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J DENT RES 2013 92: 241 originally published online 14 January 2013
DOI: 10.1177/0022034512473309

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RESEARCH REPORTS
Biological

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J Dent Res 92(3):241-246, 2013

A. actinomycetemcomitans LPS Enhances Foam Cell Formation Induced by LDL

ABSTRACT

The objective of this study was to examine whether native low-density lipoprotein (LDL) induces foam cell formation by macrophages and to examine the effect of lipopolysaccharide (LPS) on native LDL-induced foam cell formation by macrophages *in vitro*. RAW 264.7 cells were cultured with LDL or high-density lipoprotein (HDL) in the presence of LPS derived from *Aggregatibacter actinomycetemcomitans*. Foam cell formation was determined by staining with Oil-red-O to visualize cytoplasmic lipid droplet accumulation. The expression of LDL-receptor and the degree of internalization of FITC-conjugated LDL in RAW 264.7 cells were examined by immunofluorescence microscopy. The images were digitally recorded and analyzed with Image J software. Statistical analysis was performed by JMP software. Foam cell formation was induced by the addition of native LDL in dose- and time-dependent manners, whereas HDL showed no effect. LPS enhanced the foam cell formation induced by native LDL. In addition, LPS stimulated the expression of LDL-receptor protein on RAW 264.7 cells and enhanced the internalization of LDL. The enhancement of foam cell formation induced by LPS and LDL was inhibited by the depolymerizing agent nocodazole and amiloride analog 5-(N-ethyl-N-isoprophyl) amiloride (EIPA). Our findings indicate that LPS plays an important role in foam cell formation by LDL-stimulated macrophages.

KEY WORDS: periodontopathic bacteria, macrophage, LDL-receptor, macropinocytosis, atherosclerosis, periodontitis.

DOI: 10.1177/0022034512473309

Received August 17, 2012; Last revision November 6, 2012; Accepted December 10, 2012

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INTRODUCTION

Atherosclerosis shows a chronic inflammatory process initiated by the recruitment of monocyte-derived macrophages into the subendothelial arterial space and their transformation into lipid-laden foam cells (Ross, 1993). Low-density lipoprotein (LDL) is an abundant atherogenic lipoprotein in plasma (Pyörälä *et al.*, 1994) and the main component of plasma cholesterol, which enters vessel walls and then, by some mechanism, enters macrophages. Previously, macrophage foam cell formation was reported to occur only through the uptake of modified forms of LDL such as oxidized or aggregated LDL (Kruth, 2001). However, a previous study demonstrated that macrophage foam cell formation can occur *via* the uptake of native LDL (Kruth *et al.*, 2002). Chronic bacterial infections, including periodontitis, are associated with an increased risk of coronary heart diseases (Pussinen *et al.*, 2003; Spahr *et al.*, 2006; Sakurai *et al.*, 2007). However, the mechanisms by which chronic infections increase the likelihood of atherosclerosis are poorly defined.

Periodontitis is a chronic inflammatory disease characterized by the destruction of tooth-supporting structures. *Aggregatibacter actinomycetemcomitans*, a Gram-negative periodontopathic bacterium, plays a crucial role in the development of periodontal disease (Nishihara and Koseki, 2004). Lipopolysaccharide (LPS) in periodontal pockets promotes atherogenesis as an inflammatory agent by activating leukocytes. The atherogenic properties of LPS derived from *A. actinomycetemcomitans* and their effects on macrophage-like cells, namely, RAW 264.7 cells, have been examined in several studies (Pussinen *et al.*, 2004; Lakio *et al.*, 2006). In addition, previous experimental and clinical investigations have indicated that adhesion of monocytes to vascular endothelial cells is an important step in atherosclerosis (Libby *et al.*, 2002). Macrophages play an important role in the pathogenesis of atherosclerosis and are one of the major cell types involved in foam cell formation. In contrast, scant attention has been paid to the effects of periodontopathic bacterial LPS on foam cell formation induced by LDL. In the present study, we examined the synergistic effect of LPS on LDL-stimulated macrophages.

MATERIALS & METHODS

Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37°C in α-minimal essential medium (α-MEM; GIBCO BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum

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(FBS), penicillin G (100 U/mL), and streptomycin (100 μg/mL) in an atmosphere of 5% CO_2 in air.

