

RESVERATROL IN *CHLAMYDIA PNEUMONIAE*-INDUCED FOAM CELL FORMATION AND INTERLEUKIN-17A SYNTHESIS

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The involvement of *Chlamydia pneumoniae* in the pathogenesis of atherosclerosis has been suggested by numerous seroepidemiological, *in vivo* and *in vitro* studies. In particular, it has been shown that *C. pneumoniae* is able to promote the accumulation of low-density lipoproteins into macrophages, thus facilitating foam cell formation. The aim of our study was to investigate the effects of resveratrol on macrophage-derived foam cell formation induced by *C. pneumoniae*, examining its underlying biochemical mechanisms. Our results showed a relevant decrease in the number of foam cells, in the production of thiobarbituric acid reactive substances, superoxide anion and IL-17A while treating *C. pneumoniae* infected macrophages with resveratrol. Furthermore, the inhibition of Peroxisome Proliferator-Activated Receptors gamma by a specific antagonist (GW 9662), in presence of resveratrol and *C. pneumoniae*, enhanced intracellular lipid and cholesterol accumulation and the subsequent foam cell formation. In conclusion, the main result of our study is the evidence of an antiatherogenic effect of resveratrol on macrophage-derived foam cell formation and IL-17A production induced by *C. pneumoniae*.

The involvement of *Chlamydia pneumoniae*, a respiratory pathogen, in the pathogenesis of atherosclerosis has been initially suggested by numerous seroepidemiological studies and, subsequently, by the direct detection of antigens and nucleic acids (1-11). In particular, *C. pneumoniae* has been isolated from atherosclerotic plaques in a viable form (12) and it has been found in peripheral blood mononuclear cells (PBMC) of patients with atherosclerotic cardiovascular diseases (7, 13-16).

Therefore, in recent years, four large-scale randomized clinical trials (WIZARD, ACES, PROVE IT-TIMI and CLARICOR) have been undertaken to demonstrate long-term benefits in patients with cardiovascular diseases and undergoing anti-chlamydial antibiotic treatment, but

none of them showed a reduction in cardiovascular events. However, the failure of the secondary prevention trials is not enough to rule out a role of *C. pneumoniae* in the pathogenesis of atherosclerosis. In fact, the negative results of these studies may be due to several factors including the refractoriness of chlamydial chronic infections to antibiotic treatment and also the enrollment of patients with advanced coronary artery diseases (17, 18).

However, a very recent clinical trial has showed a positive association between the anti-*C. pneumoniae* therapy and the secondary prevention of cardiovascular events (19).

Nevertheless, we cannot ignore that the association between *C. pneumoniae* and atherosclerosis is also supported by numerous *in vivo* and *in vitro* studies

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(7, 20). Firstly, most of the animal-model studies have demonstrated that the *C. pneumoniae* infection could initiate or accelerate the progression of the atherosclerotic lesion (20).

Secondly, several *in vitro* studies have shown that the *C. pneumoniae* infection, as well as promoting the production of pro-inflammatory cytokines and other pro-atherogenic factors, may play a role in all the stages of the atherosclerotic lesion development, from the initial lesion to the plaque rupture and the thrombus formation which is responsible for the acute cardiovascular event (7, 20-22). In particular, it has been demonstrated that *C. pneumoniae* is able to promote the accumulation of low-density lipoproteins (LDL) into macrophages, at least in part, by affecting the Peroxisome Proliferator-Activated Receptors (PPARs), which are key regulators of macrophage lipid homeostasis (23). Furthermore, *C. pneumoniae* has been shown to induce LDL oxidation by stimulating the production of reactive oxygen species (ROS) (24) so that oxidized LDL (oxLDL) were internalized by macrophage scavenger receptors leading to foam cell formation (25).

Given the importance of the foam cells in the evolution of the atherosclerotic lesion, researchers have recently focused their attention to the antioxidant and anti-inflammatory effects of resveratrol that can help the maintenance of macrophage lipid homeostasis, as well as counteract the oxidative process involved in the pathogenesis of cardiovascular diseases (26).

Since the inflammatory process is of utmost importance in atherosclerosis, it has been recently suggested that IL-17A is pro-atherogenic and plays a role in *C. pneumoniae*-mediated acceleration of atherosclerotic lesions in presence of high lipid levels (27).

Therefore, the aim of our study was to investigate the effects of resveratrol on macrophage-derived foam cell formation induced by *C. pneumoniae*, examining its underlying biochemical mechanisms.

