Proatherogenic Macrophage Activities Are Targeted by the Flavonoid Quercetin

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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 (ox-LDL) 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 lipopolysaccharide-dependent, oxLDL-independent pathway of lipid droplet formation in macrophages. In oxLDL-stimulated macrophages, quercetin inhibited reactive oxygen species production and interleukin (IL)-6 secretion. In a system that evaluated cholesterol crystal-induced IL-1β secretion via nucleotide-binding domain and leucine-rich repeat repeat containing protein 3 inflammasome activation, quercetin also exhibited an inhibitory effect. Dyslipidemic apolipoprotein E-deficient mice chronically treated with intraperitoneal quercetin injections had smaller atheromatous lesions, reduced lipid deposition, and less macro- and T cell inflammatory infiltrate in the aortic roots than vehicle-treated animals. Serum levels of total cholesterol and the lipid peroxidation product malondialdehyde were also reduced in these mice. Our results demonstrate that quercetin interferes with both key proatherogenic activities of macrophages, namely foam cell formation and pro-oxidant/proinflammatory responses, and these effects may explain the atheroprotective properties of this common flavonoid.

Introduction

Atherosclerosis is a chronic, nonresolving 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 (TLRs), 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

ABBR eviations: apoE(−/−), apolipoprotein E-deficient; BMMo, bone marrow-derived macrophages; CVD, cardiovascular disease; DCF, dichlorofluorescein; Dil, 1,1’-dioctadecyl-3,3,3,3’-tetramethylindocarbocyanine perchlorate; DMSO, dimethyl sulfoxide; EC, endothelial cell; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; H/E, hematoxylin/eosin; HFD, high-fat diet; IL, interleukin; ISTD, internal standard; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MDA, malondialdehyde; MFI, mean of fluorescent intensity; MIP-1α, macrophage inflammatory protein 1α; MS, mass spectrometry; nd, not determined; NF-xB, nuclear factor-xB; NLRP3, nucleotide-binding domain and leucine-rich repeat containing protein 3; oxLDL, oxidized LDL; PA, phenolic acids; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TLR, Toll-like receptor; TNF, tumor necrosis factor.
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 prototypically naturally occurring flavonoid that exhibits potent antioxidant activities and protects against 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 seem to be prominent (Perez-Vizcaino and Duarte, 2010; Russo et al., 2012). Several epidemiological (Hertog et al., 1993, 1995) and clinical (Edwards et al., 2007; Eger et al., 2009) studies support the cardioprotective effects of this flavonoid in humans. Moreover, administration of quercetin to rabbits (Južwiak 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 antioxidant/cytotoxic 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 EC and smooth muscle cells 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 nonhematopoietic 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 assessed only one aspect of the oxLDL-macrophage interaction. 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.

Materials and Methods

Reagents, Animals, and Diet. Wild-type and apolipoprotein E-deficient C57BL/6 mice [apoE(−/−)] were purchased from Charles River Breeding Laboratories (Portage, MI) and The Jackson Laboratory (Bar Harbor, ME), respectively. Mice were maintained under a specific pathogen-free environment at the specific pathogen-free animal facility of the Sede de Investigacion Universitaria, Universidad de Antioquia. The institutional ethical committee approved all in vivo procedures. Animals were fed a standard mouse diet (LabDiet, Richmond, IN) or a high-fat diet (HFD; adjusted calories diet 42%; Harlan Teklad, Madison, WI). Quercetin (CAS number 117-39-5) was purchased from Merck (Whitehouse Station, NJ), and a stock solution (10 mM) was prepared in DMSO and stored at −20°C. LPS (from Escherichia coli 072:B5) and 2,7-dichlorofluorescin diacetate were from Sigma (St. Louis, MO). Cholesterol crystals were prepared for some experiments following published protocols (Duewell et al., 2010) with minor modifications. In brief, cholesterol standard (purity >99%; Sigma) was solubilized in preheated 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/polyacrylamide gel electrophoresis. LDL (500 μg/ml) was oxidized with CuSO4·5H2O (40 μM) at 37°C for 9 h. Oxidation was stopped with EDTA (1%). Effective oxLDL generation was confirmed by the increased formation of thiobarbituric acid-reactive species and enhanced mobility of apolipoprotein B100 on 0.8% agarose gels (Supplemental Fig. 1) because of increased electronegativity compared with 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′-diododecyl-3,3,3′,3′-tetramethylinodocarbonyl peroxide (DiI; Invitrogen, Carlsbad, CA; 10 μM) over 12 h as described previously (Ide et al., 2006), and the DiI-oxLDL complex was dia lysed against PBS for 12 h.

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 as described previously (Lutz et al., 1999). Nine-day cultures consisted of nonadherent 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; Invitrogen) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM β-mercaptoethanol, and 2 mM l-glutamine at 1 × 10⁶ 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% CO₂. In all cases, macrophages (5 × 10⁵ cells/ml) were pretreated with 20 μM quercetin for 12 h and subsequently incubated with 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 pilot assays where nontoxic concentrations were defined (data not shown). Parallel cultures were treated with an equivalent volume of vehicle (0.05% DMSO) 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 (data 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 Limulus amoebocyte lysate assay (QCL-1.000; Lonza Hopkinton, Inc., Hopkinton, MA) 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, Madison, WI).