LPS Preparation

LPS was extracted from lyophilized cells of *A. actinomycetemcomitans* Y4 by a hot phenol-water method (Nishihara *et al.*, 1986). The extract was treated with nuclease and washed extensively with pyrogen-free water by ultracentrifugation. The major chemical components of the extract were 41% neutral sugar, 8% hexosamine, and 31% fatty acid (Tsutsumi *et al.*, 2010).

Cell Viability Assays

Cell viability was determined with the tetrazolium salt WST-1 (4-[3-(4- iodophenyl)-2H-5-tetrazolio]-1-3-benzene disulfonate) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). RAW 264.7 cells were plated in 96-well culture plates at a concentration of 2 x 10^4/mL and treated with LDL (Sigma-Aldrich Co., St. Louis, MO, USA) or high-density lipoprotein (HDL) (Sigma-Aldrich) solution for 44 hrs. These lipoproteins were derived from human plasma, dissolved in 150 mM NaCl, pH 7.4, supplemented with 0.01% EDTA and stored at 4°C. Endotoxin in these lipoproteins was below the detection level as determined by Limulus amebocyte lysate assay (data not shown). WST-1 solution (10 μL) was added to each well, followed by incubation for 4 hrs. Absorbance at 450 nm and 630 nm was measured by means of a Multiskan JX microplate reader (Thermo ELECTRON CO., Yokohama, Japan).

Macrophage Foam Cell Formation and Lipid Staining

We examined the foam cell formation by macrophages using staining with Oil-red-O to visualize cytoplasmic lipid droplet accumulation. RAW 264.7 cells were plated in 96-well culture plates at a concentration of 2 x 10^4/mL at 1 day before stimulation, then stimulated with LDL or HDL in the presence or absence of nocodazole (Calbiochem, San Diego, CA, USA) or 5-(N-ethyl-N-isoprophyl) amiloride (EIPA, Sigma-Aldrich) for various time periods. In some experiments, the cells were precultured with LPS for 24 hrs prior to stimulation with LDL, nocodazole, and EIPA. The cells were fixed with 10% formaldehyde for 10 min, washed with phosphate-buffered saline (PBS; pH 7.2), and dehydrated with 60% isopropanol for 1 min, followed by staining with a working solution of Oil-red-O (Primary Cell Co., Ltd., Sapporo, Japan) for 20 min. After cells were washed in 60% isopropanol in PBS, foam cell formation by macrophages was observed under an optical microscope (IX71; Olympus Co., Tokyo, Japan), and images were captured digitally in real time by means of a CCD camera.

Immunofluorescent Staining

The expression of LDL-receptor (LDL-R) in RAW 264.7 cells was examined by immunofluorescence microscopy. RAW 264.7 cells were plated in 8-well chamber plates (Nalge Nunc International, Rochester, NY, USA) at a concentration of 5 x 10^4/mL, then stimulated with LDL or HDL (100 μg/mL) for various time periods. Next, the cells were rinsed with PBS and then fixed with 4% formaldehyde for 15 min at room temperature. After being washed in PBS, the cells were blocked with blocking buffer (PBS supplemented with 1% bovine serum albumin), followed by incubation with anti-LDL-R polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. Bound antibodies were detected with Alexa Fluor 488 anti-goat IgG antibody (Invitrogen, Carlsbad, CA, USA) for 2 hrs at room temperature. The cells were visualized by means of a Fluorescence Microscope BZ-9000 (KEYENCE Corp., Osaka, Japan). Images were captured digitally in real time and processed with BZ-II imaging software (KEYENCE Corp.; magnification, x 40; exposure time, 2.0 sec). The images were digitally recorded and stored as 'tagged image file format' (TIFF) files. Semi-quantitative analysis of the detected proteins was performed by histogram analysis with Image J software (http://rsbweb.nih.gov/ij, National Institutes of Health, Bethesda, MD, USA). Briefly, each image was first color-split into a single green channel image, followed by thresholding at a value of 0 to 255. The volume of the LDL-R-positive area was then measured as the sum of the pixel number multiplied by the brightness for each level. The average volume of control samples was assigned the value 1.0. At least 30 cells in 5 to 10 fields *per* dish were recorded, and values obtained from 3 dishes on different days were averaged for each time-point.