MATERIALS AND METHODS

Chemicals

Resveratrol, Low Density Lipoprotein (LDL), Oil Red-O, PPAR- γ antagonist (GW9662), Thiazolyl Blue Tetrazolium Bromide (MTT reagent), Thiobarbituric Acid

(TBA) reagent and other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cholesterol assay kit was from Abcam, Fluorescein isothiocyanate (FITC)-conjugated specific anti-chlamydial monoclonal antibody (Pathfinder Chlamydia Culture Confirmation System) was from Bio-Rad and IL-17A ELISA kit was from R&D Systems, Minneapolis, MN, USA.

Propagation of *C. pneumoniae* and culture of J774A.1 macrophages

C. pneumoniae strain AR-39 (ATCC 53592) was propagated in HEp-2 cells (ATCC CCL23) in the presence of 2 $\mu\text{g/ml}$ cycloheximide by centrifugation as previously described (28). Infected HEp-2 cells were harvested after 72 h incubation at 37°C and 5% CO₂, in sucrose-phosphate-glutamate buffer (SPG buffer) (0.2 M sucrose, 3.8 mM KH₂PO₄, 6.7 mM Na₂HPO₄, 5.5 mM glutamic acid at pH 7.4) and vortexed with glass beads for 2 to 5 min. After the removal of cell debris by centrifugation at 350 g for 10 min, the supernatant was centrifugated at 39,800 g and 4°C for 1 h and the pellet was resuspended in SPG buffer and stored at -70°C.

The infectious titers [Inclusion-Forming Units (IFU) per ml] was assessed by immunofluorescence assay. Briefly, the HEp-2 cells were infected with tenfold serial dilutions of bacterial stock, incubated for 72 h, fixed with methanol, and stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against chlamydial LPS. The total number of IFU was enumerated by counting ten microscope fields using a fluorescence microscope (100X magnification).

Murine J774A.1 macrophages (ATCC TIB-67) were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS) at 37°C and 5% CO₂.

C. pneumoniae-induced foam cell formation

J774.A.1 macrophages (1.0x10⁵ cells/well) were infected with *C. pneumoniae* at a multiplicity of infection (MOI) of 2 as above described. Next, cells were washed with phosphate buffered saline (PBS) and cultured for 2 days in the presence or absence of human LDL (100 $\mu\text{g/ml}$).

Foam cell formation was evaluated by oil red-O staining and detection of intracellular cholesterol levels.

Cytotoxicity assay of resveratrol

The cytotoxic effect of resveratrol was determined by the MTT method. Macrophages (1x10⁴ cells/well) were incubated with resveratrol at various concentrations (0, 25, 50 and 100 μM) for 48 h. Twenty μl MTT reagent (5 mg/ml) were added to each well and incubated for 4 h.

Afterwards, the medium was removed and MTT crystals were dissolved with 150 μ l isopropanol. The amount of formazan produced was detected by measuring the absorbance at 570 nm (ELISA Reader). The cytotoxic effect of resveratrol on cells were expressed as percent cell viability compared with the control cells.

Anti-chlamydial assay of resveratrol

To evaluate the anti-chlamydial effect of resveratrol, *C. pneumoniae* infected macrophages were incubated for 72 h in the presence or absence of resveratrol (25 μ M). Then macrophages were fixed and stained with FITC-conjugated monoclonal antibody against chlamydial LPS as previously described. The infectivity of bacteria was expressed as the percentage of infected cells per coverslip.

Effect of resveratrol on C. pneumoniae induced foam cell formation

J774.1 macrophages (1×10^5 cells/well) were pretreated with resveratrol (25 μ M) for 1 h at 37°C and 5%CO₂. Next, cells washed with PBS were, infected with *C. pneumoniae* (MOI=2), and incubated with resveratrol (25 μ M) and LDL (100 μ g/ml) for additional 48 h.

In some experiments, macrophages were incubated for 48 h with LDL (100 μ g/ml) in the presence and absence of *C. pneumoniae* (MOI= 2).

In other experiments, macrophages were pretreated with resveratrol for 1 h and then incubated with LDL and resveratrol (25 μ M) for additional 48 h.

For experiments with PPAR- γ antagonist, macrophages were pre-treated with GW9662 (10 μ M) at 37°C and 5% CO₂ for 2 h before resveratrol treatment (25 μ M). Macrophages were then infected with *C. pneumoniae* (MOI=2) and incubated with resveratrol (25 μ M), GW9662 (10 μ M) and LDL (100 μ g/ml) for additional 48 h.