**CD36 Expression and Foam Cell Formation.** For CD36 surface expression, quercetin-treated macrophage cultures were further stimulated with oxLDL (30 µg/ml) for 36 h, 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 h, washed, removed, and analyzed by flow cytometry to determine the amount of internalized fluorescent dye. In parallel uptake assays, quercetin-pretreated cells were exposed to oxLDL (30 µg/ml) for 36 h, and the intracellular accumulation of cholesterol was determined by gas chromatography (GC)/mass spectroscopy (MS). To evaluate oxLDL-independent foam cell formation, LPS (10, 30, or 100 ng/ml) was added to quercetin-treated macrophages (cultured onto circular coverslips) in the absence or presence of LDL (50 µg/ml) and incubated for an additional 36 h. 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-F1i; Nikon, Tokyo, Japan). The area of Oil Red O staining per macrophage was determined with the help of NIS Element BR software (400× magnification; Nikon). The amount of lipid accumulation was reported as the total area (in pixels²) in 100 cells per slide.

**ROS Production.** Quercetin-treated BMMo were washed twice with PBS and treated with oxLDL (50 µg/ml) for 1 h. Cells were then incubated with 10 µM fluorescent ROS-sensitive substrate 2,7-dichlorofluorescein diacetate for 30 min, washed twice, resuspended with PBS, and analyzed by flow cytometry, spectrophotometry, 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 h. In another set of experiments, quercetin-treated BMMo were primed with LPS (10 or 100 ng/ml) for 2 h and treated with cholesterol crystals (500 µg/ml) for the last 10 h, for a total treatment time of 24 h. 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, San Jose, CA) or an isotype control (clone M18-254; BD Biosciences) before incubation with a fluorescent isothiocyanate-rat anti-mouse secondary antibody (clone C10-3; BD Biosciences). For oxLDL uptake and ROS production, cells were resuspended in PBS and analyzed. A Beckman Coulter (Fullerton, CA) EPICS XL flow cytometer was used for all acquisitions (at least 10,000 events), and data storage and analysis were performed with WinMDI software (http://www.methods.info/software/flow/winmd.html). 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/Electron-Ionization MS-Single Ion Monitoring.** After treatments, macrophages were washed four times and resuspended in PBS at 1 × 10⁶ cells/ml. Next, 20 µl of (1000 ppm) 16-dehydropregnenolone (Sigma) was added to the suspension as an internal standard (ISTD). Cell lysate was obtained after three cycles of freezing/thawing and 30 min of 28-Hz ultrasonic disruption (Ultrasonic Bath; Dentsply, Woodbridge, Ontario, Canada). Lipid fractions were extracted with 1 ml of chloroform and subsequently dried and dissolved with 200 µl of chloroform to be analyzed by GC/MS (7890/5975C; Agilent Technologies, Santa Clara, CA). Samples (2 µl) were injected in splitless mode at 270°C by using an HP-5MS capillary column (Agilent Technologies; 5% phenyl-polydimethylsiloxane, 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 min, and the temperature was increased at a rate of 12°C/min to reach 290°C. The temperatures of the ionization source and the quadrupole were 230 and 150°C, respectively. The ionization voltage was 70 eV. For quantitation in single ion monitoring mode, ions were selected as follows: 145 (quantifier) and 275, 255, and 105 (identifier) for cholesterol (Sigma) and 314 (quantifier) and 145 and 105 (identifier) for the ISTD. Quantitation was performed by using the response factors of cholesterol standards (1–50 mg/liter). For cholesterol standard quantification, the coefficient of variation was less than 1.81%. For the ISTD, as well as for cholesterol quantification in macrophages, a maximal coefficient of variation of 3.46% 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) or the multiplex Luminex technology format (IL-1β, IL-6, IL-10, IL-12p70, IL-12p40, TNFα, MCP-1, and MIP-1α; Milliplex xMAP, Millipore Corporation, Billerica, MA). 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 ELISAAs 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 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 intraperitoneally with 50 mg/kg quercetin every other day. Two weeks later, mice were shifted to a HFD and maintained on quercetin treatments for the next 12 weeks. This dose was based on reported toxicity studies in rodents and humans (Ferry et al., 1996; Harwood et al., 2007) and preclinical bioactivity reports in rodents (Amália et al., 2007). Because it is known that bioavailability of oral quercetin is low, and this route of administration leads to negligible systemic circulation of the active aglycone form, we decided to use intraperitoneal administration. In addition, because the half-life of quercetin and its metabolites in blood is short (11–28 h; Manach et al., 2005), we defined this chronic and repetitive schedule of injections to assure permanent exposure to the flavonoid during the whole experimental period and increase the chances of observing an atheroprotective effect in 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 Fisher Scientific, Waltham, MA) and stored at −20°C. Frozen samples were processed with a cryostat (Leica, Wetzlar, Germany) to obtain 6- to 7-μm-thick sections of the aortic sinus as described previously (Paigen et al., 1987). The sections were mounted on charged glass slides (Thermo Fisher Scientific) and stained with conventional hematoxylin/eosin (H/E) or Oil Red O (Sigma) or processed for immunohistochemistry. For immunohistochemistry, sections were acetone-fixed, blocked for endogenous peroxidase activity, permeabilized, and incubated with macrophage- or T cell-specific monoclonal antibody (anti-mouse monocytic/macrophage, clone MOMA-2, or anti-CD3 antibody, clone KT3, respectively; Serotec, Oxford, UK). A secondary horseradish peroxidase-conjugated goat anti-rat IgG antibody and the chromogenic substrate 3,3′-diaminobenzidine (Serotec) were used to develop antibody binding. Finally, the sections were counterstained with hematoxylin. Micrographs (40× magnification) were taken, and the area of the atherosclerotic lesion (H/E), lipid deposition (Oil Red O) in the aortic arch and the epicardial coronary bed (Oil Red O) were measured. Blood samples were taken, and serum levels of total cholesterol, hepatic enzymes (alanine aminotransferase and aspartate aminotransferase), amylase, and creatinine were deter-
mined with dry chemistry methods (Johnson & Johnson, New Brunswick, NJ) 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 method and a standard curve as described previously (Jentzsch et al., 1996). MDA values are expressed as nanomol per milligram of protein (as determined by the bichinchoninic acid method; Thermo Fisher Scientific).

