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Chlamydia pneumoniae-Induced Macrophage Foam Cell Formation Is Mediated by Toll-Like Receptor 2[▽]

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Chlamydia pneumoniae **induces macrophage foam cell formation, a hallmark of early atherosclerosis, in the presence of low-density lipoprotein (LDL). This study examined the role that Toll-like receptor 2 (TLR2) and TLR4 may play in pathogen-induced foam cell formation. Murine macrophage RAW 264.7 cells either infected with *C. pneumoniae* or treated with the TLR4 ligand *E. coli* lipopolysaccharide (LPS) or the TLR2 ligand Pam_3-Cys-Ala-Gly-OH (Pam) became Oil Red O-stained foam cells and showed increased cholesteryl ester (CE) content when cocultured with LDL. In macrophages from $TLR2^{-/-}$ mice, foam cells were induced by *Escherichia coli* LPS but not by *C. pneumoniae* or Pam. Conversely, *C. pneumoniae* or Pam, but not *E. coli* LPS, induced foam cells in the TLR4-deficient GG2EE macrophage cell line, suggesting that *C. pneumoniae* elicits foam cell formation predominantly via TLR2. Enhancing cholesterol efflux using the liver X receptor (LXR) agonist GW3965 significantly decreased the CE content of cells exposed to each of the three TLR ligands (*C. pneumoniae*, Pam, and *E. coli* LPS). Overall, our results suggest that activation of the LXR signaling pathway may affect potentially atherogenic processes modulated by the TLR ligands.**

Chlamydia pneumoniae causes community-acquired pneumonia, bronchitis, and other respiratory tract diseases (21). Infection also is strongly associated with atherosclerosis and cardiovascular diseases by a variety of studies. Seroepidemiological studies have indicated that patients with coronary artery disease have higher titers of anti-*C. pneumoniae* antibodies compared to healthy population controls (42). Pathology studies have detected the organism within atherosclerotic lesions but not in adjacent normal tissue by immunohistochemistry, PCR, and electron microscopy (19), and the pathogen has been isolated from atherosclerotic lesions and propagated in vitro (4, 19, 27). Cell biology studies have suggested that the organism can be detected in circulating leukocytes, has the capacity to infect all atheroma cell types (13, 28, 34), and can induce the expression of inflammatory cytokines, procoagulants, matrix metalloproteinase, and adhesion molecules (8, 9, 12). Animal model studies have shown that *C. pneumoniae* can cause arterial inflammation in normolipidemic mice and rabbits and initiate lesion development or contribute to exacerbation of lesions in rabbits or mice, respectively (29, 33). Despite the numerous indications of *C. pneumoniae*'s role in atherosclerosis, human secondary prevention antibiotic treatment trials have failed to benefit heart disease patients (6, 16, 35). Although this may suggest that *C. pneumoniae* is not causally associated with atherosclerosis or heart disease, it is more likely that other reasons account for the lack of secondary prevention antibiotic efficacy. For example, all secondary prevention trial participants had advanced heart disease, making successful antibiotic intervention difficult to achieve because of substantial preexisting cardiovascular damage. In addition, a nonproductive (persistent) chlamydial growth state is possible during chronic diseases and antibiotics are not eradicative for this specific form of the pathogen (14). Finally, none of the major trials established conclusively if the antibiotics achieved appropriate penetration at the site of chlamydial infection. If this pathogen plays a causal role in the atherosclerotic process, standard chemotherapeutic measures in unselected heart disease patient populations are unlikely to reduce pathological consequences of infection, making a better understanding of the role of *C. pneumoniae* in cardiovascular pathophysiology an important public health objective.

Foam cell accumulation is a key event in early atherosclerosis (41), and it is known that *C. pneumoniae* induces human and murine macrophage foam cell formation, at least in part, by stimulating enhanced low-density lipoprotein (LDL) binding and entry (23). Furthermore, *C. pneumoniae* induces monocytes to oxidize lipoproteins, making them atherogenic (24, 25). One possible mechanism for increased LDL uptake is up-regulation of scavenger receptors (such as SR-A, CD36, etc.), which can internalize modified LDL without feedback inhibition, resulting in the accumulation of cholesterol and cholesteryl esters (CEs) in cytoplasmic storage vesicles (10). Normal cellular cholesterol homeostasis, however, involves not only specific trafficking of lipid storage vesicles but also processing of excess intracellular cholesterol such that it is effluxed from the cell via a transporter coupled to the production of high-density lipoprotein. The liver X receptors (LXRs) activate the expression of genes involved in cholesterol efflux such as *ABCA1*, *ABCG1*, etc. (48). LXRs are expressed and activated by endogenous or synthetic ligands such as GW3965, which has been shown to have potent antiatherogenic activity