Oil Red O staining

Macrophages were washed twice with PBS, fixed with 10% paraformaldehyde for 10 min, stained with 1% Oil-Red O solution (60% isopropanol) for 10 min and then counterstained with haematoxylin for two min. Cells were washed three times with tap water and examined by light microscopy (magnification: $\times 100$). The intracellular lipid droplets were stained red, and cell nuclei were stained blue.

Foam cells, defined as cells with ten Oil Red O-positive lipid droplets or more (≥ 10), were quantified and expressed as percentage of positive Oil Red O cells to total cells. Foam cell formation was also assessed by cytoplasmic lipid detection. Briefly, cells were washed with PBS and fixed with 10% paraformaldehyde for 3 h at room temperature. Cells were then washed with tap water and incubated with Oil Red-O solution for 30 min. Cells were

rinsed with tap water and allowed to air dry. Lipid-bound Oil Red O was extracted with isopropanol and absorbance was measured at 520 nm in a spectrophotometer.

Intracellular cholesterol assay

Cells were harvested and washed twice with PBS, and then disrupted by ultrasonication. The content of intracellular total cholesterol and free cholesterol was detected using the cholesterol/cholesteryl ester quantitation kit according to the manufacturer's instructions. Cholesteryl ester content was determined by subtracting free cholesterol from total cholesterol. Cholesteryl ester and cholesterol levels were expressed as μ g cholesterol/mg proteins. Foam cells were defined by cholesteryl ester/total cholesterol $\geq 50\%$. Protein concentration was determined by the Bradford method (29).

Lipoprotein oxidation assay

LDL oxidation was quantified by measuring the levels of thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde (MDA). Briefly, cells were collected into microfuge tubes and then centrifuged at 4000 rpm for 10 min 400 μ l of supernatant were incubated with 800 μ l of TBARS reagent (0.67% thiobarbituric acid in 0.1 M NaOH-trichloroacetic acid, 1:1), at 95°C for 30 min. The mixture was cooled and centrifuged at room temperature for 10 min at 500 \times g, and the absorbance of the supernatants was measured at 532 nm in a spectrophotometer. Concentrations of LDL oxidation products were determined from a standard curve constructed using MDA, which reacts with TBA to yield fluorimetric MDA-TBA adducts. Oxidation values was expressed as nmol MDA/ml.

Superoxide anion assay

Superoxide anion production by NADPH oxidase was determined by measuring the rate of superoxide dismutase-inhibitable reduction of ferrocyclochrome *c* at 550 nm ($\epsilon=21100$ M⁻¹cm⁻¹ for ferrocyclochrome *c*) as previously described (32). Superoxide anion concentration was expressed as nmol/10⁶ cells.

IL-17A measurement

IL-17A levels in the supernatants were measured by using commercially available ELISA kit in according to the manufacturer's instructions.

Statistical analysis

Results were expressed as means \pm standard deviation of at least three independent experiments performed in triplicate. Statistical significance of differences was analyzed by Student's *t*-test. Values of $p < 0.05$ were

considered statistically significant.

RESULTS

Inhibitory effect of resveratrol on C. pneumoniae induced foam cell formation

In order to evaluate the effect of resveratrol on *C. pneumoniae*-induced foam cell formation, we first performed experiments to examine the cytotoxic effect of this polyphenol on cells. Thus, macrophages were incubated for 48 h with resveratrol at the concentration of 25, 50 and 100 μ M and cell viability was determined by the MTT test. The viability of macrophages was 84% and 68% respectively at 50 and 100 μ M, whereas no significant reduction of cell viability or morphological changes were observed when macrophages were treated with resveratrol at 25 μ M.

As expected, *C. pneumoniae* infection induced macrophages-derived foam cell formation.

Specifically, compared to uninfected and LDL-treated macrophages, *C. pneumoniae* infection significantly increased the number of foam cells (4% vs 38%) ($p=0.006$) as well as the ratio of intracellular cholesteryl ester to total cholesterol (33.3% vs 66.7%) ($p<0.001$) (Fig. 1 A-D).

The treatment with resveratrol 25 μ M markedly reduced the *C. pneumoniae*-induced foam cell formation (Fig. 1 A-D). Indeed, resveratrol treatment resulted in a significant reduction of the number of foam cells (12.5% vs 38%, $p=0.03$) as well as the ratio of intracellular cholesteryl ester to total cholesterol as compared to infected and untreated cells (38.5% vs 66.7%, $p<0.001$).

