JPET Fast Forward. Published on August 6, 2012 as DOI: 10.1124/jpet.112.196147
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JPET #196147

Title page

Proatherogenic macrophage activities are targeted by the flavonoid quercetin

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Running title page

Running title: Quercetin targets proatherogenic macrophages

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42 text pages

0 tables

6 figures

60 references

223 words in the abstract

576 words in the introduction

1665 words in the discussion

Abbreviations

CVD, cardiovascular disease; BMMo, bone marrow-derived macrophages; LPS, lipopolysaccharide; LDL, low density lipoprotein; oxLDL, oxidized LDL; ROS, reactive oxygen species; apoE-/-, apolipoprotein E-deficient; SR, scavenger receptor; IL, interleukin; NLRP, nucleotide-binding domain and leucine-rich repeat containing protein; MDA, malondialdehyde; NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; AP-1, activator protein-1; EC, endothelial cell; SMC, smooth muscle cell;

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TLR, toll-like receptor; HFD, high-fat diet; TBARS, thiobarbituric acid-reactive species; DiI, 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate; TNF, tumor necrosis factor; GC/EIMS-SIM, gas chromatography electron-ionization mass spectrometry and selected ion monitoring; i.p., intraperitoneal

Section assignment: Cardiovascular

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Abstract

Many studies have demonstrated that the flavonoid quercetin protects against cardiovascular disease (CVD) and related risk factors. Atherosclerosis, the underlying cause of CVD, is also attenuated by oral quercetin administration in animal models. Although macrophages are key players during fatty streak formation and plaque progression and aggravation, little is known about the effects of quercetin on atherogenic macrophages. Here, we report that primary bone marrow-derived macrophages internalized less oxidized low-density lipoprotein (oxLDL) and accumulated less intracellular cholesterol in the presence of quercetin. This reduction of foam cell formation correlated with reduced surface expression of the oxLDL receptor CD36. Quercetin also targeted the LPS-dependent, oxLDL-independent pathway of lipid droplet formation in macrophages. In oxLDL-stimulated macrophages, quercetin inhibited reactive oxygen species production and IL-6 secretion. In a system that evaluated cholesterol crystal-induced IL-1β secretion via NLRP3 inflammasome activation, quercetin also exhibited an inhibitory effect. Dyslipidemic ApoE-deficient mice chronically treated with intraperitoneal quercetin injections had smaller atheromatous lesions, reduced lipid deposition and less macrophage and T cell inflammatory infiltrate in the aortic roots than vehicle-treated animals. Serum levels of total cholesterol and the lipid peroxidation product malondialdehyde (MDA) were also reduced in these mice. Our results demonstrate that quercetin interferes with both key proatherogenic activities of macrophages, namely foam cell formation and prooxidant/proinflammatory responses, and these effects may explain the atheroprotective properties of this common flavonoid.

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Introduction

Atherosclerosis is a chronic, non-resolving inflammatory disease caused by the accumulation of apolipoprotein B-containing lipoproteins, such as low density lipoprotein (LDL), in the vascular wall (Hansson, 2009). Dyslipidemia triggers abnormal lipoprotein retention, endothelial cell (EC) activation, the recruitment of circulating leukocytes and lipoprotein oxidation. Resident and infiltrating monocyte-derived macrophages interact with oxidized LDL (oxLDL), transform into foam cells and promote vascular inflammatory responses through innate receptors, such as CD36 and toll-like receptors (TLR), and crystal sensors, such as the NLRP3 inflammasome (Hansson and Hermansson, 2011). In the past few years, it has become clear that the interaction of lipoproteins and other endogenous molecules with macrophages and the subsequent effects on inflammation and fat metabolism constitute a central event in atherosclerosis pathogenesis (Moore and Tabas, 2011) that could be targeted for immunological intervention of cardiovascular disease (CVD).

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a prototype naturally occurring flavonoid that exhibits potent anti-oxidant activities and protects from various degenerative diseases (Chirumbolo, 2010; Perez-Vizcaino and Duarte, 2010; Mendoza and Burd, 2011). Among the diverse pharmacological activities of quercetin, those supporting its ability to protect from CVD and related risk factors appear to be prominent (Perez-Vizcaino and Duarte, 2010; Russo et al., 2012). Several epidemiological (Hertog et al., 1993; Hertog et al., 1995) and clinical (Edwards et al., 2007; Egert et al., 2009) studies support the cardioprotective effects

of this flavonoid in humans. Moreover, administration of quercetin to rabbits (Juzwiak et al., 2005), hamsters (Auger et al., 2005) and mice (Hayek et al., 1997; Leckey et al., 2010; Loke et al., 2010; Kleemann et al., 2011) inhibits atherosclerosis development, which is the underlying cause of CVD. Cellular and molecular investigations further support quercetin's bioactivity in atherosclerosis and suggest that it acts through anti-oxidant/cytoprotective and anti-inflammatory mechanisms. *In vitro*, quercetin is a potent reactive oxygen species (ROS) scavenger (Boots et al., 2008); it protects LDL from oxidation (Hayek et al., 1997; Naidu and Thippeswamy, 2002; Leckey et al., 2010) and EC from lipid peroxidation (Kleemann et al., 2011) and prevents redox imbalance (Kostyuk et al., 2011) and endothelin-1-induced dysfunction (Romero et al., 2009). *In vivo*, quercetin protects against vascular oxidative stress (Loke et al., 2010). Quercetin also has anti-inflammatory activities; it inhibits adhesion molecule expression and chemokine expression in endothelial cells and smooth muscle cells (SMC) *in vitro* (Kobuchi et al., 1999; Tribolo et al., 2008; Winterbone et al., 2009; Panicker et al., 2010; Kleemann et al., 2011) and reduces vascular and systemic markers of inflammation *in vivo* (Loke et al., 2010; Kleemann et al., 2011).

Although there is a large amount of information available regarding the atheroprotective effects of quercetin in non-hematopoietic vascular cells, little is known about its effects on atherogenic macrophages. Quercetin has been shown to interfere with oxLDL uptake by macrophages (Hayek et al., 1997; Kawai et al., 2008) and inflammatory pathways in oxLDL-stimulated leukocytes (Bhaskar et al., 2011). However, those studies were performed in transformed cell lines or heterogeneous cell populations and only assessed one aspect of the oxLDL-macrophage interaction.

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In the present study, we investigated the atheroprotective mechanisms of quercetin in macrophages by using primary cells and found that it inhibited oxLDL-dependent and -independent foam cell formation, protected macrophages from oxLDL-induced ROS production and inhibited the secretion of proinflammatory cytokines induced by oxLDL, lipopolysaccharide (LPS) or cholesterol crystals. In an *in vivo* model of atherosclerosis, we also demonstrated that parenteral administration of quercetin significantly reduced lipid deposition and inflammatory infiltrate in the aortic root.

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Materials and methods

Reagents, animals and diet: Wild-type and apolipoprotein E-deficient C57BL/6 mice (apoE-/-) were purchased from Charles River (USA) and Jackson laboratories (USA), respectively. Mice were maintained under a specific pathogen-free environment at the SPF animal facility of the Sede de Investigacion Universitaria (SIU), Universidad de Antioquia. The institutional ethical committee approved all *in vivo* procedures. Animals were fed with a standard mouse diet (LabDiet, USA) or a high-fat diet (HFD, adjusted calories diet 42%, Harlan Teklad, USA). Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; CAS number 117-39-5) was purchased from Merck (USA) and a stock solution (10 mM) was prepared in DMSO and stored at -20°C. LPS (from *E. coli* 0727:B8) and 2,7-diclorofluorescein diacetate (DCFH-DA) were from Sigma-Aldrich (USA). Cholesterol crystals were prepared for some experiments following published protocols (Duewell et al., 2010) with minor modifications. Briefly, cholesterol standard (purity > 99%, Sigma, USA) was solubilized in pre-heated acetone (60°C) and crystallized by cooling (-20°C). After six cycles, a final crystallization step was performed in the presence of 10% sterile USP water to obtain hydrated crystals. The suspension was centrifuged, and the crystals were dried in a laminar flow cabinet before they were macerated with a homogenizer and stored at room temperature until further use.

Obtaining and characterization of oxLDL: Human LDL isolation was performed following previously published protocols (Naidu and Thippeswamy, 2002). LDL fraction purity was confirmed by SDS/PAGE. LDL (500 µg/ml) was oxidized with

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$CuSO_4$-$5H_2O$ (40 μM) at 37°C for 9 hours. Oxidation was stopped with EDTA (1%). Effective oxLDL generation was confirmed by the increased formation of thiobarbituric acid-reactive species (TBARS) and enhanced mobility of apolipoprotein B100 (ApoB100) on 0.8% agarose gels (Supplemental Figure 1) due to increased electronegativity, compared to native LDL. Endotoxin levels in LDL and oxLDL preparations were always less than 0.4 ng/mg protein. For some experiments, oxLDL (1 mg/mL) was fluorescently labeled with 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen, USA; 10 μM) over 12 hours as previously described (Ide et al., 2006), and the DiI-oxLDL complex was dialyzed against PBS for 12 hours.

Macrophage generation and treatment: Macrophages were generated by culturing bone marrow precursors obtained from C57BL/6 mice in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) as previously described (Lutz et al., 1999). Nine-day cultures consisted of non-adherent dendritic cells and firmly adherent bone marrow-derived macrophages (BMMo) (Lutz et al., 1999). Adherent macrophages were removed with cell scrapers, counted and seeded in RPMI 1640 culture medium (Glutamax, Gibco, USA) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM β-mercaptoethanol and 2 mM L-glutamine at 1×10^6 cells/ml for further use. We evaluated the *in vitro* effects of quercetin on macrophage proatherogenic responses (CD36 surface expression, foam cell formation, production of ROS and proinflammatory cytokine secretion). Assays were first carried out to optimize the experimental conditions. All experiments were performed at 37°C under 5% CO_2. In all cases, macrophages (5×10^5 cells/ml) were pre-treated with 20 μM quercetin for 12 hours and subsequently incubated with

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different stimuli (LDL, oxLDL, LPS or cholesterol crystals) as indicated in the following sections. Quercetin concentration for macrophage treatment was based on previous in vitro bioactivity work (Kawai et al., 2008; Winterbone et al., 2009; Choi et al., 2010) and on pilot assays where not toxic concentrations were defined (not shown). Parallel cultures were treated with an equivalent volume of vehicle (DMSO 0.05%) for use as negative controls. None of the macrophage proatherogenic responses evaluated here were affected by the treatment with vehicle in both basal or stimulated conditions (not shown). Likewise, quercetin alone did not induce any detectable effect on resting macrophages (not shown). All reagents used during macrophage differentiation and treatments were endotoxin free, as certified by commercial suppliers. Regular testing of endotoxin contamination to macrophage cultures was performed by using a colorimetric LAL assay (QCL-1.000, Lonza, USA) with negative results (less than 0.2 EU/ml). *In vitro* toxicity of the different treatments was assessed by commercial viability tests (LDH assay kit, Promega, USA).

CD36 expression and foam cell formation: For CD36 surface expression, quercetin-treated macrophage cultures were further stimulated with oxLDL (30 µg/ml) for 36 hours, washed, removed from the plate with a cell scraper and cold PBS and submitted to flow cytometry. To assess oxLDL uptake, cells were further treated with DiI-labeled oxLDL (50 µg/ml) for 6 hours, washed, removed, and analyzed by flow cytometry to determine the amount of internalized fluorescent dye. In parallel uptake assays, quercetin-pre-treated cells were exposed to oxLDL (30 µg/ml) for 36 hours, and the intracellular accumulation of cholesterol was determined by gas chromatography/mass spectroscopy (GC/MS). To evaluate oxLDL-independent foam cell formation, LPS (10, 30 or 100 ng/ml) was added to quercetin-treated

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macrophages (cultured onto circular coverslips) in the absence or presence of LDL (50 µg/mL) and incubated for an additional 36 hours. Cells on coverslips were fixed in paraformaldehyde (4%), stained with Oil Red O (0.5%) and mounted on microscope slides. Representative micrographs were taken with the aid of a digital camera (Nikon DS-Fi1, Japan). The area of Oil Red O staining per macrophage was determined with the help of NIS Element BR software (400X magnification; Nikon, Japan). The amount of lipid accumulation was reported as the total area (in pixels^2) in 100 cells per slide.

ROS production: Quercetin-treated BMMo were washed twice with PBS and treated with oxLDL (50 µg/ml) for 1 hour. Cells were then incubated with 10 µM fluorescent ROS-sensitive substrate DCFH-DA for 30 minutes, washed twice, resuspended with PBS, and finally analyzed by flow cytometry, spectroflurometry or fluorescence microscopy to assess intracellular ROS production.

Cytokine secretion: After quercetin treatment, macrophages were stimulated with oxLDL (25 µg/ml) or LPS (10 µg/mL) for an additional 12 hours. In another set of experiments, quercetin-treated BMMo were primed with LPS (10 or 100 ng/ml) for 2 hours and treated with cholesterol crystals (500 µg/ml) for the last 10 hours, for a total treatment time of 24 hours. Macrophage culture supernatants were collected and used to quantify the levels of IL-1β, IL-6, IL-10, IL-12p70, IL-12p40, TNFα, MCP-1 and MIP-1α by ELISA or Luminex.

Flow cytometry: For CD36 surface expression, cells were first incubated with a purified anti-mouse CD36 antibody (clone CRF D-2712, BD Biosciences, USA) or an

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isotype control (clone M18-254, BD Biosciences, USA) before incubation with a FITC-rat anti-mouse secondary antibody (clone C10-3, BD Biosciences, USA). For oxLDL uptake and ROS production, cells were resuspended in PBS and analyzed. A Beckman-Coulter EPICS XL flow cytometer was used for all acquisitions (at least 10,000 events), and data storage and analysis was performed with WinMDI software. The results were reported as the percentage of CD36+, DiI+ or DCF+ cells and the mean of fluorescent intensity (MFI).