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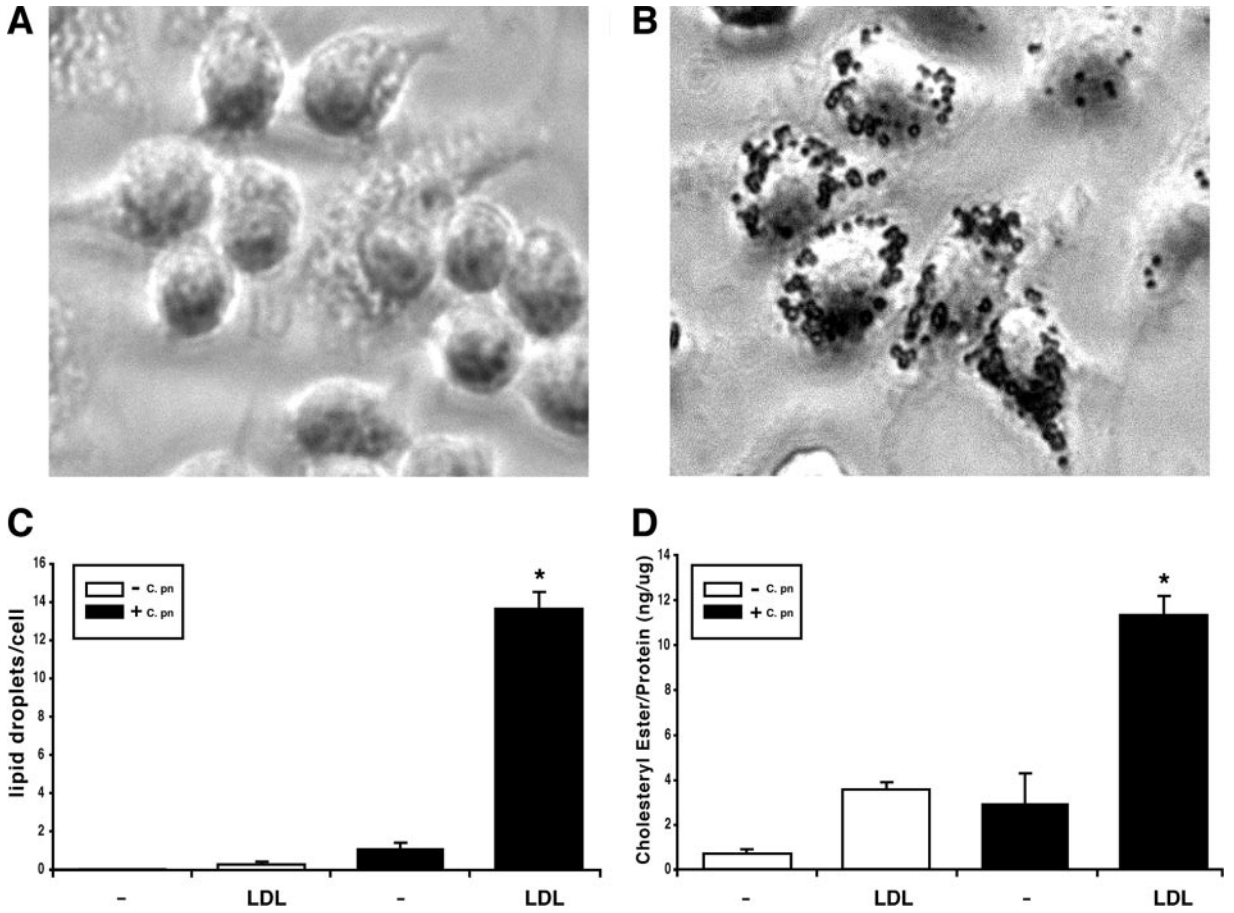