To determine whether the reduction in the number of foam cells were due to an anti-chlamydial activity of resveratrol, *C. pneumoniae* infected macrophages were incubated for 72 h in the presence of this polyphenol (25 μ M) and the subsequent number of infected cells was determined. There was

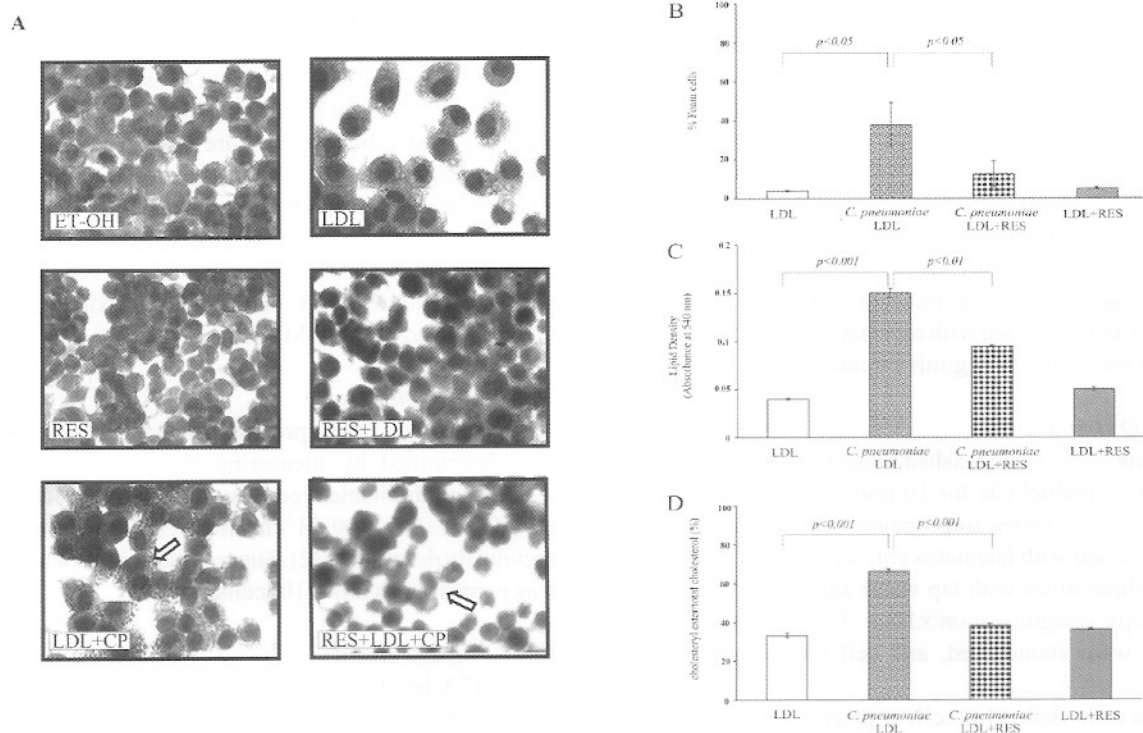


Fig. 1. Inhibitory effect of resveratrol on *C. pneumoniae* induced foam cell formation. (A) Macrophages were incubated with ethanol, LDL (100 μ g/ml), RES (25 μ M), RES/LDL, *C. pneumoniae*/LDL and *C. pneumoniae*/LDL/RES for 48 h, fixed and stained with Oil Red O to detect intracellular lipid content. Cellular nuclei were stained with haematoxylin. The white arrow indicates intracellular lipid droplets stained by Oil Red O. (B) Foam cells expressed as percentage of positive Oil Red O cells compared to total macrophages. (C) Lipid content measured by alcohol extraction of Oil Red O-stained lipid, with absorbance at 540 nm. (D) The ratio of intracellular cholesteryl ester to total cholesterol. CP: *C. pneumoniae*; RES: resveratrol.