Quantitation of cellular cholesterol by GC/EIMS-SIM: After treatments, macrophages were washed four times and resuspended in PBS at 1×10^6 cells/mL. Next, 20 µL (1000 ppm) 16-dehydropregnenolone (Sigma, USA) was added to the suspension as an internal standard (ISTD). Cell lysate was obtained after three cycles of freezing/thawing and 30 minutes of 28-Hz ultrasonic disruption (Ultrasonic bath, Ney ULTRAsonik,™ USA). Lipid fractions were extracted with 1 mL chloroform and subsequently dried and dissolved with 200 µL chloroform to be analyzed by GC/MS (7890/5975C, Agilent Technologies, USA). Samples (2 µL) were injected in splitless mode at 270°C using an HP-5MS capillary column (5% phenyl-polymethylsiloxane, 30 m × 0.25 mm in diameter × 0.25 mm) with helium as the carrier gas. The oven was programmed at 190°C for 3 minutes, and the temperature was increased at a rate of 12°C/minute to reach 290°C. The temperatures of the ionization source and the quadrupole were 230°C and 150°C, respectively. The ionization voltage was 70 eV. For quantitation in single ion monitoring (SIM) mode, ions were selected as follows: 145 (quantifier) and 275, 255 and 105 (identifier, ID) for cholesterol (Sigma, USA) and 314 (quantifier) and 145 and 105 (ID) for the ISTD. Quantitation was performed by using the response factors of cholesterol standards

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(1-50 mg/L). For cholesterol standard quantification, CV was less than 1.81%. For ISTD, as well as for cholesterol quantification in macrophages, a maximum CV of 1.46% was permitted.

ELISA and Luminex: Cytokine concentrations were determined with commercial sandwich ELISA kits (IL-1β, IL-6, IL-10, IL-12p70 and TNFα; mouse OptEIA ELISA kits, BD Biosciences, USA) or multiplex Luminex technology format (IL-1β, IL-6, IL-10, IL-12p70, IL-12p40, TNFα, MCP-1 and MIP-1α; Milliplex xMAP, Millipore, USA). The detection thresholds were as follows: 15.6 pg/mL for IL-1β, 31.2 pg/mL for IL-6, 31.3 pg/mL for IL-10, 62.5 pg/mL for IL-12p70 and 15.6 pg/mL for TNFα for ELISA assays and 3.2 pg/mL for all analytes in the xMAP system.

***In vivo* experiment:** A mouse model of atherosclerosis was used to evaluate whether systemic exposure to quercetin promotes atheroprotection in vivo and whether this effect associates to lipid deposition and inflammatory response. ApoE-/- mice spontaneously develop atherosclerotic lesions in several parts of the arterial vascular tree, which grow to form stable large lesions in aged mice (Meir and Leitersdorf, 2004). Lesion development, however, can be accelerated by feeding apoE-/- mice a HFD**.** Male apoE-/- mice (n=9) were treated i.p. with 50 mg/kg quercetin every other day. Two weeks later, mice were shifted to a HFD and maintained on quercetin treatments for the following 12 weeks. This dose was based on reported toxicity studies in rodents and humans (Ferry et al., 1996; Harwood et al., 2007) and on preclinical bioactivity reports in rodents (Amalia et al., 2007). Because it is known that bioavailability of oral quercetin is low, and that this route of administration leads to none/little systemic circulation of the active aglycone form, we

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decided to use ip administration. Additionally, since half life of quercetin and its metabolites in blood is short (from 11 to 28 hours; (Manach et al., 2005)), we defined this chronic and repetitive schedule of injections in order to assure permanent exposure to the flavonoid during the whole experimental period and increase the chances to observe an atheroprotective effect in the case it exists. Control mice (n=9) were treated with vehicle (0.1% DMSO). After 14 weeks, mice were sacrificed, and the hearts and aortas were removed. Samples were fixed with paraformaldehyde, immersed in 30% sucrose, embedded with Shandon Cryomatrix™ (Thermo scientific, USA) and stored at -20°C. Frozen samples were processed with a cryostat (Leica Microsystems, Germany) to obtain 6-7-µm-thick sections of the aortic sinus as previously described (Paigen et al., 1987). The sections were mounted on charged glass slides (Thermo Fisher scientific, USA) and stained with conventional hematoxylin/eosin (H/E), Oil Red O (Sigma-Aldrich, USA) or processed for immunohistochemistry (IHC). For IHC, sections were acetone-fixed, blocked for endogenous peroxidase activity, permeabilized and incubated with macrophage- or T cell-specific mAb (anti-mouse monocyte/macrophage, clone MOMA-2, or anti-CD3 antibody, clone KT3, respectively; Serotec, UK). A secondary horseradish peroxidase (HRP)-conjugated goat anti-rat IgG antibody and the chromogenic substrate DAB (Serotec, UK) were used to develop Ab binding. Finally, the sections were counterstained with hematoxylin. Micrographs (40X magnification) were taken, and the area of the atherosclerotic lesion (H/E), lipid deposition (oil red+), macrophage infiltration (MOMA-2+) and T cell infiltration (CD3+) were calculated (in μm^2) by using NIS Element BR image analysis software (Nikon, Japan). The results were reported as the mean area of 9-12 sections per each animal. Blood samples were taken and serum levels of total cholesterol, hepatic enzymes (ALT and AST), amylase and

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creatinine were determined with dry chemistry methods (Johnson and Johnson, USA) at the Universidad de Antioquia Veterinary School. Circulating levels of the lipid peroxidation product malondialdehyde (MDA) were also determined by using the thiobarbituric acid-reactive species (TBARS) method and a standard curve as previously described (Jentzsch et al., 1996). MDA values are expressed as nmol per mg protein (as determined by BCA method, Pierce, USA).

Statistical analysis: For *in vitro* assays, experiments were performed in triplicate and the mean ± SEM was reported. One-way analyses of variance and Newman-Keuls tests were used for multiple group comparisons with GraphPad Prism software (GraphPad, USA). For *in vivo* experiments, results are expressed as the mean ± SEM. Treated and control groups were compared with unpaired Mann-Whitney student t test. Statistically significant differences are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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RESULTS

1. Quercetin modulates oxLDL-dependent foam cell formation in BMMo

Given the importance of oxLDL uptake in foam cell formation during atherosclerosis (Hansson and Hermansson, 2011), we first investigated whether quercetin influences the internalization of fluorescently labeled oxLDL and subsequent cholesterol accumulation in macrophages. Efficient oxLDL internalization by BMMo was confirmed by fluorescent microscopy and flow cytometry (Figure 1A, top). Quercetin was a potent inhibitor of oxLDL uptake by BMMo; the percentage of DiI-oxLDL+ cells and the amount of incorporated ligand per cell was significantly reduced in quercetin-treated macrophages (Figure 1A, bottom). Chromatographic quantitation of intracellular cholesterol content confirmed that oxLDL (Figure 1B, top), but not LDL (not shown) induced cholesterol accumulation. Interestingly, when cells were treated with quercetin, cholesterol accumulation in BMMo was reduced to basal levels (Figure 1B, bottom). To further understand the mechanisms of reduced oxLDL uptake in quercetin-treated macrophages, we used flow cytometry to monitor the surface expression of CD36, one of the most important oxLDL receptors in this cell type. Although basal expression of CD36 on BMMo was high (approximately 80% CD36+ cells with relatively high density; figure 2A), exposure to oxLDL further increased in a dose-dependent manner (Figure 2B). As expected, native LDL was unable to promote CD36 expression (not shown). When BMMo were pre-treated with quercetin and subsequently exposed to oxLDL, we observed a significant down regulation of CD36 expression (Figure 2C). These results demonstrate that quercetin

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inhibits oxLDL-mediated foam cell formation, apparently via down regulation of its receptor on macrophages.

2. Quercetin modulates TLR-dependent lipid body formation in macrophages

In addition to the classical oxLDL-scavenger receptor (SR)-dependent pathway of foam cell formation, a novel TLR-dependent mechanism of lipid body formation that leads to cholesterol and triglyceride accumulation in macrophages has been described (Nicolaou and Erridge, 2010). This alternative pathway is believed to be important to atherosclerosis pathogenesis and as a potential target for intervention (Nicolaou and Erridge, 2010). Therefore, we asked whether quercetin also inhibited lipid body formation in BMMo through this pathway. We first confirmed that LPS promotes lipid “droplet” in a dose-dependent manner and that quercetin did not induce any lipid body deposition in BMMo (Figure 3A). Interestingly, quercetin significantly prevented lipid body formation and accumulation in macrophages under conditions of low (10 ng/ml) and moderate LPS (30 ng/ml) stimulation. Quercetin was unable to inhibit droplet formation when macrophages were stimulated at 100 ng/ml LPS (conditions in which 100% BMMo were heavily charged with lipid droplets, figure 3B). As reported (Funk et al., 1993), supplementation of LPS-stimulated cultures with LDL (50 μg/ml) enhanced lipid body formation (3-, 42- and 99-fold increase for cultures stimulated with 10, 30 and 100 ng/ml LPS, respectively, compared to non-supplemented cultures). In these LDL-supplemented cultures, quercetin exhibited essentially the same activity (inhibition of lipid droplet formation at a low but not at a high LPS-stimulating dose; not shown). Thus, we showed, for the first time, that a

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TLR-dependent non-conventional pathway of foam cell formation is also targeted by quercetin.

3. ROS production in oxLDL-stimulated macrophages is also prevented by quercetin treatment

Recognition of oxLDL by CD36 and other receptors on macrophages trigger pro-oxidative events, such as ROS production, which contribute to atherosclerosis progression and aggravation (Hulsmans and Holvoet, 2010; Levitan et al., 2010; Park and Oh, 2011). By using a fluorometer-based assay, we found that oxLDL recognition by BMMo rapidly induces ROS production, with maximal levels produced as soon as 1-hour post-incubation (not shown). ROS production was also monitored by fluorescence microscopy and quantified by flow cytometry (Figure 4A). These methods allowed the demonstration that fluorescence could not be quenched by cobalt, which is indicative of intracellular ROS production (Figure 4A). We observed that basal ROS production in BMMo was negligible, whereas oxLDL stimulation induced dose-dependent ROS production (Figure 4B). Native LDL did not induce detectable ROS (not shown) indicating that only the modified lipoprotein exerts a pro-oxidative effect in macrophages. Notably, quercetin exhibited a potent inhibitory effect on oxLDL-induced ROS production as indicated by the significant reduction in the percentage of DCF+ cells and the MFI in quercetin-treated cultures compared with vehicle-treated cells (Figure 4C). Collectively, these experiments are the first demonstration that ROS production triggered by oxLDL in macrophages is modulated by quercetin.

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4. Macrophage proinflammatory cytokine response induced by oxLDL and cholesterol crystals is inhibited by quercetin treatment

Because proinflammatory cytokine and chemokine production by macrophages in the arterial wall is essential for atherosclerosis development (Tedgui and Mallat, 2006; Tabas, 2010; Hansson and Hermansson, 2011), we investigated the effect of quercetin on oxLDL-stimulated macrophages. By using a sensitive Luminex method in a multiplex format, we found that sub-lethal concentrations of oxLDL, did not induce TNFα, IL-1β, IL-10, IL-12p40, IL-12p70, MIP-1α or MCP-1 (not shown). In contrast, IL-6 was readily induced in a dose-dependent manner (Figure 5A, top). Interestingly, IL-6 secretion was significantly inhibited when quercetin was present in oxLDL-stimulated BMMo cultures (Figure 5A, bottom). As expected (Comalada et al., 2006), quercetin also inhibited IL-1β, TNFα, IL-12p70 and MCP-1 secretion in LPS-stimulated BMMo (Figure 5B). A novel proinflammatory pathway triggered by cholesterol crystals in macrophages that leads to NLRP3 inflammasome activation and IL-1β maturation/release was recently shown to be important during atherosclerosis (Duewell et al., 2010; Moore and Tabas, 2011). *In vitro*, NLRP3 activation and IL-1β secretion can be induced by addition of cholesterol crystals to LPS-primed macrophages (Duewell et al., 2010). To evaluate the effect of quercetin on this proinflammatory pathway, we first defined the conditions under which IL-1β release was mediated by the presence of cholesterol crystals in the BMMo culture medium (Figure 5C, left). Under those conditions, quercetin exerted a potent inhibitory effect, reducing the secreted amount of active IL-1β (Figure 5C, right). This suggested a potential inhibitory activity of quercetin on cholesterol crystal-induced NLRP3 inflammasome activation. Collectively, these results demonstrate that

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quercetin targets multiple innate inflammatory pathways that are known to operate in macrophages during atherosclerosis.

5. **Nontoxic doses of parenterally administered quercetin are atheroprotective in apoE-/- mice**

Having demonstrated that quercetin inhibits important proatherogenic macrophage activities *in vitro*, it was important to evaluate the *in vivo* relevance of these findings. We therefore used apoE-/- mice to evaluate the atheroprotective effect of intraperitoneal (i.p.) quercetin, a route of administration that is expected to improve bioavailability and systemic exposure to active aglycone (Russo et al., 2012). As shown in figure 6A, mice treated with quercetin had smaller atheromatous plaques with reduced lipid deposition, as well as reduced macrophages and T cell inflammatory infiltrate in the aortic roots compared to vehicle-treated mice. Interestingly, quercetin-treated mice also had significantly lower plasma cholesterol levels than vehicle-treated mice (Figure 6B, left). Moreover, MDA serum levels were also reduced (Figure 6C, right), indicating that quercetin-treated mice were protected from dyslipidemia-induced oxidative stress. Notably, these atheroprotective effects were observed in the absence of any sign of toxicity, since no animal presented weight loss, piloerection, dehydration, secretions, changes in mucosal surfaces or abnormal behavior, locomotion or activity during or following the experiment (not shown). Additionally, no evidence of nephrotoxicity, hepatotoxicity or pancreatic toxicity was found with serum clinical biochemical tests (Supplemental Figure 2). These results are in accordance with the *in vitro* effects of quercetin described in

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previous sections and demonstrated that parenteral administration of the quercetin aglycone is atheroprotective.