FIG. 1. *C. pneumoniae* induces foam cell formation and CE accumulation in RAW 264.7 macrophages when cocultured with LDL. (A, B) Micrographs of Oil Red O-stained macrophages treated with LDL (100 μg/ml) (A) or infected with *C. pneumoniae* (C. pn; MOI = 1) in the presence of LDL for 24 h (B) (magnification, ×300; light microscopy). (C) Lipid droplet levels were quantified in uninfected (open bars) and infected (solid bars) cells. Cells were incubated with LDL (100 μg/ml) for 24 h. Lipid droplet levels were normalized to cell numbers. (D) CE levels were determined after the same treatment described for panel C. CE levels were normalized to protein content. The data are representative of three experiments and are means of triplicates with standard deviations. An asterisk indicates a statistically significant difference compared with uninfected samples cultured with LDL ($P < 0.05$; *t* test).

in modified LDL-loaded macrophages in vitro, as well as in the aortas of hyperlipidemic mice, by inducing the cholesterol efflux genes *ABCA1* and *ABCG1* (20).

Induction of LXR-activated genes also has been shown to influence pathogen pattern recognition Toll-like receptor 3/4 (TLR3/4) activity (5). The discovery of mammalian TLRs as critical sensors of the innate immune system provides a mechanistic link among infection, inflammation, and atherosclerosis (31). Studies have shown the expression of TLR1, -2, and -4 in human atherosclerotic lesions (11, 49). Animal studies (2, 18, 32) have shown that the loss of TLR4 or myeloid differentiation factor 88 (MyD88), the common TLR signaling adaptor molecule, reduces disease severity in atherosclerosis-prone, apolipoprotein E-deficient (apoE$^{-/-}$) mice (32).

Recent studies also have indicated a proatherogenic effect of local TLR2 activation (44) and established a clear role for TLR2 in modulating the severity of experimental atherosclerosis in mice, where complete loss of TLR2 results in decreased lesion size whereas systemic exposure to an exogenous TLR2 agonist dramatically exacerbated atherosclerosis (36), providing insight into a potential mechanism by which recurrent microbial infections might influence disease severity. TLR2 detects a large range of microbial components, such as gram-positive organism-derived lipoteichoic acid, bacterial lipoproteins, and zymosan (1). Of the 11 characterized TLRs, TLR2 is unique by virtue of its ability to heterodimerize with TLR1 or TLR6 (38), resulting in a relatively broad ligand specificity (45, 46). The recent discovery of CD36 as a coreceptor for TLR2 raises the prospect that a novel proinflammatory pathway exists between endogenously derived lipids and activation of innate immunity (17).

Chlamydiae express a variety of ligands that could serve as potential TLR ligands. For example, both TLR2 and TLR4 are involved in the recognition of *C. pneumoniae* by host cells, although host cell activation occurs predominantly via TLR2 (39). Several studies have reported that chlamydial heat shock protein 60 activates TLR2 and/or TLR4 (7, 26, 37, 39, 47) and that chlamydial lipopolysaccharide (LPS) induces macrophage foam cell formation in the presence of LDL (22). The objectives of the present study were to determine the role that TLR2 or TLR4 may play in *C. pneumoniae*-induced foam cell formation and to initiate an investigation of the cross talk between