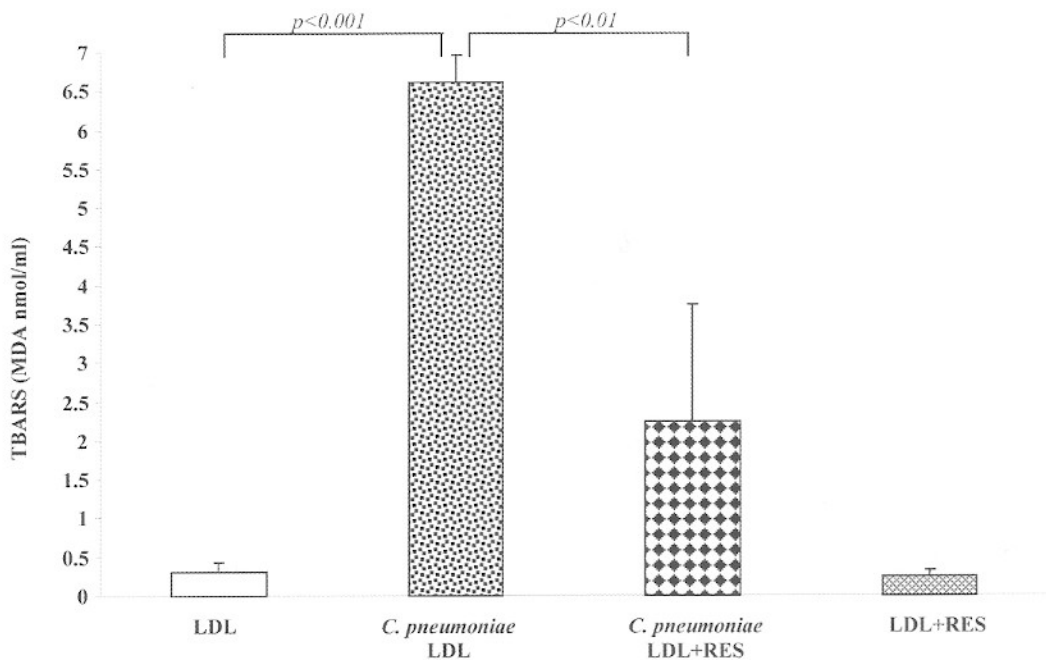


Fig. 2. Inhibitory effect of resveratrol on LDL oxidation induced by *C. pneumoniae* infection in the presence of LDL. Macrophages were incubated with LDL (100 $\mu\text{g/ml}$), *C. pneumoniae*/LDL, *C. pneumoniae*/RES/LDL and LDL/RES (25 μM) for 48 h. Then, levels of thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA), produced in the culture medium were measured as described in Materials and Methods. RES: resveratrol.

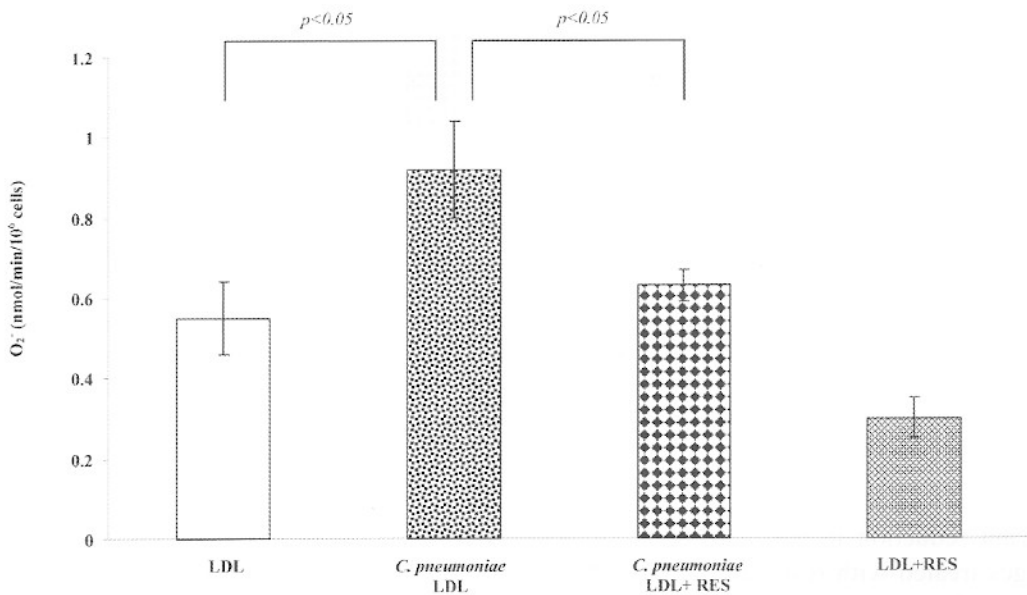


Fig. 3. Inhibitory effect of resveratrol on superoxide anion production induced by *C. pneumoniae* infection in the presence of LDL. Macrophages were incubated with LDL (100 $\mu\text{g/ml}$), *C. pneumoniae*/LDL, *C. pneumoniae*/RES/LDL and LDL/RES (25 μM) for 48 h. Then, superoxide anion levels (O_2^-) in culture medium were determined by measuring the rate of superoxide dismutase-inhibitable reduction of ferrocytochrome c at 550 nm. RES: resveratrol.