Discussion

Macrophages play a central role during all stages of atherosclerosis development (Moore and Tabas, 2011) and quercetin metabolites are present in plaque foam cells *ex vivo* (Kawai et al., 2008). This suggests that lesional macrophages could be *in vivo* quercetin targets that mediate atheroprotection. The work reported here shows that quercetin interferes with two key proatherogenic properties of macrophages: foam cell formation and prooxidant/proinflammatory activity (Supplemental Table 1). Our results provide new insights into the atheroprotective mechanism of this flavonoid.

Non-regulated accumulation of lipoproteins in macrophages is a constant process during atherosclerosis. oxLDL are internalized via scavenger receptors (SR) leading to foam cell formation (Levitan et al., 2010; Silverstein et al., 2010). We found that quercetin inhibited oxLDL uptake and cholesterol accumulation in BMMo (Figure 1), and this effect paralleled a reduced surface CD36 expression (Figure 2). Accordingly, apoE-/- mice treated with quercetin had smaller atherosclerotic lesions with less lipid deposition (Figure 6). Previous reports showed reduced foam cell formation and CD36 expression in oxLDL-stimulated mouse RAW264 (Kawai et al., 2008) and J774A1 (Choi et al., 2010) cell lines treated with the quercetin metabolite quercetin-3-glucuronide or the quercetin glycoside quercitrin, respectively. In those reports, however, CD36 gene expression was assessed at the messenger level by RT-PCR and at the protein level by western blot. Our results demonstrated for the first time, the inhibitory effect of quercetin aglycone on functional surface CD36 receptor expression and subsequent foam cell formation in primary macrophages. The molecular mechanisms of quercetin-mediated CD36 reduction appear to involve peroxisome proliferator-activated receptor-γ and protein kinase C (Choi et al., 2010)

but require further characterization. In pioneering work (Hayek et al., 1997), the atheroprotective activity of quercetin *in vivo* was associated with reduced atherogenic ligand (oxLDL) formation. Here, we complement by demonstrating a reduced atherogenic receptor formation on macrophages. Because other flavonoids have been shown to inhibit CD36 expression and oxLDL uptake (Lian et al., 2008), it is likely that this represents a common atheroprotective mechanism of many anti-oxidant flavonoids.

A TLR-dependent, CD36-independent pathway that mediates lipid body formation via alterations in macrophage lipid metabolism has been recently proposed as an alternative route for foam cell formation during atherosclerosis (Nicolaou and Erridge, 2010). We found that LPS-induced lipid droplet formation was significantly reduced by quercetin (Figure 3). Although the mechanisms underlying this inhibitory effect were not investigated here, quercetin is known to inhibit NF-κB (Nam, 2006), a major signal transduction pathway for TLR. However, taking into account the growing complexity and convergence of metabolic and inflammatory pathways and the variety of cellular processes targeted by quercetin, it would not be surprising if other pathways are involved. In a report being published during the revision of this manuscript, researchers elegantly demonstrated that quercetin stimulated cholesterol efflux in macrophages, via p38 MAP kinase-mediated upregulation of the ABCA1 transporter (Chang et al., 2012), providing mechanistic insights into the molecular processes implicated in the reduction of cholesterol accumulation and foam cell formation promoted by this flavonoid and reported here. Beyond the complexity of the mechanisms involved, our results indicate that quercetin targets multiple pathways for macrophage foam cell formation.

JPET Fast Forward. Published on August 6, 2012 as DOI: 10.1124/jpet.112.196147
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Cytokines and ROS are essential proatherogenic factors, and macrophages in the atheroma are a rich source (Tedgui and Mallat, 2006). Recognition of modified LDL by macrophages triggers ROS and proinflammatory cytokine production (Hulsmans and Holvoet, 2010; Park and Oh, 2011). Two major pathways of ROS/inflammatory response to modified LDL have been described in macrophages, which involve either a CD14/TLR4/MD-2 (Bae et al., 2009) or a CD36/TLR4/TLR6 (Stewart et al., 2010) receptor complex. When we stimulated BMMo with oxLDL, we found a dose-dependent induction of ROS and IL-6 but not of other cytokines/chemokines (Figure 5). This result was in agreement with reports showing vigorous ROS induction and weak cytokine/chemokine response in oxLDL-stimulated macrophages (Miller et al., 2005; Stewart et al., 2010). Interestingly, we found a significant modulation of oxLDL-induced ROS production and IL-6 secretion by quercetin in BMMo (Figures 4 and 5), suggesting that this flavonoid could impact the vicious circle of LDL oxidation and inflammation observed during atherosclerosis progression. In line with this, apoE-/- mice treated with quercetin had lower plasma levels of MDA and less aortic inflammatory infiltrate (Figure 6). Quercetin was previously shown to inhibit ROS, nitric oxide and proinflammatory cytokine production by LPS-stimulated macrophages (Comalada et al., 2006; Ciz et al., 2008); Figure 5B), indicating that these effects are not restricted to oxLDL stimulation. This is in accordance with the broad spectrum of antinflammatory diseases that have been ameliorated by quercetin administration in animal models. ROS production is implicated in macrophage apoptosis, secondary necrosis and defective efferocytosis, which are features of advanced and vulnerable atherosclerotic lesions (Moore and Tabas, 2011). Future studies are required to determine whether quercetin inhibits macrophage apoptosis/necrosis and late atherosclerosis progression. IL-6 plays an

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important role in local and systemic inflammation during atherosclerosis, and together with CRP are well-established CVD risk factors (Hansson, 2005). Our (Figure 5) and others' (Kleemann et al., 2011) results indicate that quercetin interferes with the inflammatory cascade that occurs during atherosclerosis. A recent report showed that quercetin inhibits several inflammatory mediators in oxLDL-stimulated human PBMCs, including IL-6 cytokine secretion via interference with NF-κB signaling (Bhaskar et al., 2011). Interestingly, this study also showed reduced TLR2 and TLR4 expression, suggesting that this flavonoid integrally affects TLR/NF-κB inflammatory signal transduction in oxLDL-stimulated leukocytes.

IL-1β is abundantly produced by lesion macrophages (Galkina and Ley, 2009), and its role in atherosclerosis has been established (Tedgui and Mallat, 2006). Active IL-1β secretion requires the production of pro-IL-1β via NF-κB-mediated transcriptional up-regulation, followed by a Caspase-1-dependent proteolytic maturation via inflammasome assembly (Strowig et al., 2012). Whereas oxLDL primes for pro-IL-1β production via CD36-TLR4-TLR6 (Stewart et al., 2010), further cholesterol accumulation and crystallization activates the NLRP3 inflammasome via phagolysosomal damage (Duewell et al., 2010). Because cholesterol crystals are present from the early stages of atherosclerosis, NLRP3 inflammasomes are currently considered key triggers of atherogenic inflammatory responses (Hansson and Hermansson, 2011). We found that quercetin is inhibitory in an *in vitro* system that assessed cholesterol crystal-induced IL-1β secretion (Figure 5) (Duewell et al., 2010), pointing to this flavonoid as a potential inhibitor of NLRP3 inflammasomes in macrophages. Because quercetin also inhibited LPS-induced IL-1β production in the absence of crystals (Figure 5B), we cannot rule out the possibility that results are due to reduced availability of the inflammasome substrate (namely, pro-IL-1β) instead of

direct inhibition of inflammasome assembly. Future work is required to clarify this mechanism.

Finally, we demonstrated that dyslipidemic apoE-/- mice chronically treated with non-toxic doses of i.p. quercetin presented significant reductions in all indicators of atherosclerosis progression, including lesion size, foam cell accumulation, oxidative stress and inflammatory response *in situ* (Figure 6). These results strikingly mirrored those obtained *in vitro* (Figures 1-5), suggesting that the effects of quercetin on macrophages might be operating *in vivo* and could explain at least some of the atheroprotective activities of this flavonoid. Our results are in line with previous reports showing atheroprotective effects of oral quercetin in various animal models (Hayek et al., 1997; Auger et al., 2005; Juzwiak et al., 2005; Leckey et al., 2010; Loke et al., 2010; Kleemann et al., 2011) and further extend them by demonstrating a significant level of protection following i.p chronic administration. Furthermore, we demonstrated reduced inflammatory macrophage and T cell infiltrate in the atheromatous plaque (Figure 6), which could have been a consequence of the anti-oxidant/anti-inflammatory activity of quercetin on macrophages (Figures 4, 5). In contrast to previous reports (Hayek et al., 1997; Leckey et al., 2010; Loke et al., 2010; Kleemann et al., 2011), we observed that quercetin lowered cholesterol levels in mice (Figure 6). This indicates that the spectrum of atheroprotective activities that this flavonoid exhibits could be influenced by altering pharmacological properties with different administration routes and/or dosing regimens. An interesting question is whether quercetin, in addition to attenuate atherosclerosis progression, is able to reverse or cure advanced lesions or to stabilize vulnerable plaques. This is particularly interesting when considering potential applications in humans, where interventions that target established atherosclerotic lesions are required. Appropriate

animal models of advanced/vulnerable lesions will permit to address these questions in the future.

Despite the clear cardioprotective effects of quercetin *in vivo*, no or very little quercetin aglycone circulates in blood after oral intake. Paradoxically, the circulating conjugated forms exhibit limited bioactivity. These contradictory observations have been clarified with recent studies showing that circulating quercetin conjugates are locally de-conjugated prior to cell uptake (Perez-Vizcaino et al., 2012). Because absorption after i.p. injection leads to both systemic and portal circulation, animals in our experiments most likely were chronically exposed to the conjugated metabolites in addition to the aglycone. However, these metabolites were most likely de-conjugated in the vasculature before entering lesional macrophages and foam cells and promoting atheroprotection. In this context, the observation that deconjugation is more active in inflammatory macrophages (Kawai et al., 2008; Perez-Vizcaino et al., 2012) further supports our results and reinforces the importance of macrophage- and foam cell-intrinsic mechanisms in the atheroprotective activity of quercetin. The field has grown more complex with studies showing that some *in vivo* biological activities of quercetin are dependent on its transformation to phenolic acids (PA) by colonic microorganisms (Vissiennon et al., 2012). Several PA were recently shown to inhibit CD36 expression, oxLDL-induced foam cell formation, LPS-induced proinflammatory cytokine production and to promote cholesterol efflux in a macrophage cell line (Xie et al., 2011). Therefore, an interesting possibility that requires further investigation is that part of the atheroprotection observed *in vivo* in our and others' work is mediated by the effect of quercetin and quercetin-derived PA on lesional macrophages.

In summary, we have shown that quercetin is an active molecule in atherogenic macrophages and that it is plausible that atheroprotective effects of this flavonoid are

JPET Fast Forward. Published on August 6, 2012 as DOI: 10.1124/jpet.112.196147
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mediated through this cell type. A better knowledge of quercetin pharmacology, together with the use of delivery systems to specific cell targets will allow an intelligent exploitation of this intriguing molecule.

JPET Fast Forward. Published on August 6, 2012 as DOI: 10.1124/jpet.112.196147
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Acknowledgements

We thank Maria Zorro for preparing the cells and C. Fraga and F. Perez-Vizcaino for reviewing of the manuscript.

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JPET Fast Forward. Published on August 6, 2012 as DOI: 10.1124/jpet.112.196147
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Footnotes

This work was supported by Colciencias (grants 1115-343-19225 and 1115-519-28906) and the Universidad de Antioquia (CODI CPT-0607). OJLG was the recipient of “Young investigator” grant and is currently recipient of a doctoral fellowship grant from Colciencias.

Reprint requests

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FIGURE LEGENDS

Figure 1. Quercetin inhibits oxLDL uptake by BMMo. Macrophages were incubated with oxLDL (A, B, upper panel) or DiI-oxLDL (A, upper panel), and particle uptake (A) or cellular cholesterol content (B) was determined by fluorescent microscopy/flow cytometry and GC/MC, respectively (for details see Materials and methods). The amount of oxLDL uptake was graphed as the percentage of DiI+ cells or the MFI in oxLDL- or DiI-oxLDL-treated macrophages cultured in the presence or absence of 20 μM quercetin (A, lower panel). Cholesterol content was reported based on the peak area of the ion 145 (B, upper panel), and the effect of quercetin treatment was evaluated (B, bottom). No toxicity, as assessed by LDH release, was observed for any of the treatments. All experiments were performed in triplicate, and bars represent the mean ± SD. ***, $p < 0.001$, compared to vehicle-treated cells. The results are representative of at least 3 independent experiments.

Figure 2. Quercetin modulates CD36 expression in BMMo. Macrophages were incubated with oxLDL, as described in Materials and methods, and CD36 surface expression was determined by flow cytometry. A representative histogram of CD36 expression is shown in (A). Graphs representing the percentage of CD36+ cells or the MFI in macrophage cultures incubated with the indicated amount of oxLDL are also shown (B). The effect of quercetin (20 μM) was evaluated in macrophages stimulated with 30 μg/mL oxLDL (C). No toxicity, as assessed by LDH release, was observed for any of the treatments. All experiments were performed in triplicate, and

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bars represent the mean±SD. ***, $p < 0.001$, compared to vehicle-treated cells. The results are representative of at least 3 independent experiments.

Figure 3. LPS-induced lipid body formation is inhibited by quercetin treatment in macrophages. BMMo were cultured in the presence of LPS and/or quercetin (20 μM) as indicated, and cellular lipid body formation was assessed by Oil Red O staining. Representative photographs of macrophage cultures are shown (A). The area of red staining in triplicate cultures was calculated and graphed as the mean±SD (B). No toxicity, as assessed by LDH release, was observed for any of the treatments. ***, $p < 0.001$, compared to vehicle-treated cells. The results are representative of two independent experiments.