LDL Labeling

LDL (1 mg/mL) in 0.1 M $NaHCO_3$ (pH 9.0) was gently mixed with 10 μL/mL of FITC (2 mg/mL in dimethyl sulfide), then unbound dye was removed by gel filtration on a Sephadex G-25 column equilibrated with 150 mM NaCl (pH 7.4) and 0.01% EDTA buffer (Brinkley, 1992). RAW 264.7 cells (2.5 x 10^3/mL) were incubated with LPS for 24 hrs and added to FITC-conjugated LDL. After being cultured for an additional 12 hrs, the cells were visualized by means of a Fluorescence Microscope BZ-9000 (KEYENCE Corp.). Images were captured digitally in real time and processed with BZ-II imaging software (KEYENCE Corp.). Semi-quantitative analysis of the internalization was performed by histogram analysis with Image J software. The stained area was measured for 30 cells in 10 fields *per* dish and averaged for each time-point. The percentage of positive cells was calculated as internalized cell number/total cell number × 100.

Statistical Analysis

Statistical analysis of foam cell formation by macrophages was conducted with JMP software, version 8.0.2 (SAS Institute Inc., Cary, NC, USA). Results are expressed as the mean ± standard deviation (SD). One-way analysis of variance was used to analyze the manner in which the distribution of each continuous variable differed among the groups. A Tukey-Kramer HSD (honestly significant difference) test was utilized to examine differences in regard to group means. In immunofluorescence staining analysis, statistical significance was determined by the Dunnett test.

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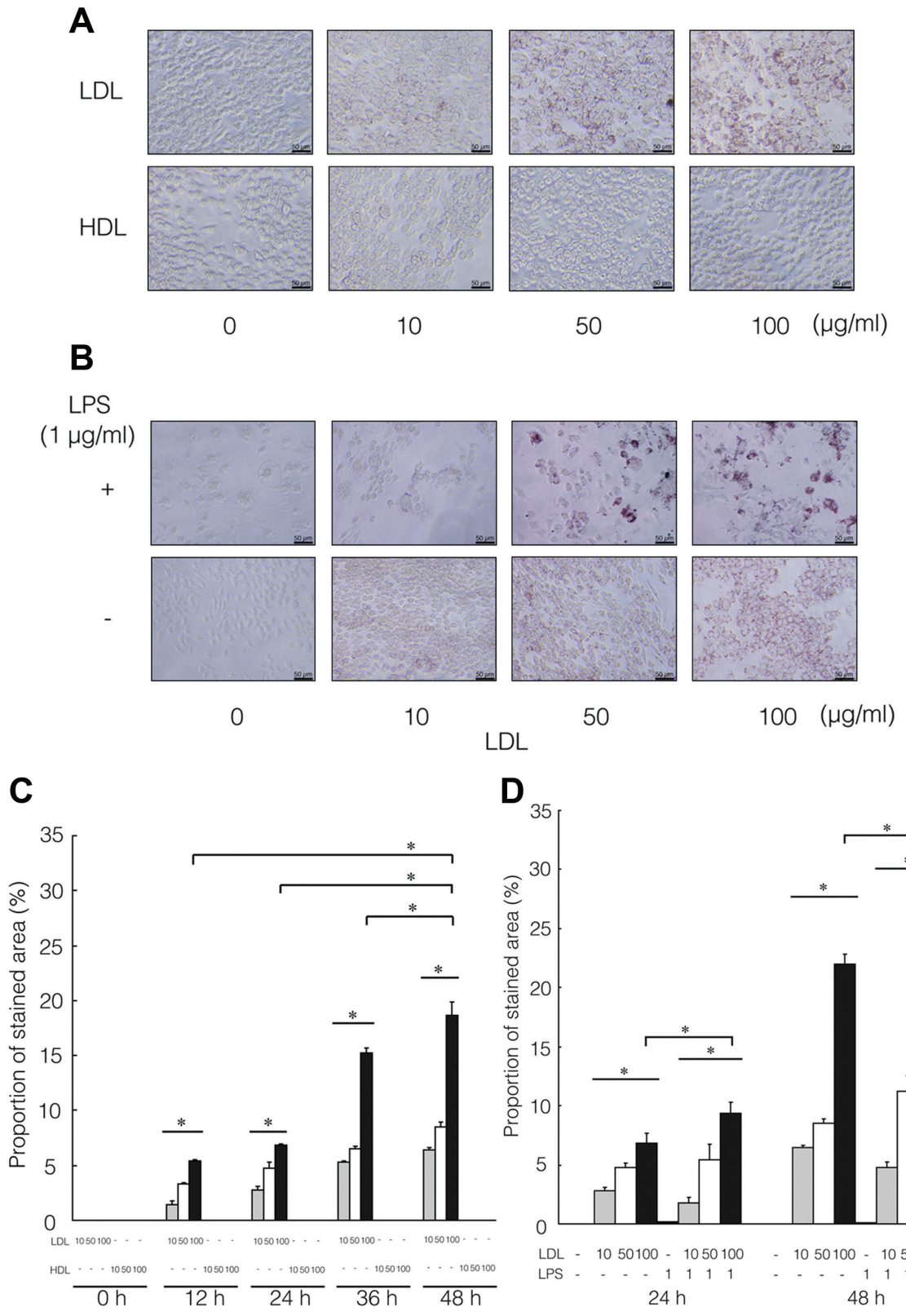