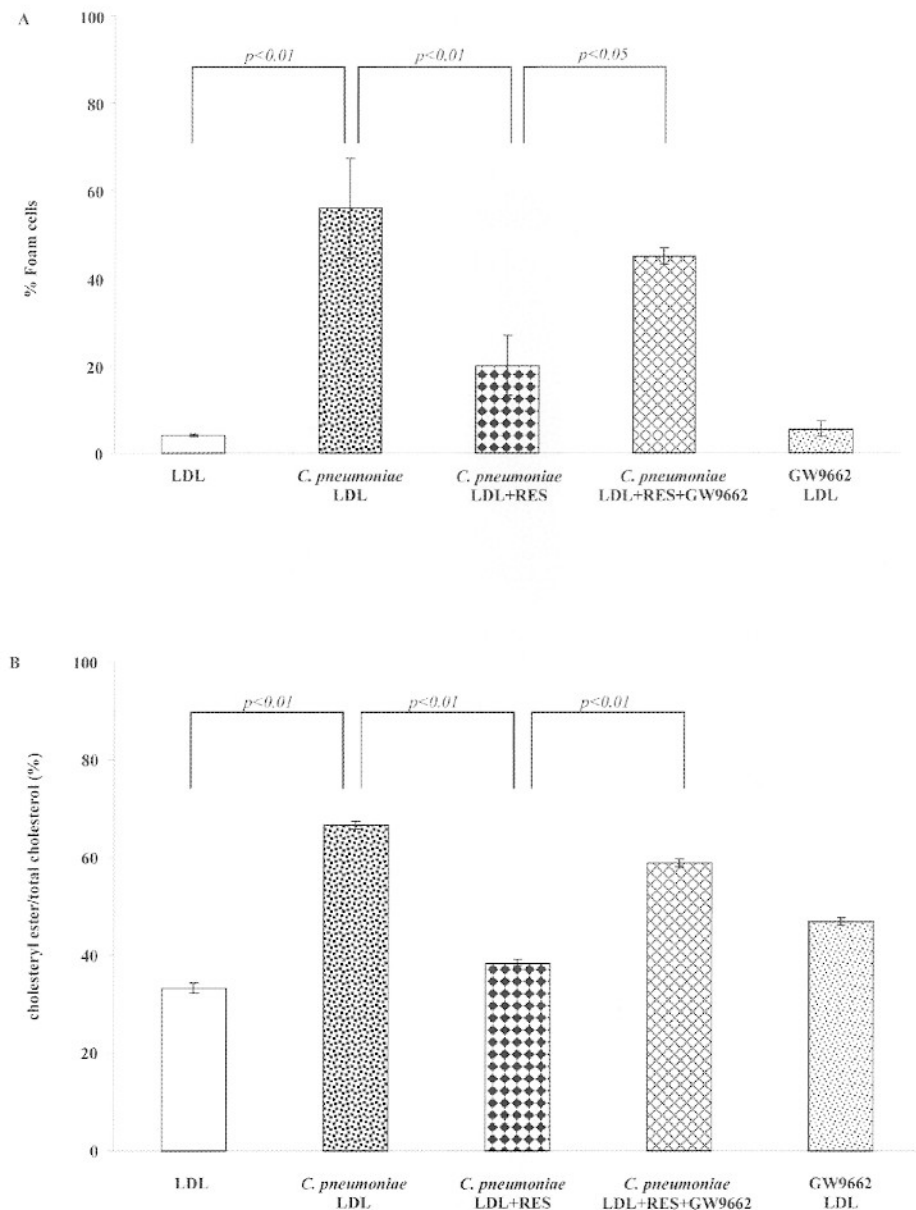


Fig. 4. Inhibitory effect of resveratrol on *C. pneumoniae* induced foam cell formation via PPAR- γ dependent pathway. Macrophages were pre-treated for 2 h with the PPAR- γ antagonist (GW 9662) (10 μ M), before the addition of resveratrol (25 μ M), and then further incubated for 48 h with resveratrol (25 μ M), GW 9662 (10 μ M), and LDL (100 μ g/ml) in the presence or absence of *C. pneumoniae*. (A) Foam cells expressed as percentage of positive Oil Red O cells compared to total macrophages. (B) The ratio of intracellular cholesteryl ester to total cholesterol. RES: resveratrol.

no significant reduction in the number of infected macrophages treated with resveratrol ($75 \pm 2.3\%$) as compared to infected and untreated cells ($69 \pm 3.1\%$).