Figure 4. Quercetin is a potent inhibitor of ROS production in oxLDL-stimulated macrophages. BMMo were treated with oxLDL or oxLDL and quercetin, as indicated, and the intracellular production of ROS was monitored with a ROS-sensitive fluorescent dye (see Materials and methods for details). Representative fluorescence microscopy photographs or flow cytometry dot plots from oxLDL-treated macrophages are shown (A). The effect of Co^{2+}-quenching is also shown (A, right). ROS production in macrophages in response to oxLDL is dose-dependent, as evidenced by the percentage of DCF+ macrophages or the MFI (B). The effect of quercetin (20 μM) was evaluated in macrophages stimulated with 50 μg/mL oxLDL (C). No toxicity, as assessed by LDH release, was observed for any of the treatments. All experiments were performed in triplicate, and bars represent the

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mean±SD. ***, p < 0.001, compared to vehicle-treated cells. The results are representative of at least 3 independent experiments.

Figure 5. Cytokine proinflammatory responses of macrophages to oxLDL, LPS and cholesterol crystals are modulated by quercetin. Macrophages were treated with oxLDL or LPS with/without cholesterol crystals in the absence or presence of quercetin (20 μM, for details see Materials and methods), and the secretion of proinflammatory cytokines or chemokines was evaluated by ELISA (C, left) or Luminex (A, B, C; right). oxLDL induced IL-6 secretion by macrophages in a dose-dependent manner (A, top). The effect of quercetin on IL-6 secretion was then evaluated in oxLDL- (25 μg/mL) stimulated BMMo (A, bottom). IL-1β, IL-12, TNFα and MCP-1 secretion was also evaluated in macrophages that were pre-treated with quercetin and stimulated with LPS (10 ng/mL) (B). Cholesterol crystals induced IL-1β secretion in a dose-dependent manner when BMMo were primed with LPS (2 hours), followed by 10 hours of crystals exposure (C, left). The effect of quercetin on LPS-primed (10 ng/ml) and cholesterol crystal- (500 μg/mL) stimulated IL-1β secretion was evaluated (C, right). Cytokine levels in supernatants are represented as the mean±SD from triplicate cultures. No toxicity, as assessed by LDH release, was observed for any of the treatments. ***, p < 0.001, compared to vehicle-treated cells. The results are representative of at least 3 independent experiments.

Figure 6. Intraperitoneal injection of quercetin attenuates atherosclerotic development in apoE-/-. ApoE-/- mice were treated with i.p. quercetin and fed a HFD diet. Vehicle-treated mice were used as negative controls. After 14 weeks of

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treatment, sections of the aortic sinuses were stained with H/E, Oil Red, or immunostained for macrophage (MOMA-2) or T cells (CD3) (A). Representative micrographs for each treatment group are shown (A, left). The size of atherosclerotic lesions, lipid deposition or leukocyte infiltrate was calculated with image analysis software (see Materials and methods for details). Each point represents the average lesion size per mouse (out of 9-12 sections), and bars represent the mean size of the group (A, right). Serum samples were also taken and used to quantify total cholesterol levels (B) and MDA concentration (C). The results were analyzed by using the Mann-Whitney U test to determine statistical significance (*: $p<0,05$, **: $p<0,01$ *** $p<0,005$, compared with control group). These results are representative of two independent experiments. The repetition experiment resulted in essentially similar conclusions.

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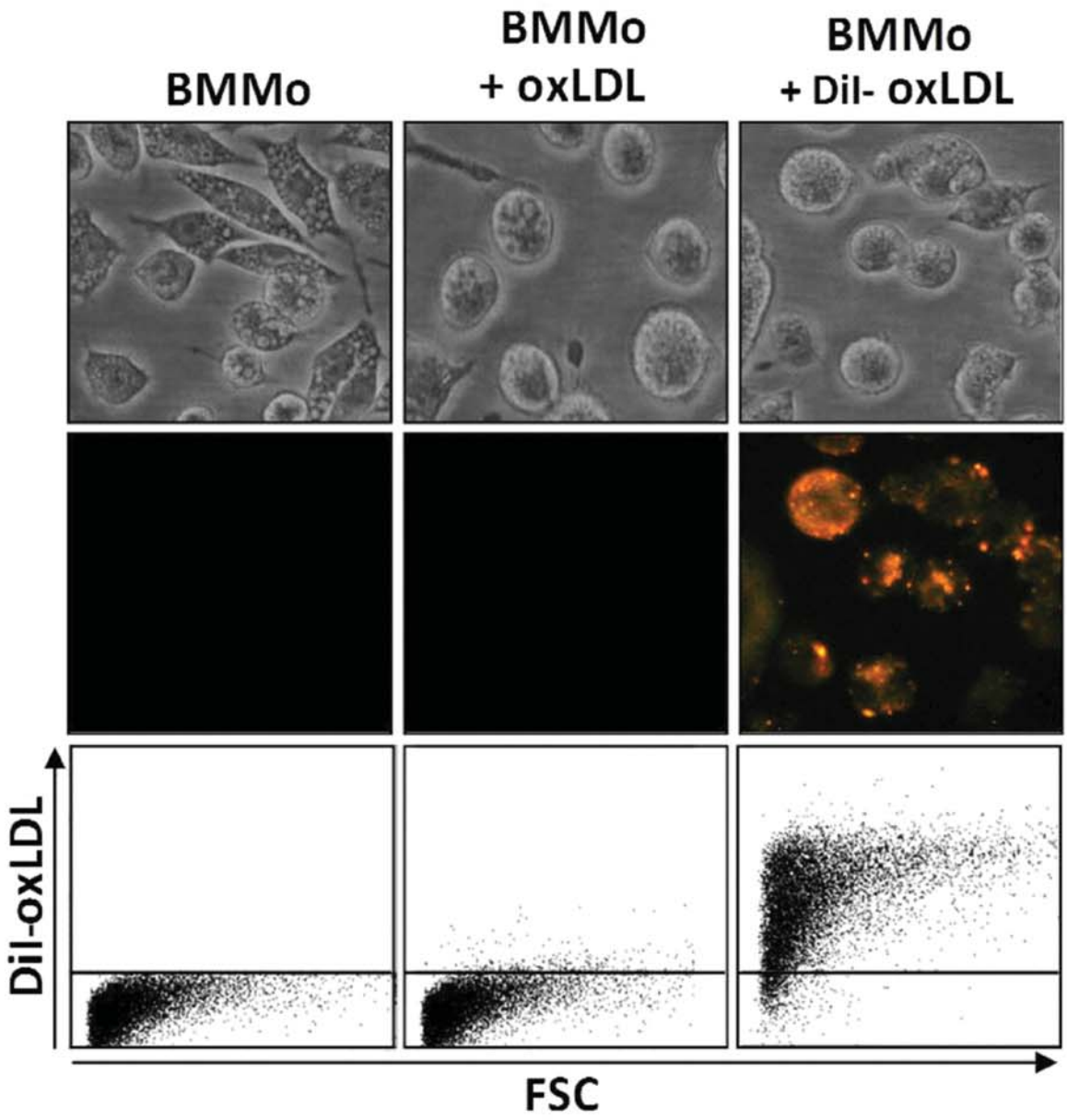
FIGURE 1A



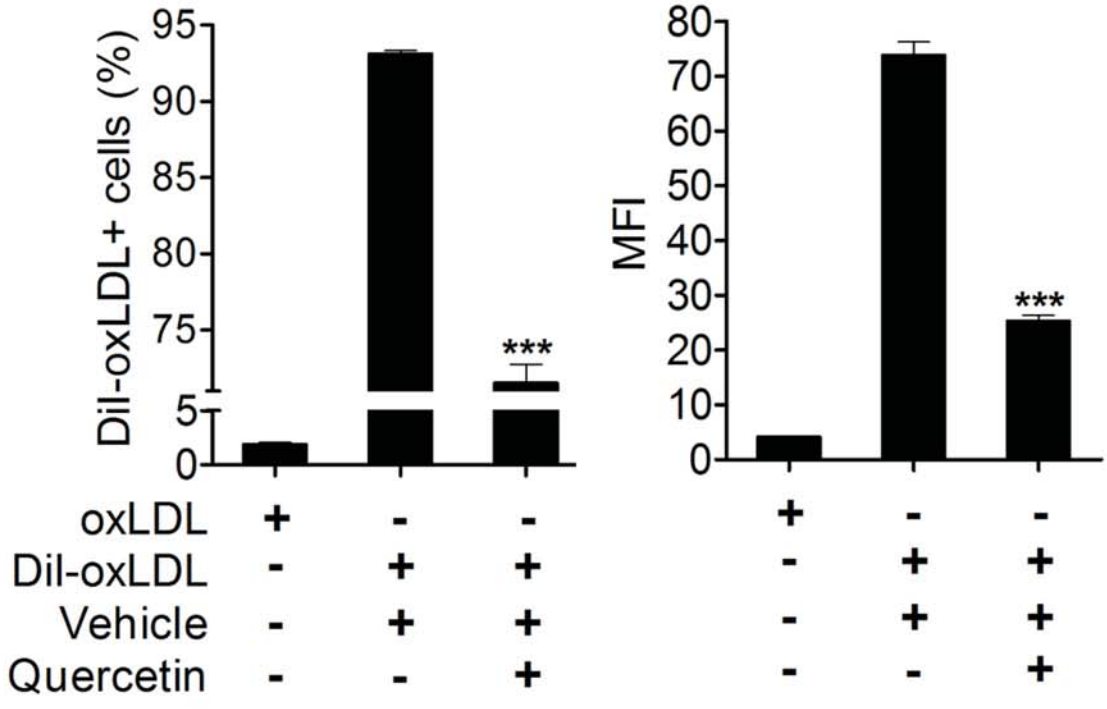


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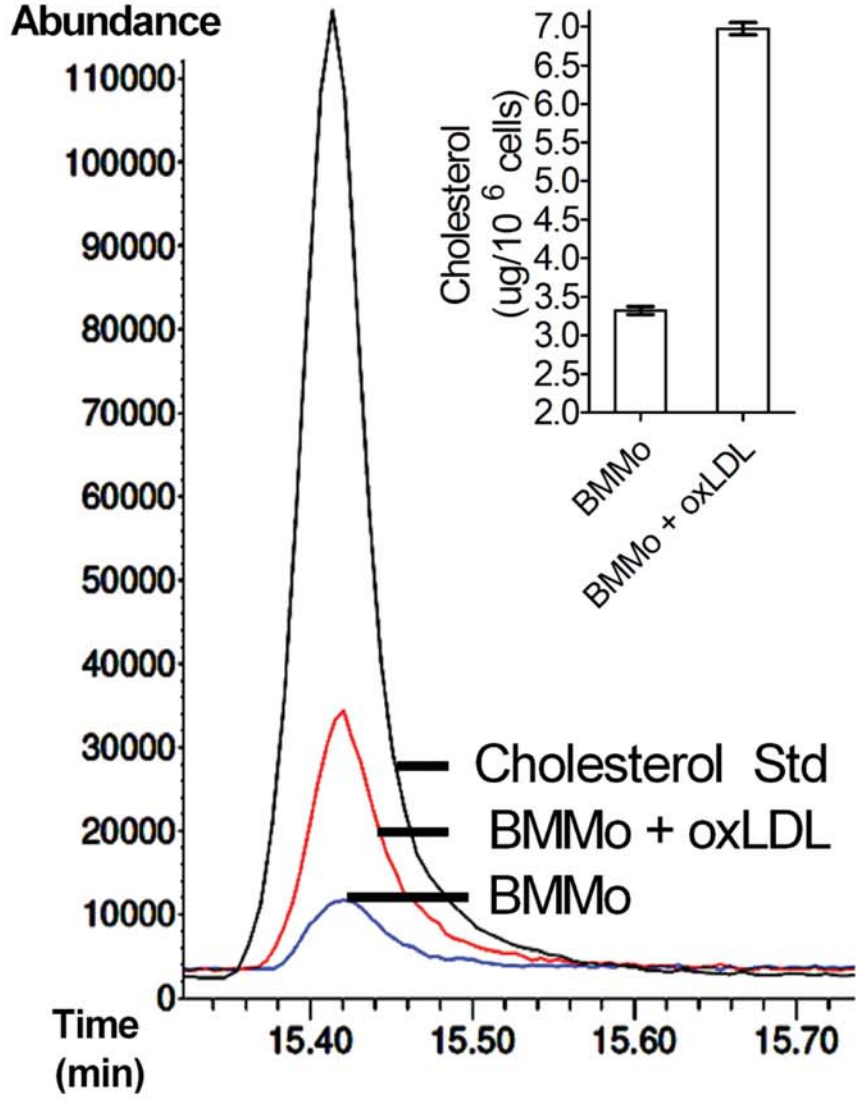
FIGURE 1B