Figure 1. Representative microscope images of foam cell formation by macrophages. **(A)** RAW 264.7 cells were cultured with LDL or HDL at 37°C for 48 hrs, then fixed, stained with Oil-red-O, and visualized by optical microscopy. **(B)** RAW 264.7 cells were treated with LPS (1 μg/mL) for 24 hrs, then incubated with LDL for 48 hrs and analyzed by Oil-red-O staining for detection of foam cell formation. **(C, D)** The proportion (%) of stained areas in RAW 264.7 cells was calculated as described in Materials & Methods. Data are expressed as the mean ± SD of 3 experiments, with similar results obtained in each experiment. *Significant difference between mean stained areas of 2 groups, as assessed by the Tukey-Kramer HSD test ($p < 0.05$).

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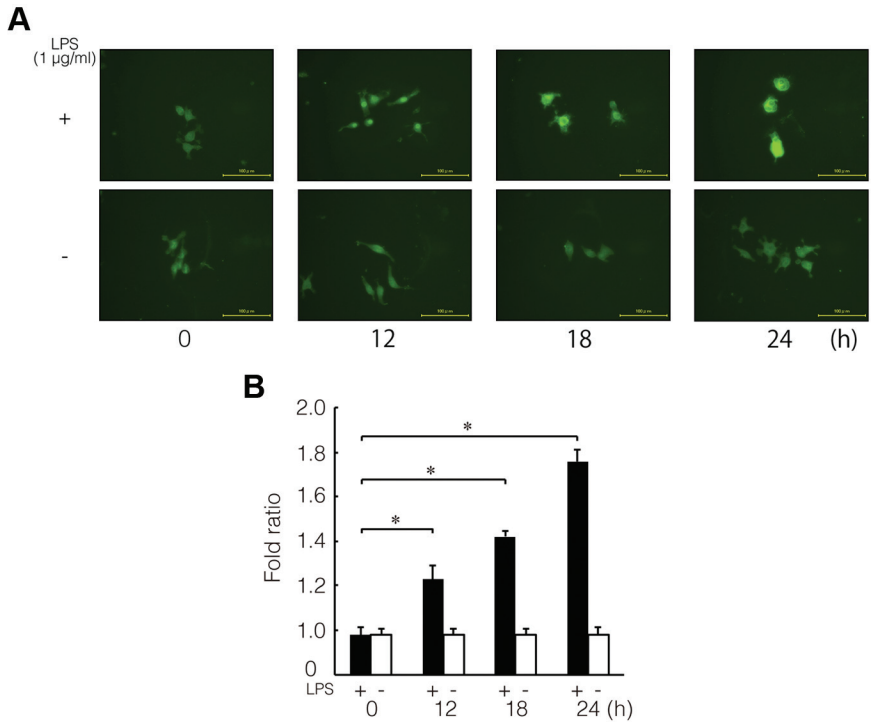

Figure 2. Expression of LDL-R in RAW 264.7 cells stimulated with LPS. (**A**) Cells were treated with LPS (1 μg/mL) at 37°C for the indicated times, then fixed and immunostained for LDL-R, and visualized by fluorescence microscopy as described in Materials & Methods. (**B**) Morphometric analysis of changes in LDL-R in RAW 264.7 cells. Data are expressed as the mean ± SD of 3 experiments, with similar results obtained in each. *Significant difference for mean of fold ratio between 2 groups, as assessed by the Dunnett test ($p < 0.05$).