Inhibitory effect of resveratrol on *C. pneumoniae* induced LDL oxidation

As previously reported, *C. pneumoniae* induces

foam cell formation partly by stimulating LDL oxidation (25). As shown in Fig. 2, a significantly increased production of TBARS, expressed as MDA, was observed in infected macrophages as compared to uninfected cells ($p < 0.001$). In contrast, in the presence of resveratrol, the LDL oxidation was significantly inhibited as shown by the reduction of

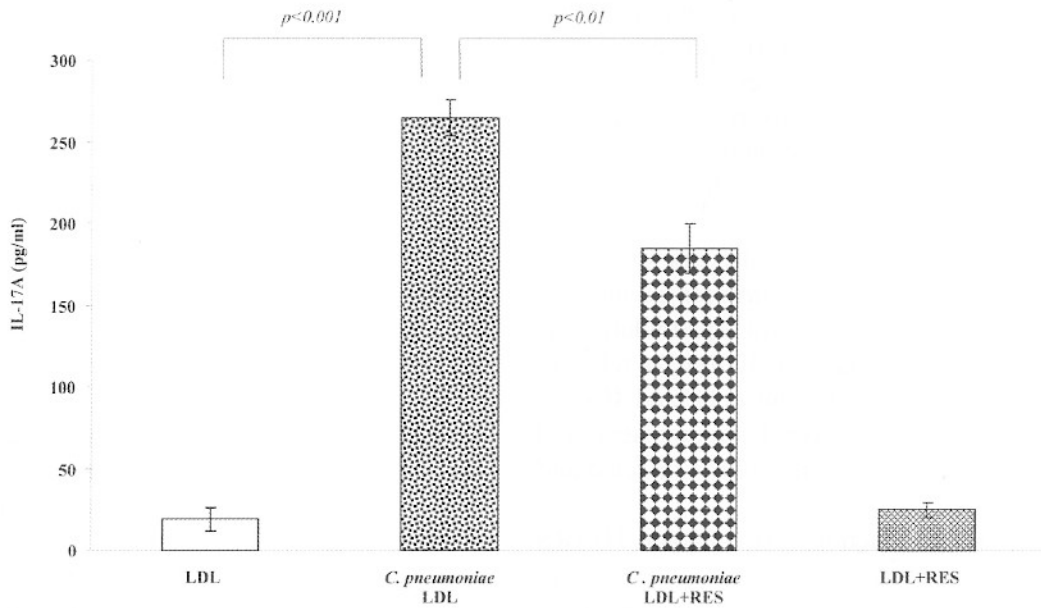


Fig. 5. Inhibitory effect of resveratrol on *C. pneumoniae* induced IL-17A production. IL-17A levels in supernatants of macrophages incubated with LDL (100 μ g/ml), *C. pneumoniae*/LDL, *C. pneumoniae*/RES/LDL and LDL/RES (25 μ M) for 48 h were measured by ELISA assay. RES: resveratrol.

MDA levels as compared to infected and untreated cells ($p=0.008$).

Inhibitory effect of resveratrol on *C. pneumoniae* induced production of superoxide anion

In order to evaluate the antioxidant effect of resveratrol on the production of superoxide anion (O_2^-), we measured the amount of O_2^- in LDL treated macrophages after a 48 h incubation with resveratrol in the presence or absence of *C. pneumoniae*. As shown in Fig. 3, *C. pneumoniae* strongly induced the production of superoxide anion ($p=0.01$) and such production was significantly inhibited by the treatment with resveratrol (25 μ M) ($p=0.02$).

Inhibitory effect of resveratrol on *C. pneumoniae* induced foam cell formation via PPAR- γ dependent pathway

As previously reported, *C. pneumoniae* induces foam cell formation via PPAR- γ dependent pathway (23). Therefore, we examined whether the inhibitory effect of resveratrol on *C. pneumoniae* induced foam cell formation was dependent upon PPAR- γ .