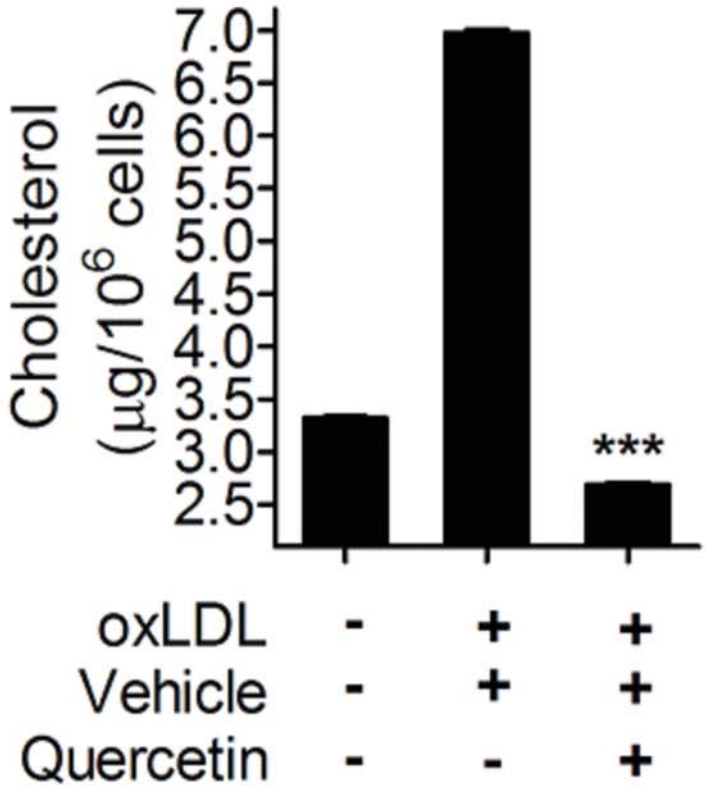
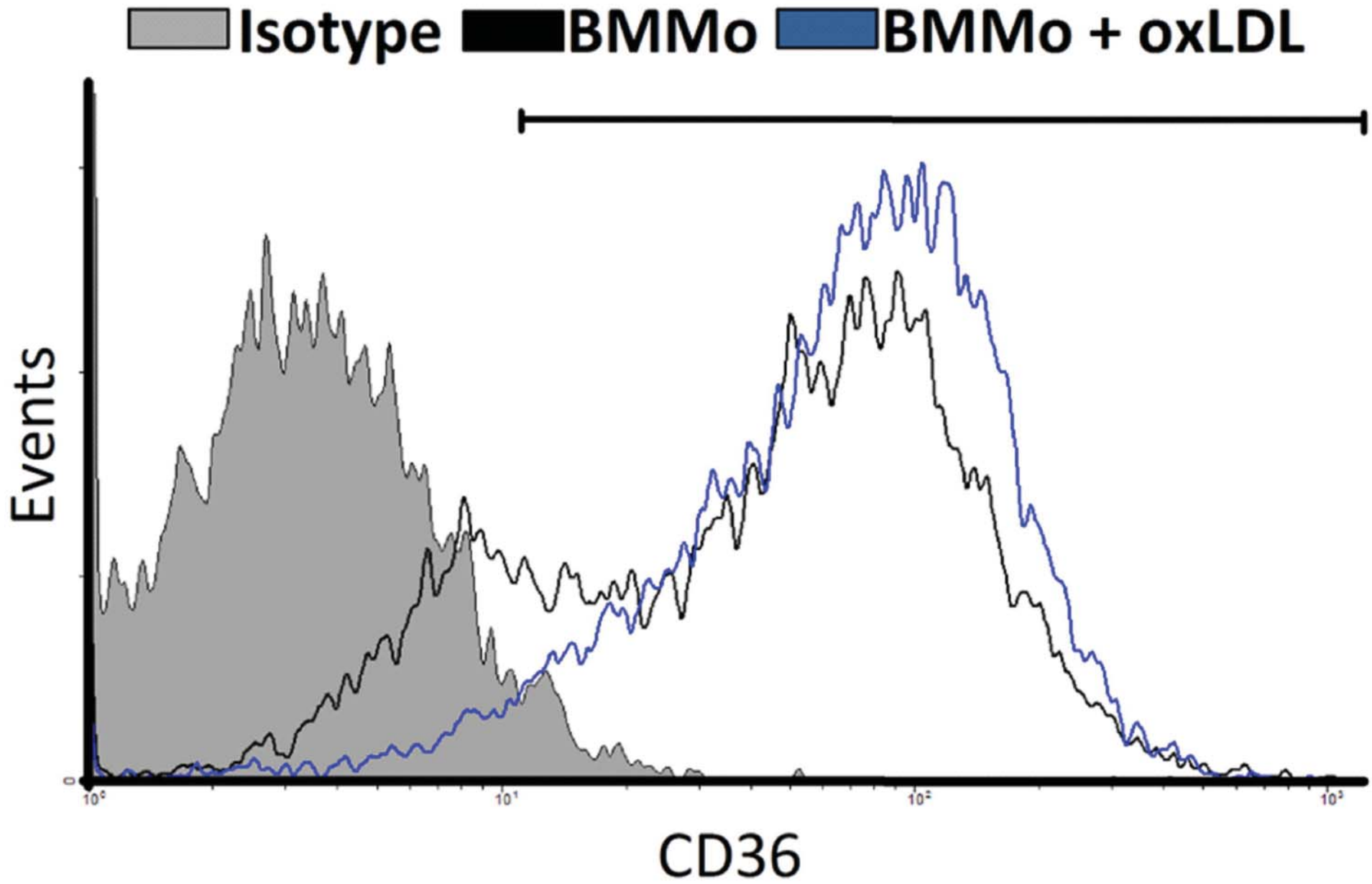


FIGURE 2A



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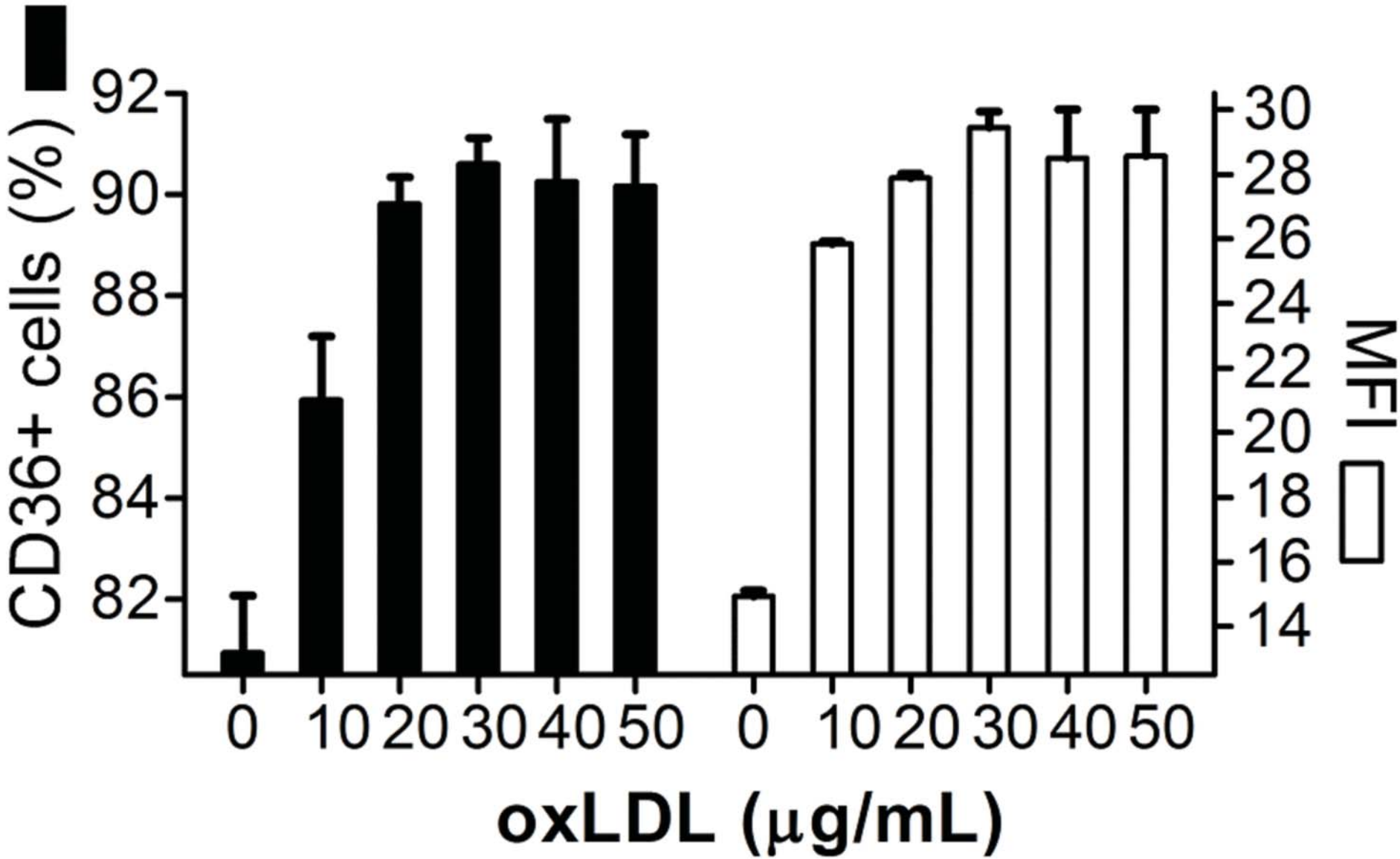
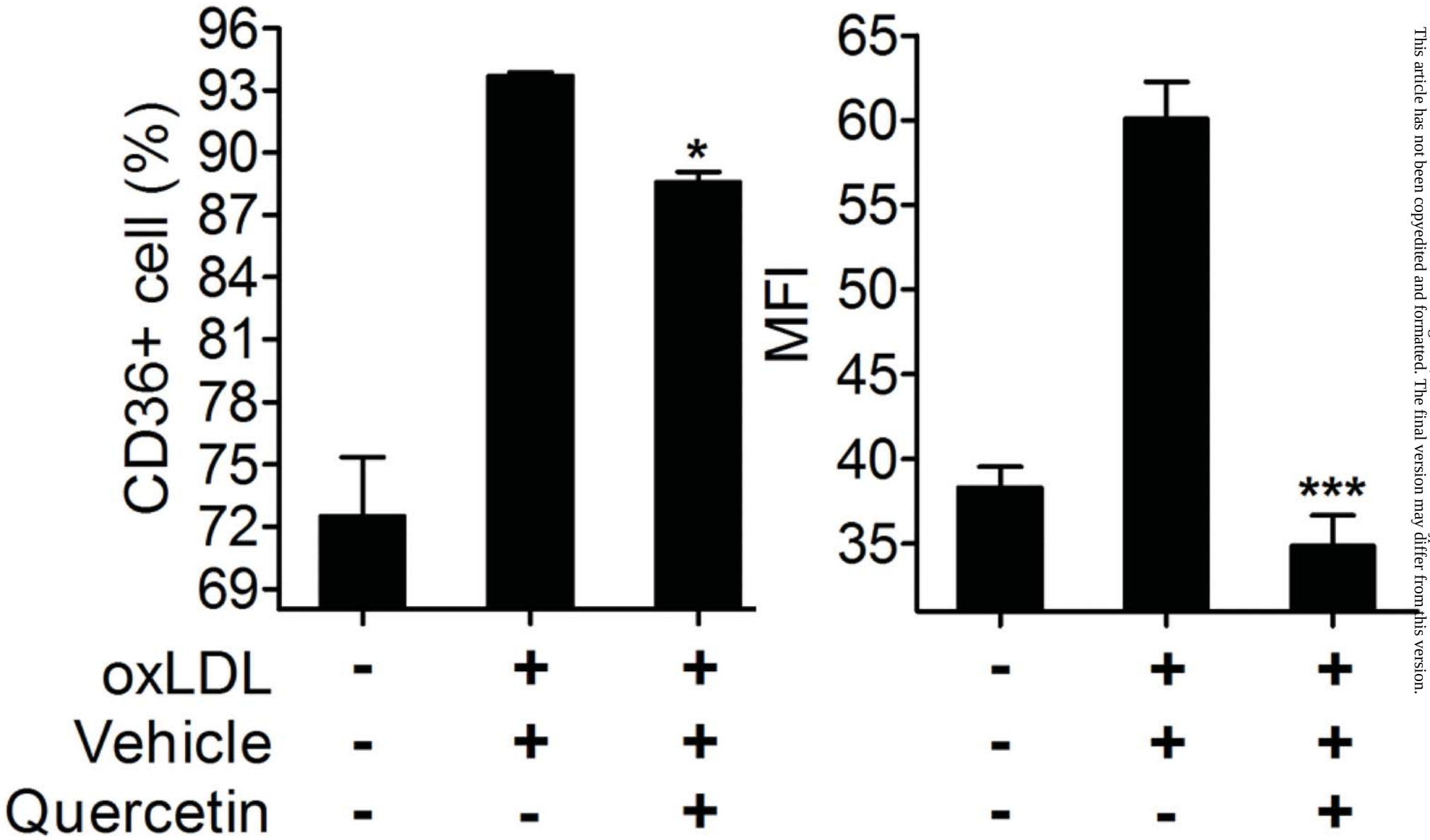


FIGURE 2C



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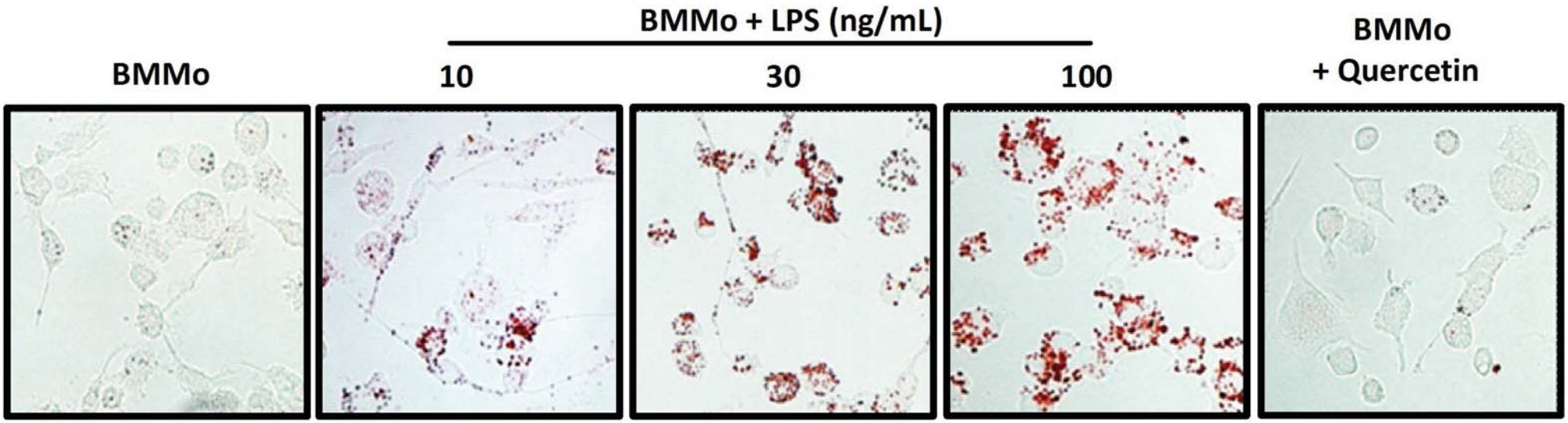