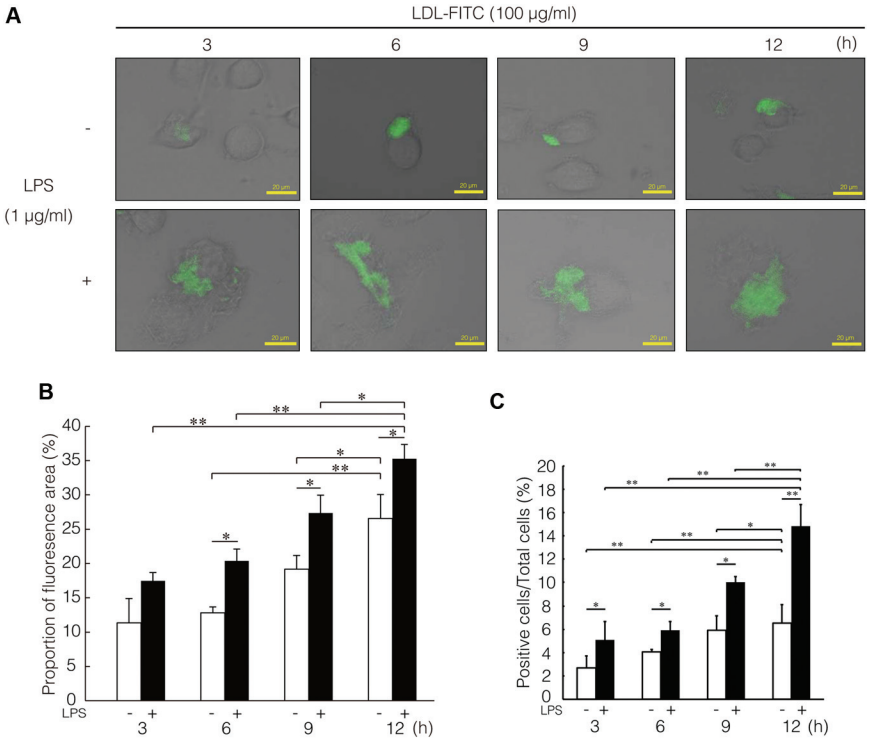

Figure 3. Internalization of LDL by macrophages. (**A**) RAW 264.7 cells were cultured with FITC-conjugated LDL in the presence of LPS and visualized by fluorescence microscopy as described in Materials & Methods. (**B**) The presence of LDL in RAW 264.7 cells was calculated as described in Materials & Methods. (**C**) The results were also expressed as a percentage (means ± SD) of the LDL-internalized cells. Data are expressed as the mean ± SD of 3 experiments, with similar results obtained in each.

RESULTS

Foam Cell Formation by Macrophages

We examined the effect of LDL on foam cell formation by macrophages. Treatment with LDL visually increased foam cell formation dose- and time-dependently, whereas HDL had almost no effect (Figs. 1A, 1C). To elucidate the effect of LPS on the foam cell formation induced by LDL, we treated RAW 264.7 cells with LPS (1 μg/mL) for 24 hrs, and then cultured them with LDL for 48 hrs. Treatment with LPS enhanced the foam cell formation by RAW 264.7 cells induced by LDL in a time-dependent manner up to 48 hrs, compared with the formation by RAW 264.7 cells stimulated with LDL in the absence of LPS (Figs. 1B, 1D).

Effect of LPS on LDL-R Expression

We examined the expression levels of LDL-R on RAW 264.7 cells stimulated with LPS. Immunofluorescence microscopic analysis revealed that the treatment with LPS (1 μg/mL) increased LDL-R expression in a time-dependent manner up to 24 hrs (Fig. 2).