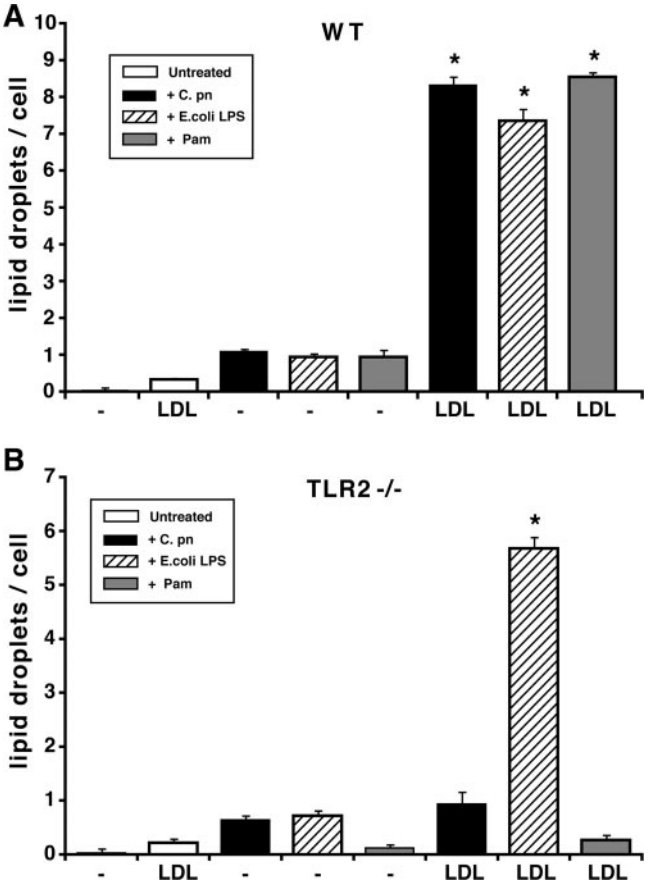

FIG. 2. *C. pneumoniae* fails to induce foam cell formation in peritoneal macrophages from TLR2$^{-/-}$ mice. Lipid droplet levels in peritoneal macrophages from wild-type (WT) (A) and TLR2$^{-/-}$ (B) mice were enumerated. Cells were left untreated (open bars), infected with *C. pneumoniae* (C. pn; MOI = 1) (solid bars), or treated with the TLR4 ligand *E. coli* LPS (500 ng/ml) (diagonal bars) or the TLR2 ligand Pam (2 μg/ml) (gray bars) in the presence or absence of LDL (100 μg/ml) for 24 h. Lipid droplet levels were normalized to cell numbers. The data are representative of three experiments and are means of triplicates with standard deviations. An asterisk indicates a statistically significant difference compared with uninfected samples cultured with LDL ($P < 0.05$; *t* test).

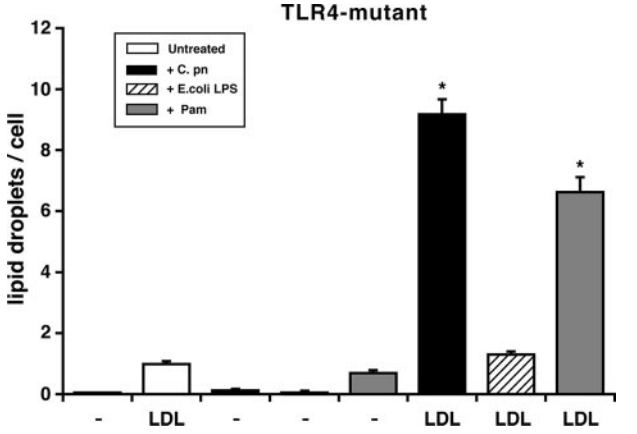

FIG. 3. *C. pneumoniae* induces foam cell formation in the TLR4-mutant murine macrophage GG2EE cell line. Lipid droplet levels in untreated GG2EE cells (open bars) or cells treated with the TLR4 ligand *E. coli* LPS (500 ng/ml) (diagonal bars) or the TLR2 ligand Pam (2 μg/ml) (grey bars) or cells infected with *C. pneumoniae* (C. pn; MOI = 1) (solid bars) are represented. Cells were incubated with or without LDL (100 μg/ml) for 24 h. Lipid droplet levels were normalized to cell numbers. The data are representative of three experiments and are means of triplicates with standard deviations. An asterisk indicates a statistically significant difference compared with uninfected samples cultured with LDL ($P < 0.05$; *t* test).

LXRs and TLRs in *C. pneumoniae*-induced foam cell formation.