FIGURE 3B

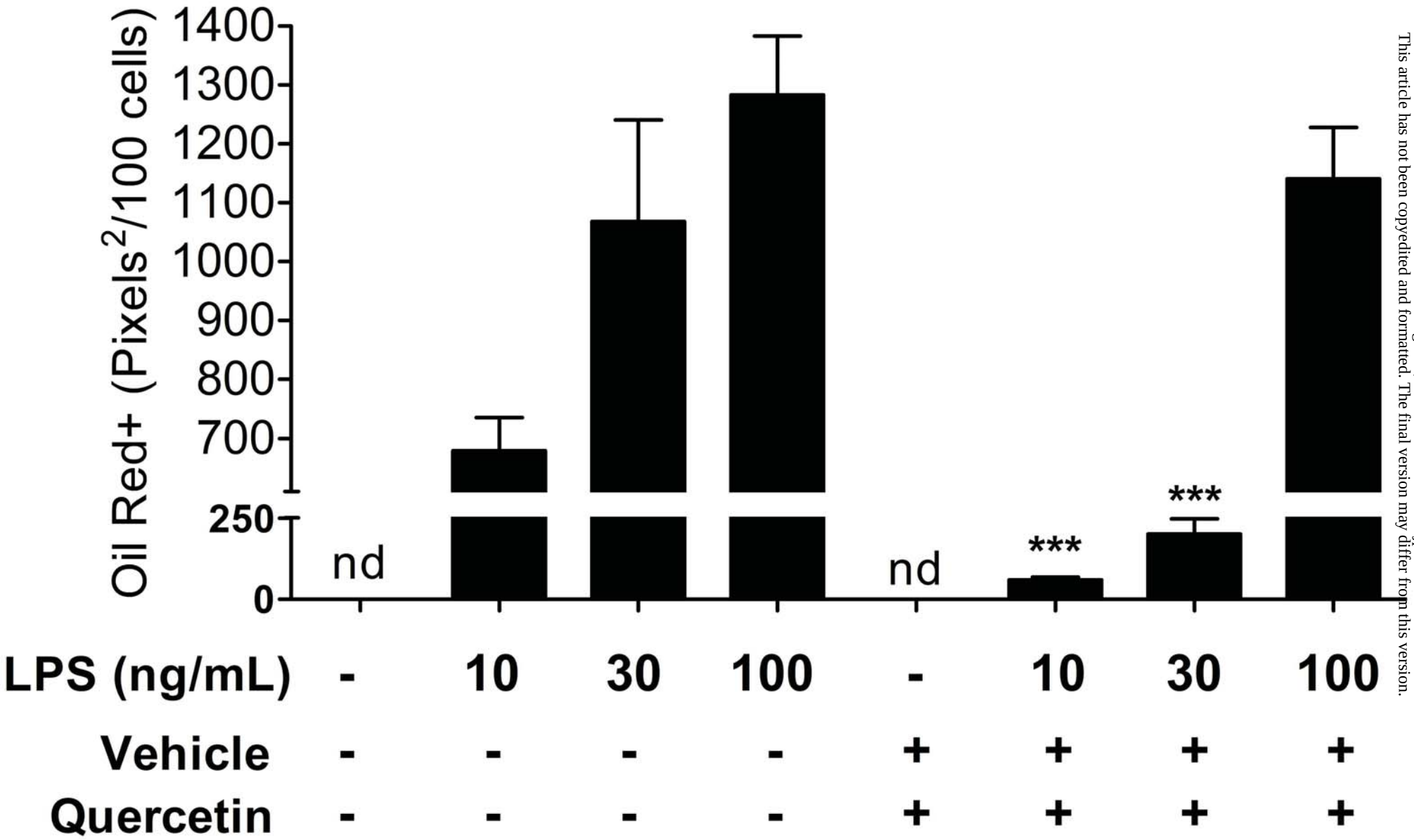

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FIGURE 4A

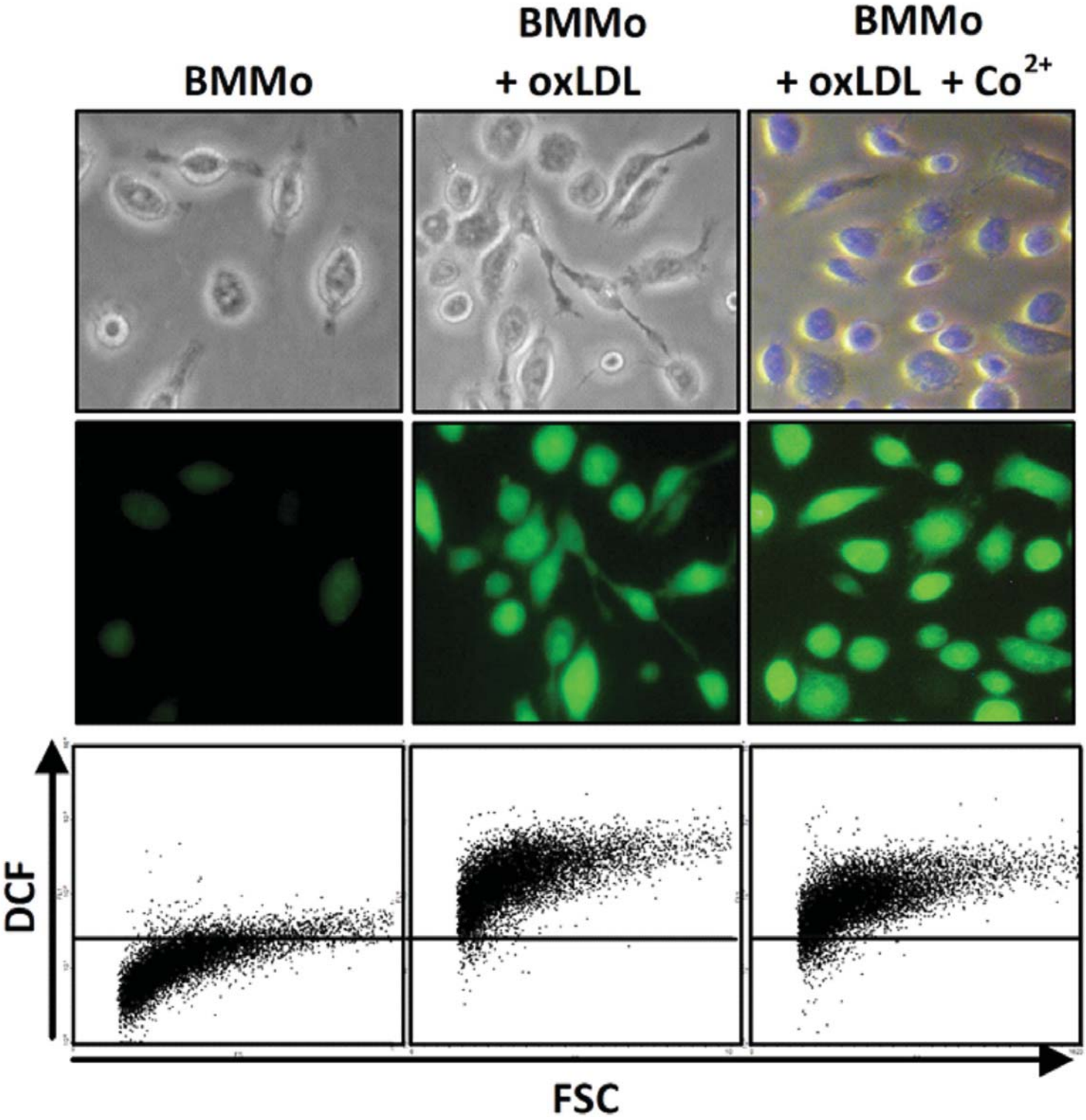

FIGURE 4B

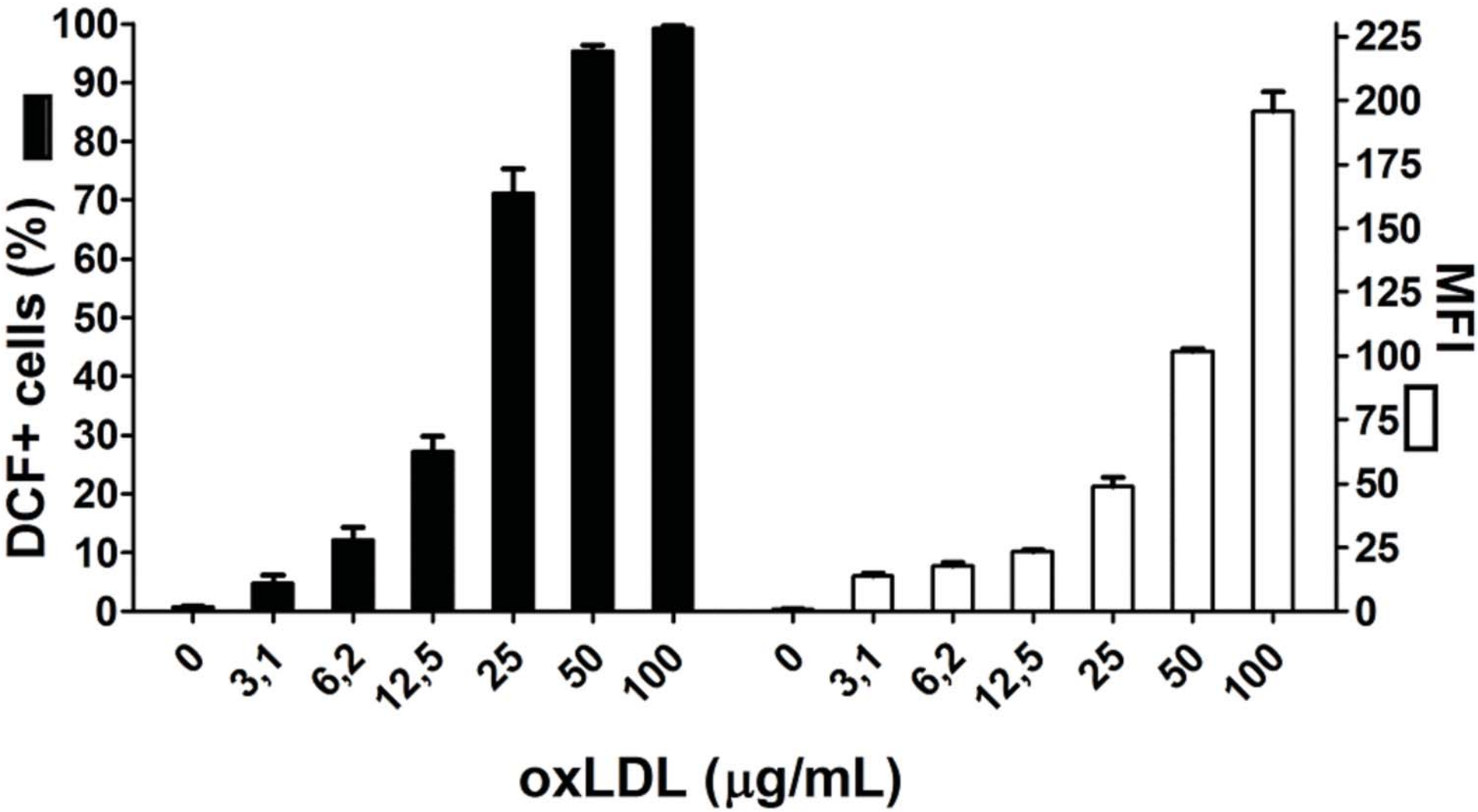

FIGURE 4C

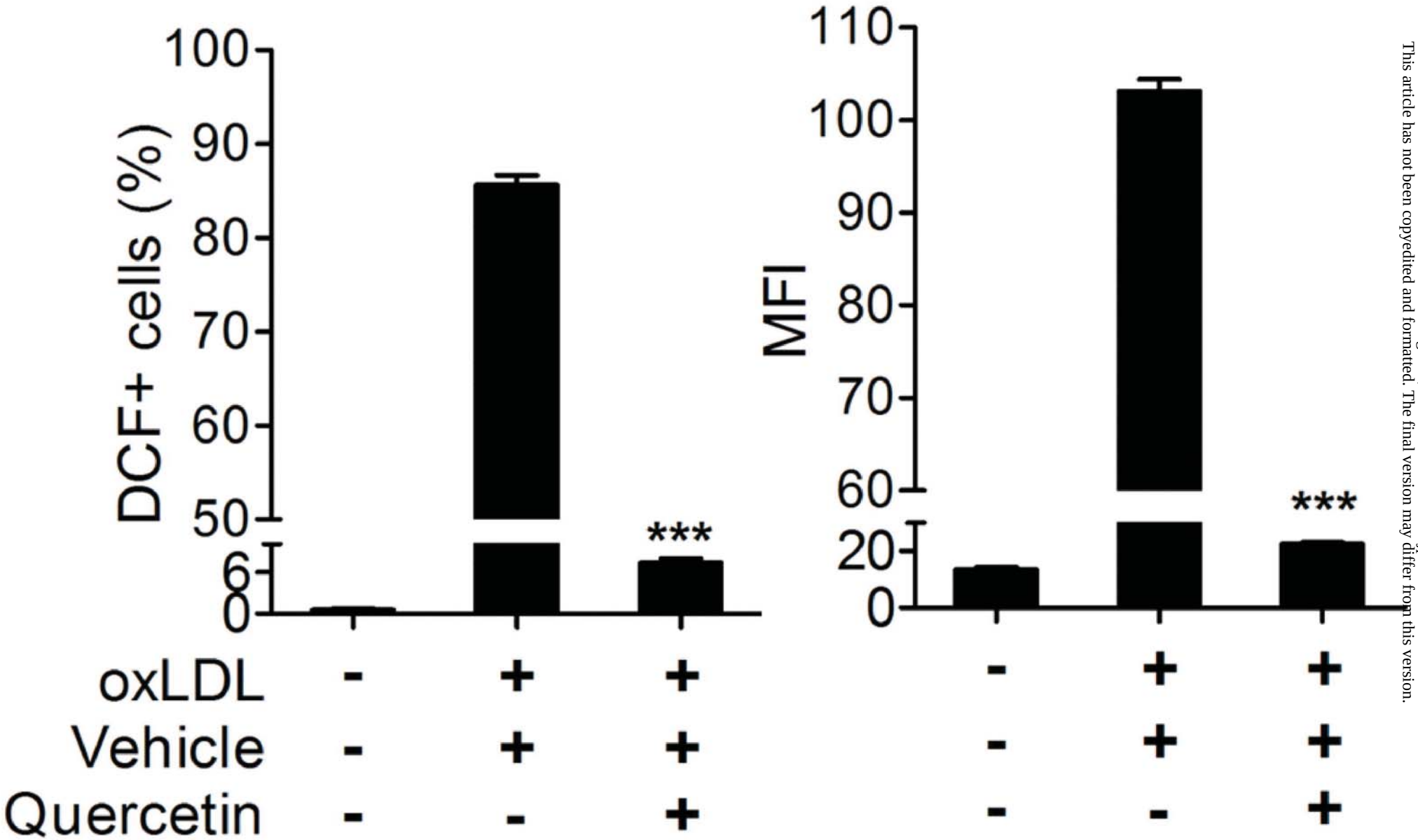

