. Akira. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN- $\beta$  promoter in the Toll-like receptor signaling. *J. Immunol.* 169: 6668–6672.
23. Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol.* 4: 1144–1150.
24. Beutler, B., Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann, X. Du, and K. Hoebe. 2006. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annu. Rev. Immunol.* 24: 353–389.
25. Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, et al. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* 101: 3516–3521.
26. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21: 335–376.
27. Michelsen, K. S., M. H. Wong, P. K. Shah, W. Zhang, J. Yano, T. M. Doherty, S. Akira, T. B. Rajavashisth, and M. Arditi. 2004. Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc. Natl. Acad. Sci. USA* 101: 10679–10684.
28. Gibson, F. C., III, C. Hong, H. H. Chou, H. Yumoto, J. Chen, E. Lien, J. Wong, and C. A. Genco. 2004. Innate immune recognition of invasive bacteria accelerates atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 109: 2801–2806.
29. Mullick, A. E., K. Soldau, W. B. Kiösses, T. A. Bell, III, P. S. Tobias, and L. K. Curtiss. 2008. Increased endothelial expression of Toll-like receptor 2 at sites of disturbed blood flow exacerbates early atherogenic events. *J. Exp. Med.* 205: 373–383.
30. Mullick, A. E., P. S. Tobias, and L. K. Curtiss. 2005. Modulation of atherosclerosis in mice by Toll-like receptor 2. *J. Clin. Invest.* 115: 3149–3156.
31. Bjorkbacka, H., V. V. Kunjathoor, K. J. Moore, S. Koehn, C. M. Ordija, M. A. Lee, T. Means, K. Halmen, A. D. Luster, D. T. Golenbock, and M. W. Freeman. 2004. Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. *Nat. Med.* 10: 416–421.
32. Naiki, Y., K. S. Michelsen, N. W. Schroder, R. Alsabeh, A. Slepkin, W. Zhang, S. Chen, B. Wei, Y. Bulut, M. H. Wong, et al. 2005. MyD88 is pivotal for the early inflammatory response and subsequent bacterial clearance and survival in a mouse model of *Chlamydia pneumoniae* pneumonia. *J. Biol. Chem.* 280: 29242–29249.
33. Joyee, A. G., and X. Yang. 2008. Role of Toll-like receptors in immune responses to chlamydial infections. *Curr. Pharm. Des.* 14: 593–600.
34. Kalayoglu, M. V., B. Hoerneman, D. LaVerda, S. G. Morrison, R. P. Morrison, and G. I. Byrne. 1999. Cellular oxidation of low-density lipoprotein by *Chlamydia pneumoniae*. *J. Infect. Dis.* 180: 780–790.
35. Cao, F., A. Castrillo, P. Tontonoz, F. Re, and G. I. Byrne. 2007. *Chlamydia pneumoniae*-induced macrophage foam cell formation is mediated by Toll-like receptor 2. *Infect. Immun.* 75: 753–759.
36. Zelcer, N., and P. Tontonoz. 2006. Liver X receptors as integrators of metabolic and inflammatory signaling. *J. Clin. Invest.* 116: 607–614.
37. Joseph, S. B., A. Castrillo, B. A. Laffitte, D. J. Mangelsdorf, and P. Tontonoz. 2003. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat. Med.* 9: 213–219.
38. Castrillo, A., S. B. Joseph, S. A. Vaidya, M. Haberland, A. M. Fogelman, G. Cheng, and P. Tontonoz. 2003. Crosstalk between LXR and toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism. *Mol. Cell* 12: 805–816.
39. Bradley, M. N., C. Hong, M. Chen, S. B. Joseph, D. C. Wilpitz, X. Wang, A. J. Lusis, A. Collins, W. A. Hseuh, J. L. Collins, et al. 2007. Ligand activation of LXR  $\beta$  reverses atherosclerosis and cellular cholesterol overload in mice lacking LXR  $\alpha$  and apoE. *J. Clin. Invest.* 117: 2337–2346.
40. Gordon, S. 2003. Do macrophage innate immune receptors enhance atherogenesis? *Dev. Cell* 5: 666–668.
41. Peterson, E. M., L. M. de la Maza, L. Brade, and H. Brade. 1998. Characterization of a neutralizing monoclonal antibody directed at the lipopolysaccharide of *Chlamydia pneumoniae*. *Infect. Immun.* 66: 3848–3855.
42. Pal, S., C. J. Luke, A. G. Barbour, E. M. Peterson, and L. M. de la Maza. 2003. Immunization with the *Chlamydia trachomatis* major outer membrane protein, using the outer surface protein A of *Borrelia burgdorferi* as an adjuvant, can induce protection against a chlamydial genital challenge. *Vaccine* 21: 1455–1465.