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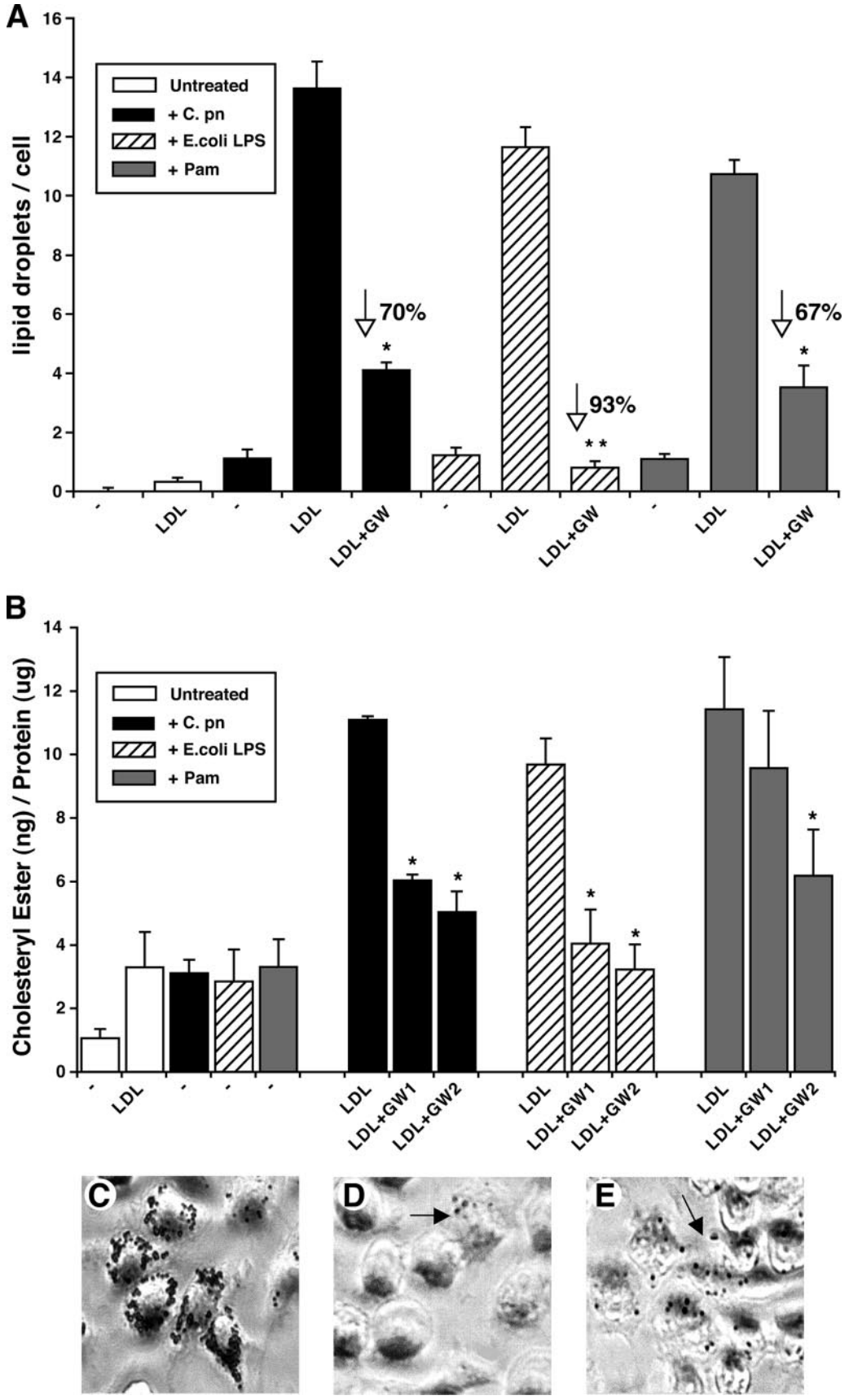
MATERIALS AND METHODS

Cell culture and growth of *C. pneumoniae*. The murine macrophage cell line RAW 264.7 was purchased from the American Type Culture Collection (ATCC; Manassas, VA) and grown in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 50 μg of vancomycin per ml, and 10 μg of gentamicin per ml. Cells were maintained in a 37°C, 5% CO_2 incubator and subcultured every 2 days by gentle scraping. The murine macrophage TLR4-mutant cell line GG2EE, derived from C3H/HeJ mice, expresses a TLR4 containing a proline-to-histidine mutation at amino acid residue 714 (3) and was grown in the same medium as RAW 264.7. *C. pneumoniae* strain AR-39 (ATCC) was propagated in HEp-2 cells (ATCC) and purified by Renografin gradient centrifugation as previously described (6). Purified organisms were suspended in sucrose-phosphate-glutamic acid buffer (0.22 M sucrose, 3.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 5 mM L-glutamic acid; pH 7.4) and stored in stock suspensions at −80°C until they were used. *Chlamydia* titers were determined by infecting HEp-2 cells with 10-fold dilutions of thawed stock, incubating them for 36 h, fixing the cells with methanol, and fluorescent staining chlamydial inclusions with fluorescein isothiocyanate-conjugated anti-chlamydial LPS antibody (Fitzgerald Industries International Inc., Concord, MA). Titers are reported as multiplicities of infection (MOI), where an MOI of 1 equals one infectious *Chlamydia* organism per HEp-2 cell.