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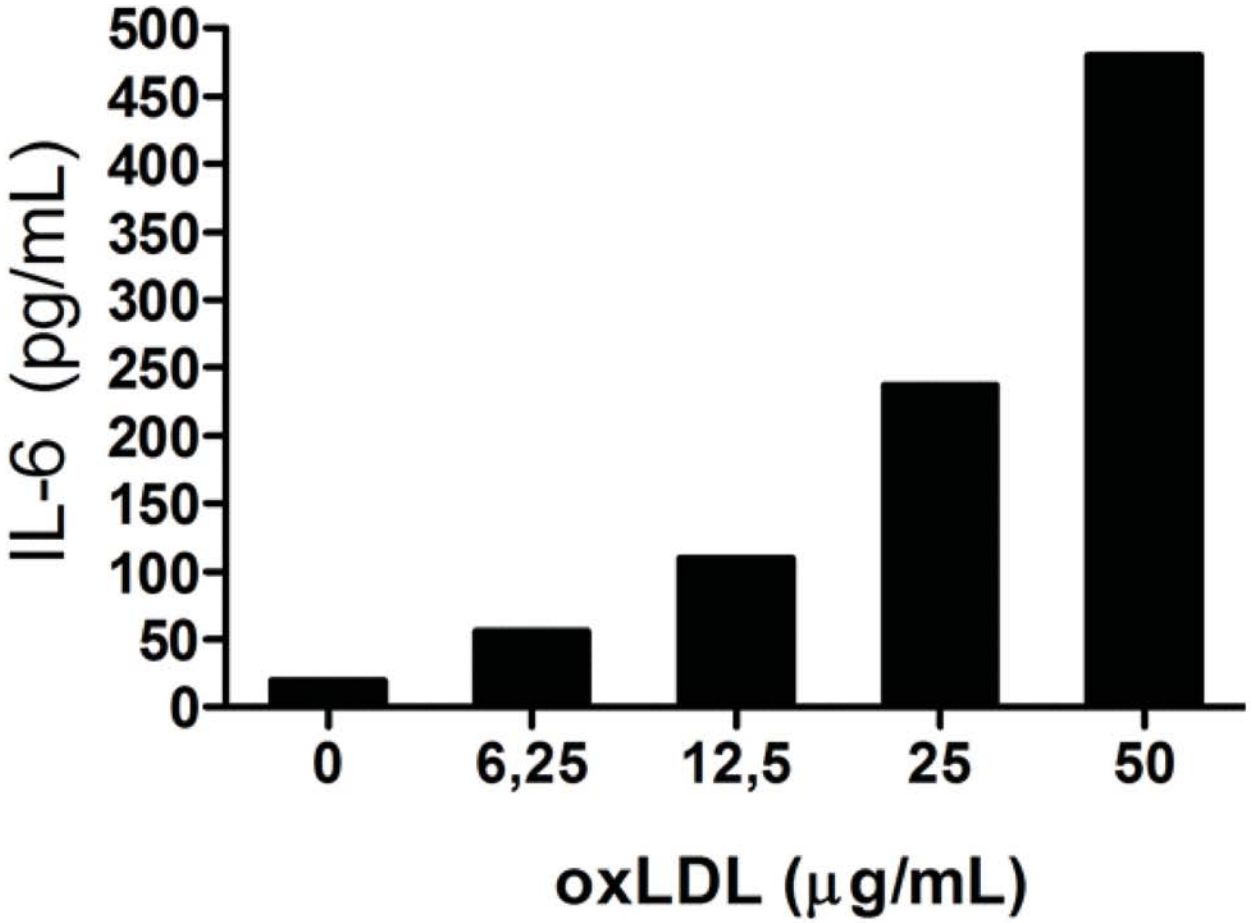
FIGURE 5A



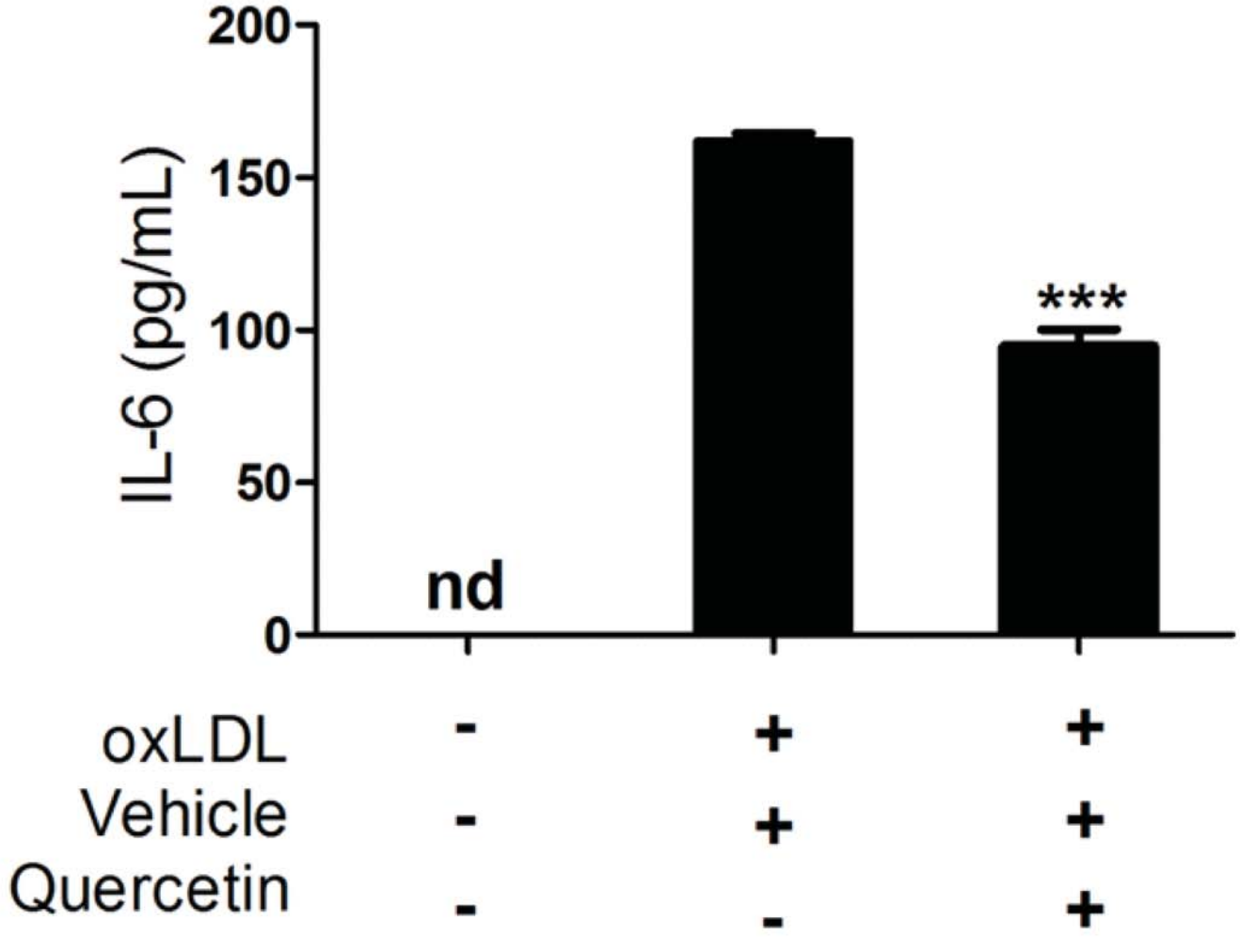


FIGURE 5B

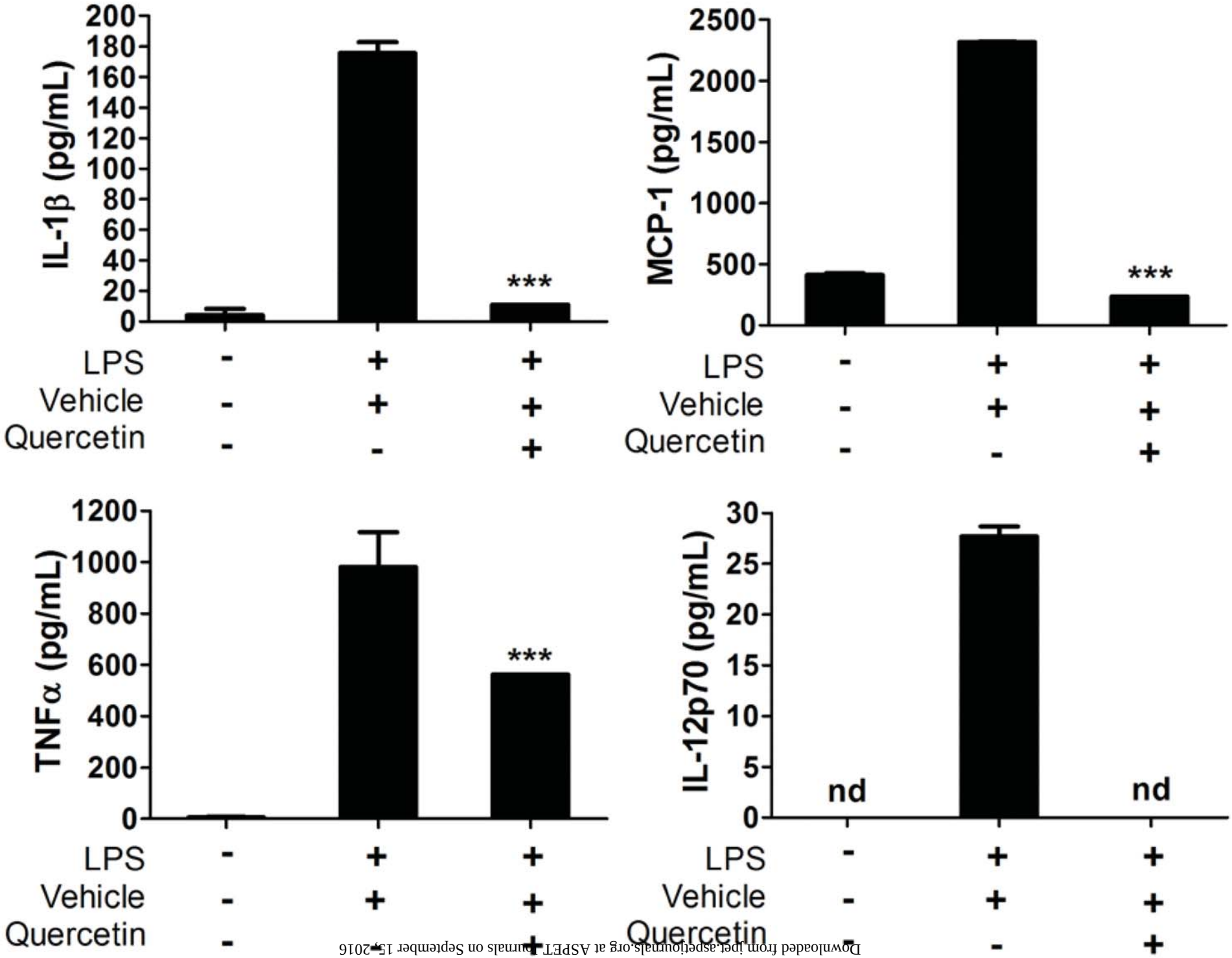
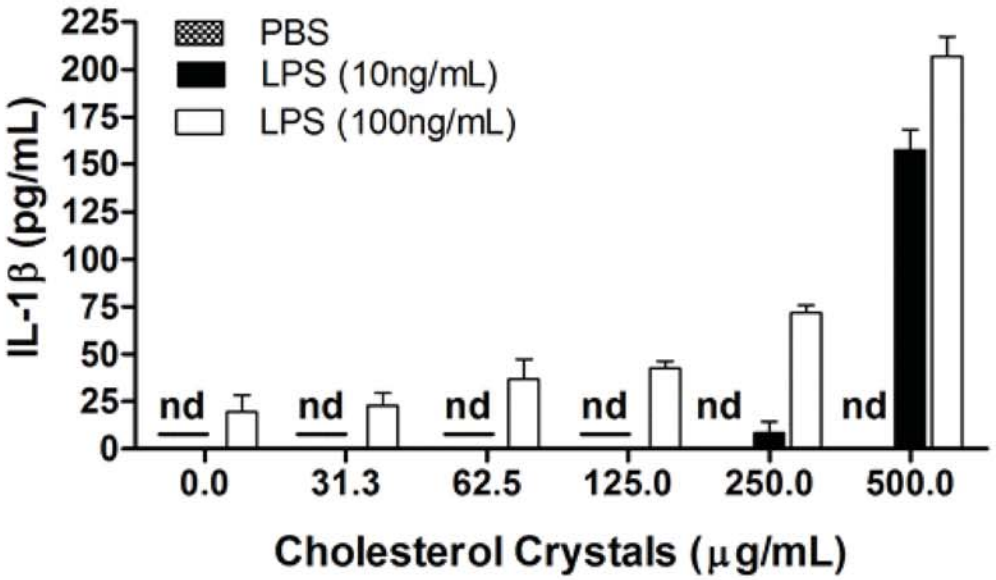