Internalization of LDL into Macrophages

The degree of internalization of LDL into RAW 264.7 cells was examined by fluorescence microscopy. RAW 264.7 cells were pre-cultured with LPS (1 μg/mL) for 24 hrs, following the incubation with FITC-conjugated LDL (100 μg/mL) for an additional 12 hrs. FITC-conjugated LDL was internalized into macrophages when the cells were stimulated with LPS (Fig. 3).

Effects of Interfering Microtubes on Foam Cell Formation Induced by LDL

Enhancement of foam cell formation of RAW 264.7 cells induced by LDL and LPS was dramatically suppressed dose-dependently by the treatment with nocodazole (Fig. 4A) and EIPA (Fig. 4B). In contrast, nocodazole and EIPA had no effect on the cell viability of RAW 264.7 cells (data not shown).

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DISCUSSION

Periodontitis is a bacterial infection with tooth-supporting tissue destruction, eventually leading to tooth loss. Gram-negative *A. actinomycetemcomitans* is involved in the pathology of several kinds of periodontitis. *A. actinomycetemcomitans* activates leukocytes because of expressing 2 exotoxins, a cytolethal distending toxin and a leukotoxin (Johansson, 2011). There is increasing evidence that foam cells present in atheromatous lesions are macrophages derived from circulating monocytes (Schaffner *et al.*, 1980; Gerrity, 1981). Several micro-organisms have been implicated in the infectious etiology of atherosclerosis (Haraszthy *et al.*, 2000), and several investigations have attempted to relate periodontitis to cardiovascular disease (Mattila, 2003; Cairo *et al.*, 2004). In the present study, we used a homogeneous clonal population of murine RAW 264.7 cells to elucidate the role of LPS derived from *A. actinomycetemcomitans* in the formation of foam cells by macrophages. This cell line is known to exhibit lipid accumulation and foam cell formation *via* scavenger receptor-mediated endocytosis and constitutive macropinocytosis (Yao *et al.*, 2009).

Although macrophage foam cell formation was reported to occur only through the uptake of modified forms of LDL, such as oxidized or aggregated LDL (Kruth, 2001), our results revealed that foam cell formation occurred through the uptake of native LDL (Fig. 1). A previous study reported that oxidized LDL induced apoptosis, reduced the viability of RAW 264.7 cells, and enhanced cell aggregation (Li *et al.*, 2006). Neither LDL nor HDL used in this study had an effect on the cell viability of RAW 264.7 cells. These results may exclude the possible involvement of the phenomenon that the oxidation of native LDL enhanced foam cell formation through cell aggregation. In addition, LPS was shown to induce further foam cell formation by macrophages in the presence of exogenous LDL, suggesting that periodontopathic bacteria are associated with increased risk of cardiac damage. *A. actinomycetemcomitans* is an important etiological agent of several kinds of periodontitis as well as endocarditis (Suzuki *et al.*, 2001). Chronic periodontitis is characterized by multiple episodes of bacteremia, which is an event that allows this pathogenic organism to migrate to areas of atherosclerotic plaque.

LDL-R functions as a primary receptor for binding and internalization of plasma-derived LDL cholesterol and regulates plasma LDL concentrations. The expressions of LDL receptors are subject to feedback control by intracellular cholesterol levels (Ye *et al.*, 2009). In the present study, we demonstrated that LPS significantly increased LDL-R protein expression (Fig. 2). Quantitative real-time reverse-transcription polymerase chain-reaction analysis also revealed that LPS stimulation increased in LDL-R mRNA copies as compared with untreated control RAW 264.7 cells (data not shown). This is consistent with a previous study which demonstrated that LPS significantly increased native LDL accumulation in THP-1 macrophages, specifically *via* the LDL-R pathway (Ye *et al.*, 2009). Taken together, these findings suggest that chronic inflammation may fundamentally modify cholesterol homeostasis by disrupting LDL receptor feedback regulation.