Infection and culture of macrophages. Macrophages were plated at a density of 2×10^5/well in 24-well plates (1.9-cm^2 growth area; Corning Costar Corporation, Cambridge, MA) and incubated overnight prior to infection. Macrophages were infected with *C. pneumoniae* (MOI = 1) or treated with the TLR2 ligand Pam (Pam_3-Cys-Ala-Gly-OH, a synthetic lipopeptide, 2 μg/ml; BACHEM) or the TLR4 ligand *Escherichia coli* LPS (500 ng/ml) in 500 μl of RPMI 1640 to 10% FBS in the presence or absence of LDL (100 μg/ml; INTRACEL) for 24 h at 35°C, 5% CO_2. In some experiments, macrophages were cultured with LDL in the presence or absence of the synthetic LXR agonist GW3965 (1 or 2 μM). Trypan blue staining showed that none of the treatments affected macrophage viability at 24 h (>95% of the macrophages were viable).

Staining and quantitation of foam cells. Macrophages were washed twice with phosphate-buffered saline (PBS), fixed for 15 min in 2% paraformaldehyde (in PBS), and stained for 30 min in 1% Oil Red O (in 60% isopropanol). Cells were then washed three times with PBS and examined by light microscopy (magnification, ×200; Diaphot 200 or Optiphot microscope; Nikon, Garden City, NY) (23). Foam cells were defined as cells with ≥10 Oil Red O-positive droplets (43). Alternatively, staining with Nile Red, a fluorescent dye for neutral lipid bodies, was used. After fixation (the same as described above), cells were stained with Nile Red (3 μg/ml; Molecular Probes N-1142, dissolved in 70% ethanol, diluted in PBS) for 3 min, washed three times with PBS, and then examined by fluorescence microscopy.

Quantitation of macrophage CE content. Macrophage CE content was quantitated by the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR). Macrophages were fixed in 2% paraformaldehyde for 15 min, washed three times with PBS, and incubated with 200 μl/well of absolute ethanol for 30 min at 4°C to extract cellular lipids. Cholesterol content was determined by incubating 20 μl of ethanol-extracted lipids diluted in 30 μl of 1× reaction buffer (0.1 M K_2HPO_4, pH 7.4, 0.05 M NaCl, 5 mM cholic acid, 0.1% Triton X-100) with 50 μl of assay solution (total cholesterol) or 50 μl of assay solution lacking cholesterol esterase (free cholesterol), for 30 min at 37°C in the dark and then measuring fluorescence (HTS-7000 microplate fluorometer; 535-nm excitation, 595-nm


A
lipid droplets / cell
Untreated
+ C. pn
+ E.coli LPS
+ Pam
70%
93%
67%
-
LDL
LDL+GW
B
Cholesteryl Ester (ng) / Protein (ug)
LDL+GW1
LDL+GW2
C
D
E

emission). The total and free cholesterol contents of each sample were calculated by using a cholesterol standard. CE content was calculated by subtracting free cholesterol from total cholesterol for each sample. Lipid-extracted cells were dissolved in 0.1% sodium dodecyl sulfate–0.1 M NaOH for 30 min, and total cell protein was determined by the bicinchoninic acid protein assay. CE levels under different treatments were normalized to total cellular protein content.

Animals and preparation of peritoneal macrophages. Six-week-old male C57BL/6J and B6.129-TLR2$^{tm1kir/J}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice were maintained on a standard diet in an IACUC-approved facility. Mice were injected intraperitoneally with 3 ml of 10% proteose peptone (Becton Dickinson and Company, Sparks, MD). Three days later, mice were euthanized and peritoneal exudate cells were harvested by lavage from the peritoneal cavity with ice-cold Hanks' balanced salt solution (GIBCO, Grand Island, NY). Cells were cultured for 24 h and washed with Hanks' balanced salt solution to remove nonadherent cells. Adherent cells were cultured for 3 days before use. Peritoneal macrophages (1×10^6/ml) were cultured in RPMI 1640 medium supplemented with 10% FBS.