FIGURE 5C



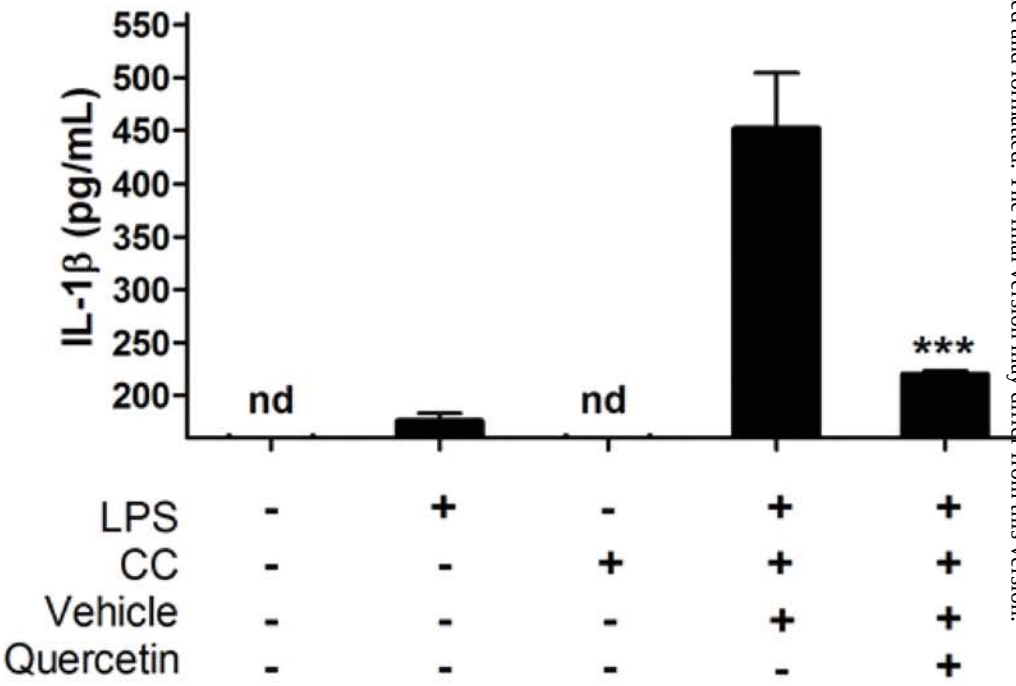


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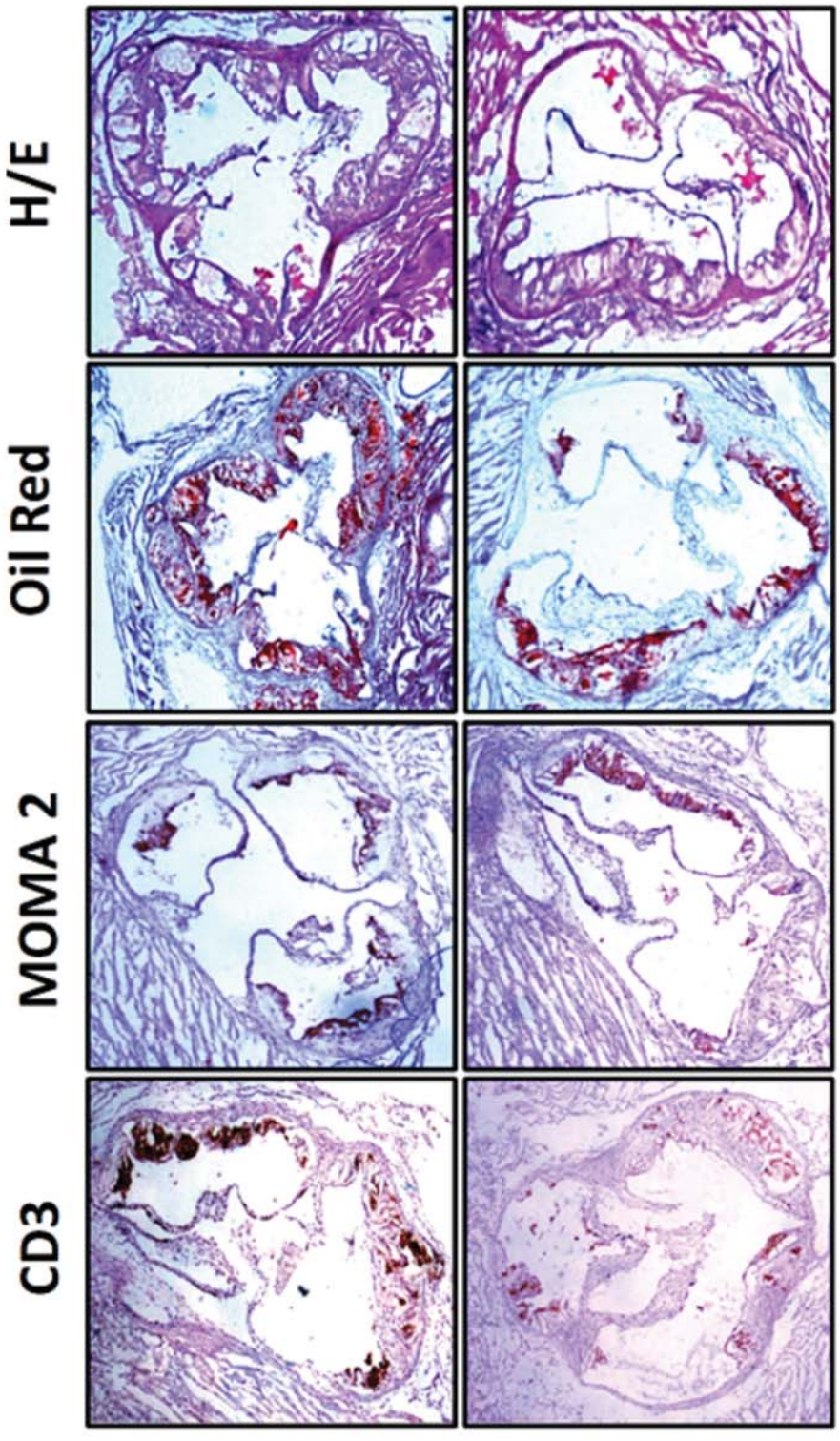
FIGURE 6A



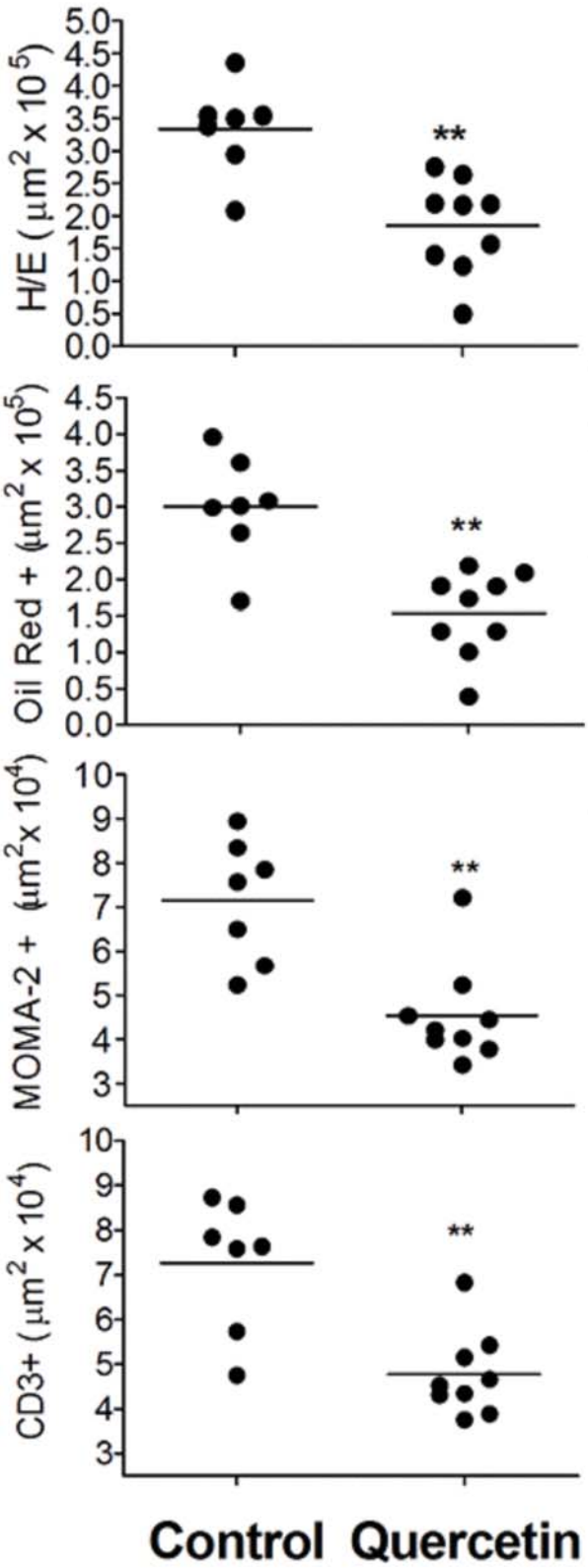


FIGURE 6B

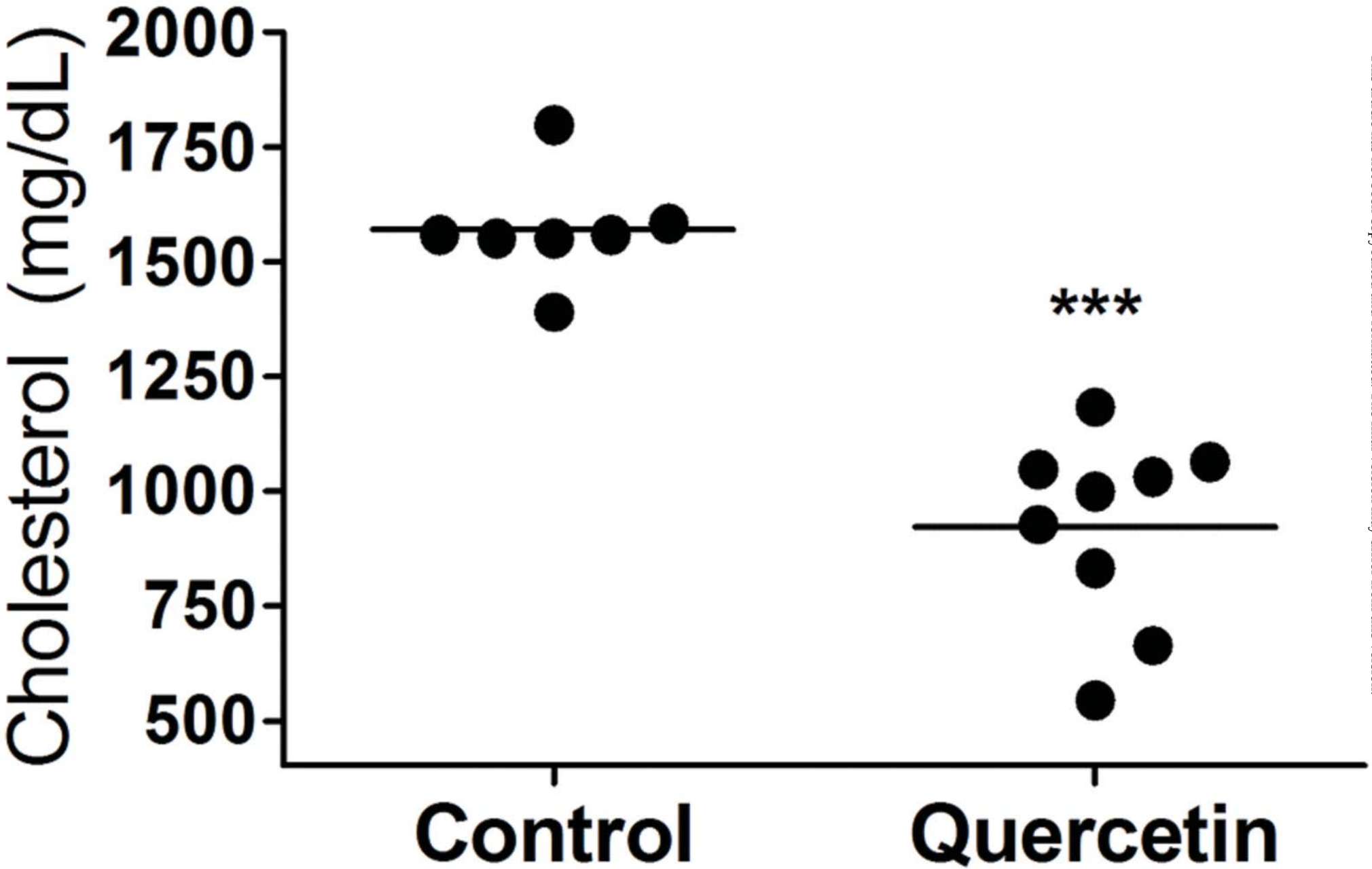


FIGURE 6C