In addition to receptor-mediated endocytosis, macrophages possess high-level macropinocytosis activity (Steinman *et al.*, 1983). Foam cell formation by macrophages was previously thought to occur only by receptor-mediated uptake of LDL. However, recent studies have revealed that macropinocytosis is one of the important mechanisms for native LDL uptake and foam cell formation by macrophages (Kruth *et al.*, 2002, 2005; Anzinger *et al.*, 2010), as well as an actin-dependent process, including membrane ruffling and formation of membrane vesicles (Lee and Knecht, 2002). Nocodazole has been shown to partially inhibit macropinocytosis in mouse-derived macrophages (Racoosin and Swanson, 1992). It is well-known that nocodazole disrupts associations between microtubules and the plasma membrane, which normally permit macropinocytosis to occur. Moreover, the addition of EIPA, a specific inhibitor of macropinocytosis, Na^+/H^+ exchange blocker, also attenuated foam cell formation induced by LDL and LPS (Feng *et al.*,

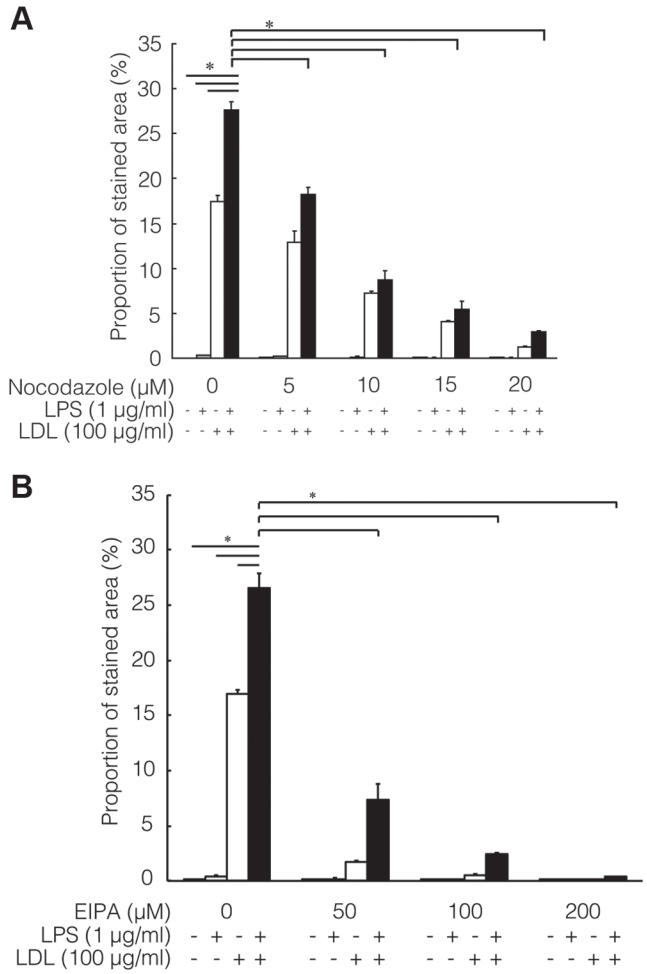

Figure 4. Inhibitory effects of nocodazole and EIPA on LDL-induced foam cell formation by macrophages. RAW 264.7 cells were precultured with LPS (1 μg/mL) for 24 hrs, following the incubation with LDL (100 μg/mL) in the presence or absence of nocodazole and EIPA for 48 hrs. Foam cell formation was then analyzed by Oil-red-O staining. Staining rates are expressed as the mean ± SD of 3 experiments, with similar results obtained in each. *Significant difference between mean proportion of staining area in 2 groups, as assessed by the Tukey-Kramer HSD test ($p < 0.05$).

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2010). We found that not only nocodazole, but also EIPA, remarkably inhibited the effect of LPS on LDL-induced foam cell formation by RAW 264.7 cells (Fig. 4). On the basis of these findings, we speculate that enhancement of LDL-induced foam cell formation by LPS is partially dependent on macropinocytosis.

In conclusion, our results show that LPS enhances the foam cell formation induced by LDL by activating LDL-R expression and macropinocytosis in RAW 264.7 cells. Although additional studies are needed to examine the correlation of native LDL and LPS with regard to etiology of atherosclerosis, by *in vivo* experiments, we propose that LPS derived from periodontopathic bacteria is involved in the induction of atherogenesis mediated by LDL.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science. The authors have received nothing of value from a commercial company or institution. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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