RESULTS

***C. pneumoniae* induced foam cell formation and CE accumulation in RAW 264.7 macrophages in the presence of LDL.** The RAW 264.7 macrophages were treated with LDL or infected with *C. pneumoniae* in the presence or absence of LDL, and foam cell formation was measured by assessment of Oil Red O staining. Lipid accumulation, a hallmark of foam cell formation, was observed only in cells infected with *C. pneumoniae* plus LDL but not in the other treatments (Fig. 1). This phenomenon occurred in a dose-dependent manner (data not shown). These observations were consistent with a previous report (22) and verified the effect of *C. pneumoniae* on foam cell formation in the presence of exogenous LDL.

***C. pneumoniae*-induced macrophage foam cell formation is dependent on TLR2 but not TLR4.** It is known that *C. pneumoniae* is capable of activating both TLR2 and TLR4. To determine if TLR2 and/or TLR4 are involved in *C. pneumoniae*-induced foam cell formation, peritoneal macrophages from TLR2$^{-/-}$ mice were studied. In this cell type, foam cell formation was induced by the TLR4 ligand *E. coli* LPS but not by *C. pneumoniae* or the TLR2 ligand Pam in the presence of LDL (Fig. 2B). In contrast, *C. pneumoniae* or Pam retained the ability to induce foam cells in the TLR4-deficient macrophage cell line GG2EE. As expected, *E. coli* LPS cultured with LDL was unable to induce foam cell formation in this cell line (Fig. 3). Taken together, these data suggest that *C. pneumoniae*-induced foam cell formation is dependent on TLR2 and does not require TLR4.

Effect of LXR agonist on foam cell formation and CE accumulation. Since cholesterol homeostasis involves not only cholesterol uptake but also cholesterol efflux, foam cell formation may be influenced by enhanced cholesterol uptake coupled with decreased cholesterol efflux. In response to lipid loading, the LXR expressed in macrophages activates a compensatory pathway for cholesterol efflux. To examine whether activation of the LXR pathway could reverse foam cell formation, the LXR agonist GW3965 was added to RAW 264.7 macrophages infected with *C. pneumoniae* or treated with Pam or *E. coli* LPS in the presence of LDL. GW3965 treatment drastically decreased the number of lipid droplets in cells treated with *E. coli* LPS and to a lesser extent in cells exposed to *C. pneumoniae* or Pam (Fig. 4A). In addition, GW3965 treatment inhibited CE accumulation in cells exposed to each of the three ligands (*E. coli* LPS, *C. pneumoniae*, and Pam) in the presence of LDL (Fig. 4B). Microscopic examination of cells confirmed the ability of GW3965 to inhibit foam cell formation induced by *E. coli* LPS (Fig. 4D), and, to a lesser degree, by *C. pneumoniae* or Pam (Fig. 4E). Taken together, these data suggest that activation of the LXR signaling pathway inhibits foam cell formation induced by TLR2 and TLR4.

DISCUSSION

Atherosclerosis is a disorder of lipid metabolism and a chronic inflammation (40), indicating that infectious agents may contribute to atherogenesis and heart disease. Macrophages play a key role in both lipid metabolism and immune responses (15, 30). Macrophage foam cell formation in the arterial intima is the hallmark of early atherosclerosis (41), and important steps in foam cell formation include excessive cholesterol uptake from high levels of serum LDL, accumulation of intracellular triglycerides, and reduced cholesterol efflux via the ATP-binding cassette transporter pumps (*ABCA1*, *ABCG1*, etc.).

Previous studies have shown that *C. pneumoniae* induces macrophage foam cell formation in the presence of LDL (23), consistent with a causal role for the organism in atherogenesis. Several mechanisms may account for the ability of *C. pneumoniae* to induce macrophage foam cell formation. It is known that *C. pneumoniae* can cause macrophage activation, resulting in LDL oxidation and scavenger receptor up-regulation (24), which in turn may lead to an increase in the uptake of the oxidized LDL by macrophages. Cell activation by *C. pneu-*