43. Hume, D. A., D. M. Underhill, M. J. Sweet, A. O. Ozinsky, F. Y. Liew, and A. Aderem. 2001. Macrophages exposed continuously to lipopolysaccharide and other agonists that act via Toll-like receptors exhibit a sustained and additive activation state. *BMC Immunol.* 2: 11.
44. Prebeck, S., C. Kirschning, S. Durr, C. da Costa, B. Donath, K. Brand, V. Redecke, H. Wagner, and T. Miethke. 2001. Predominant role of toll-like receptor 2 versus 4 in *Chlamydia pneumoniae*-induced activation of dendritic cells. *J. Immunol.* 167: 3316–3323.
45. Blessing, E., C. C. Kuo, T. M. Lin, L. A. Campbell, F. Bea, B. Chesebro, and M. E. Rosenfeld. 2002. Foam cell formation inhibits growth of *Chlamydia pneumoniae* but does not attenuate *Chlamydia pneumoniae*-induced secretion of proinflammatory cytokines. *Circulation* 105: 1976–1982.
46. Robbesyn, F., R. Salvayre, and A. Negre-Salvayre. 2004. Dual role of oxidized LDL on the NF- $\kappa$ B signaling pathway. *Free Radical Res.* 38: 541–551.
47. Derbigny, W. A., S. C. Hong, M. S. Kerr, M. Temkit, and R. M. Johnson. 2007. *Chlamydia muridarum* infection elicits a  $\beta$  interferon response in murine oviduct epithelial cells dependent on interferon regulatory factor 3 and TRIF. *Infect. Immun.* 75: 1280–1290.
48. Nagarajan, U. M., D. M. Ojcius, L. Stahl, R. G. Rank, and T. Darville. 2005. *Chlamydia trachomatis* induces expression of IFN- $\gamma$ -inducible protein 10 and IFN- $\beta$  independent of TLR2 and TLR4, but largely dependent on MyD88. *J. Immunol.* 175: 450–460.
49. Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, et al. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signaling. *Nature* 424: 743–748.
50. Watson, C., and N. J. Alp. 2008. Role of *Chlamydia pneumoniae* in atherosclerosis. *Clin. Sci. (Lond.)* 114: 509–531.
51. Beaven, S. W., and P. Tontonoz. 2006. Nuclear receptors in lipid metabolism: targeting the heart of dyslipidemia. *Annu. Rev. Med.* 57: 313–329.
52. Kalayoglu, M. V., G. S. Miranpuri, D. T. Golenbock, and G. I. Byrne. 1999. Characterization of low-density lipoprotein uptake by murine macrophages exposed to *Chlamydia pneumoniae*. *Microbes Infect.* 1: 409–418.
53. Bobryshev, Y. V., M. C. Killingsworth, D. Tran, and R. Lord. 2008. Amalgamation of *Chlamydia pneumoniae* inclusions with lipid droplets in foam cells in human atherosclerotic plaque. *Virchows Arch.* 453: 69–77.
54. Kalayoglu, M. V., R. P. Indrawati, S. G. Morrison, Y. Morrison, Yuan, and G. I. Byrne. 2000. Chlamydial virulence determinants in atherogenesis: the role of chlamydial lipopolysaccharide and heat shock protein 60 in macrophage-lipoprotein interactions. *J. Infect. Dis.* 181 (Suppl 3): S483–S489.
55. Bulut, Y., E. Faure, L. Thomas, H. Karahashi, K. S. Michelsen, O. Equils, S. G. Morrison, R. P. Morrison, and M. Arditi. 2002. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J. Immunol.* 168: 1435–1440.
56. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17: 1–14.
57. Trumstedt, C., E. Eriksson, A. M. Lundberg, T. B. Yang, Z. Q. Yan, H. Wigzell, and M. E. Rottenberg. 2007. Role of IRAK4 and IRF3 in the control of intracellular infection with *Chlamydia pneumoniae*. *J. Leukocyte Biol.* 81: 1591–1598.
58. Venkateswaran, A., B. A. Laffitte, S. B. Joseph, P. A. Mak, D. C. Wilpitz, P. A. Edwards, and P. Tontonoz. 2000. Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR  $\alpha$ . *Proc. Natl. Acad. Sci. USA* 97: 12097–12102.
59. Joseph, S. B., E. McKilligin, L. Pei, M. A. Watson, A. R. Collins, B. A. Laffitte, M. Chen, G. Noh, J. Goodman, G. N. Hagger, et al. 2002. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc. Natl. Acad. Sci. USA* 99: 7604–7609.