The treatment with PPAR- γ -specific antagonist, GW 9662, significantly diminished the effect of resveratrol increasing the number of foam cells ($p<0.05$) and ratio of intracellular cholesteryl ester to total cholesterol ($p<0.01$) (Fig. 4, A-B).

Inhibitory effect of resveratrol on *C. pneumoniae* induced IL-17A synthesis

Compared to uninfected and LDL-treated macrophages, *C. pneumoniae* infection significantly increased the IL-17A production ($p<0.001$). On the contrary, IL-17A levels were markedly lower in infected macrophages and treated with resveratrol as compared to infected and untreated cells ($p=0.002$) (Fig. 5).

DISCUSSION

C. pneumoniae association with cardiovascular diseases and especially with atherosclerosis, as well as the involvement of this microorganism in the pathogenesis of atherosclerotic plaque, has been suggested by numerous studies (3-8, 20). *C.*

pneumoniae may promote the development and progression of the atherosclerotic process whose first step consists in the foam cell formation. *C. pneumoniae* is well known to play a causative role in the foam cell formation mainly through the stimulation of LDL oxidation as well as the uptake of oxLDL by macrophages (24, 25).

Our results demonstrate for the first time that resveratrol contrasts the *C. pneumoniae*-induced intracellular lipid and cholesterol accumulation as well as the IL-17A production. Indeed, a relevant decrease in the number of foam cells and IL-17A levels was observed in infected macrophages and treated with resveratrol as compared to infected and untreated cells.

The decreased production of IL-17A and TBARS observed in our study suggests that resveratrol prevents the inflammatory response and the LDL oxidation induced by *C. pneumoniae*. Several studies have suggested that resveratrol exhibits cardio-protective and anti-inflammatory properties, since this polyphenol has been shown to decrease the synthesis of several cytokines including IL-17A (30, 31) as well as to efficiently contrast the LDL oxidation which contributes to the pathogenesis of atherosclerosis (31).

Among the several mechanisms involved in resveratrol-mediated inhibition of foam cell formation, our study shows that resveratrol treatment may prevent *C. pneumoniae* induced foam cell formation by regulating the cholesterol efflux in macrophages and decreasing superoxide anion production and thus LDL oxidation. Indeed, it is well understood that cholesterol efflux through reverse cholesterol transporters (ABCA1 and ABCG1) directly contributes to macrophage transformation into foam cells (32, 33). More interestingly, it has been recently reported that *C. pneumoniae* infection down-regulates the ABCA1/ABCG1 expression via PPAR- γ (23). Our results reveal that the inhibition of PPAR- γ by a specific antagonist (GW 9662), in the presence of resveratrol and *C. pneumoniae*, enhanced intracellular lipid and cholesterol accumulation and the subsequent foam cell formation. As a matter of fact, GW 9662 both simulates *C. pneumoniae* infection and prevents the positive effects of resveratrol by blocking the PPAR- γ pathway.

Particularly intriguing, in our study, is the effect

of resveratrol on IL-17A synthesis. This cytokine was first believed to be totally produced by T cells, but it is now known to be secreted by other innate immune cell types including macrophages (34).

Our results demonstrate, for the first time, the *C. pneumoniae*-induced synthesis of IL-17A by macrophages suggesting its involvement in atherogenesis. In fact, in our study, a decrease was observed in the production of IL-17A following resveratrol treatment of infected macrophages exposed to LDL, along with a reduced foam cell formation. These findings are supported by *in vivo* studies showing the pro-atherogenic effects of IL-17A since elevated serum concentrations of IL-17A were associated with *C. pneumoniae*-mediated atherosclerosis (27). In addition, concerning the pro-atherogenic properties of IL-17A, a recent study demonstrated that this cytokine activates vascular cells to secrete cytokines that in turn enhance foam cell formation (35).

Overall, our data demonstrate an anti-atherogenic effect of resveratrol in *C. pneumoniae*-induced foam cell formation by reducing LDL oxidation, regulating the macrophage lipid homeostasis and decreasing IL-17A production.

In conclusion, the main result of our study is the evidence of an anti-atherogenic effect of resveratrol on macrophage-derived foam cell formation and IL-17A production induced by *C. pneumoniae*. Therefore, it will be important to pursue further studies in order to identify an effective treatment with resveratrol for the prevention of *C. pneumoniae*-associated cardiovascular events in high-risk patients.

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