FIG. 4. Effects of GW3965 on foam cell formation in RAW 264.7 macrophages. (A) Lipid droplet levels in untreated RAW264.7 macrophages (open bars) or cells treated with the TLR4 ligand *E. coli* LPS (500 ng/ml) (diagonal bars) or the TLR2 ligand Pam (2 μg/ml) (gray bars) or infected with *C. pneumoniae* (C. pn; MOI = 1) (solid bars). Cells were incubated with or without LDL (100 μg/ml) in the presence or absence of 2 μM GW3965 (GW) for 24 h. Lipid droplet levels were normalized to cell numbers. Levels of inhibition are shown. The data are representative of three experiments and are reported as means of triplicates with standard deviations. An asterisk indicates a statistically significant difference compared with *C. pneumoniae*-infected or Pam-treated samples cultured with LDL ($P < 0.05$; *t* test). Double asterisks indicate a statistically significant difference compared with *E. coli* LPS-treated samples cultured with LDL ($P < 0.01$; *t* test). (B) CE levels in untreated (open bars), TLR4 ligand *E. coli* LPS (500 ng/ml)-treated (diagonal bars), *C. pneumoniae* (MOI = 1)-infected (solid bars), and TLR2 ligand Pam (2 μg/ml)-treated (gray bars) RAW 264.7 macrophages. Cells were incubated with or without LDL (100 μg/ml) in the presence or absence of 1 or 2 μM GW3965 (GW1 or GW2) for 24 h. CE levels were normalized to protein content. The data shown are representative of three experiments and are means of triplicates with standard deviations. An asterisk indicates a statistically significant difference compared with foam cells induced by *E. coli* LPS, *C. pneumoniae*, and Pam when cultured with LDL, respectively ($P < 0.05$; *t* test). (C to E) Micrographs of Oil Red O-stained macrophages containing cytoplasmic neutral lipid droplets (magnification, ×300; light microscopy). (C) Cells infected with *C. pneumoniae* plus LDL. (D) Cells treated with *E. coli* LPS plus LDL in the presence of 2 μM GW3965. (E) Cells treated with *C. pneumoniae* plus LDL in the presence of 2 μM GW3965. The arrow indicates lipid droplets stained by Oil Red O.

moniae has been demonstrated to be mediated by TLR2 or TLR4 (7, 26, 37, 39, 47), although TLR2 seems to play the predominant role. In data reported here, we showed that foam cell formation induced by *C. pneumoniae* is mediated by TLR2 but not TLR4, although pure agonists of both receptors are equally able to trigger foam cell formation and CE accumulation in the presence of LDL. Interestingly, in animal models of atherosclerosis, TLR2 activation has been shown to be proatherogenic (44). Loss of TLR2 was reported to result in decreased atherosclerotic lesion size, whereas systemic exposure to a TLR2 ligand was found to dramatically increase lesion severity (36), establishing a clear role for TLR2 in modulating the severity of experimental atherosclerosis in mice. A role for TLR4 in modulating atherosclerosis has also been proposed (32). Other studies have shown that *C. pneumoniae* also affects metabolism in murine macrophages via TLR2 but not TLR4 (50). Our results reinforce this notion and link *C. pneumoniae*'s ability to induce foam cell formation in a TLR2-dependent fashion and the proven role of TLR2 in atherogenesis.

LXRs are highly expressed in macrophages. Activation of LXR induces cholesterol efflux from lipid-loaded macrophages (48), and a synthetic LXR agonist, GW3965, has been shown to decrease atherosclerotic lesions by 50% in both $LDLR^{-/-}$ and $apoE^{-/-}$ mice (20). Viral and bacterial pathogens have been reported to antagonize LXR transcriptional activity and cholesterol efflux through activation of TLR3/4 (5). The LXR and TLR cross signaling represents a direct link between innate immunity and macrophage cholesterol metabolism and is a potential way for pathogens to contribute to cardiovascular disease (5). As shown in our data, GW3965 treatment reduced the CE content of foam cells induced by *C. pneumoniae* infection or Pam or *E. coli* LPS treatment to the same extent. Despite stimulating cholesterol efflux to the same degree in all cell populations, GW3965 inhibited lipid droplet content more prominently in cells treated with *E. coli* LPS than in cells infected with *C. pneumoniae* or treated with Pam. The reason for this difference is unclear.

Overall, our results suggest that activation of the LXR signaling pathway may affect potentially atherogenic processes modulated by the TLR ligands. Interestingly, LXR-induced genes affect not only cholesterol efflux but also fatty acid synthesis. It is possible that *C. pneumoniae* infection and TLR activation may differentially affect genes of the LXR-induced response. This process will be investigated by quantitatively measuring LXR-induced transcription in infected and uninfected cells to further our understanding of the role of this important human pathogen in atherogenic events.

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