**Statistical Analysis.** For in vitro assays, experiments were performed in triplicate, and the mean ± S.E.M. was reported. One-way analyses of variance and Newman-Keuls tests were used for multiple group comparisons with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). For in vivo experiments, results are expressed as the mean ± S.E.M. Treated and control groups were compared with unpaired Mann-Whitney Student’s *t* test.

**Results**

**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 (Fig. 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 (Fig. 1A, bottom). Chromatographic quantitation of intracellular cholesterol content confirmed that oxLDL (Fig. 1B, top), but not LDL (data not shown), induced cholesterol accumulation. It is noteworthy that when cells were treated with quercetin cholesterol accumulation in BMMo was reduced to basal levels (Fig. 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; Fig. 2A), exposure to oxLDL further increased in a dose-dependent manner (Fig. 2B). As expected, native LDL was unable to promote CD36 expression (data not shown). When BMMo were pretreated with quercetin and subsequently exposed to oxLDL, we observed a significant down-regulation of CD36 expression (Fig. 2C). These results demonstrate that quercetin inhibits oxLDL-mediated foam cell formation, apparently via down-regulation of its receptor on macrophages.

**Quercetin Modulates TLR-Dependent Lipid Body Formation in Macrophages.** In addition to the classic oxLDL-scavenger receptor-dependent pathway of foam cell formation, a novel TLR-dependent mechanism of lipid body formation that leads to cholesterol and triglyceride accumu-

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**Fig. 1.** Quercetin inhibits oxLDL uptake by BMMo. Macrophages were incubated with oxLDL (A and B, top) or DI-oxLDL (A, top), and particle uptake (A) or cellular cholesterol content (B) was determined by fluorescent microscopy/flow cytometry and GC/MS, 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 DI-oxLDL-treated macrophages cultured in the presence or absence of 20 μM quercetin (A, bottom). Cholesterol content was reported based on the peak area of the ion 145 (B, top), 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 ± S.D. *** *p < 0.001, compared with vehicle-treated cells. The results are representative of at least three independent experiments. FSC, forward scatter.
lation in macrophages has been described previously (Nico-
laou 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-depen-
dent manner and quercetin did not induce any lipid body
deposition in BMMo (Fig. 3A). It is noteworthy that quercetin
significantly prevented lipid body formation and accumula-
tion 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 stimu-
lated at 100 ng/ml LPS (conditions in which 100% BMMo
were heavily charged with lipid droplets; Fig. 3B). As re-
ported previously (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 with nonsupplemented cultures). In these LDL-
supplemented cultures, quercetin exhibited essentially the
same activity (inhibition of lipid droplet formation at a low
but not a high LPS-stimulating dose; data not shown). Thus,
we showed, for the first time, that a TLR-dependent noncon-
ventional pathway of foam cell formation is also targeted by
quercetin.

**ROS Production in oxLDL-Stimulated Macrophages Is Also Prevented by Quercetin Treatment.** Recognition of oxLDL by CD36 and other receptors on macrophages trig-
ger 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 produc-
tion, with maximal levels produced as soon as 1-h postincu-
bation (data not shown). ROS production was also monitored
by fluorescence microscopy and quantified by flow cytometry
(Fig. 4A). These methods allowed the demonstration that
fluorescence could not be quenched by cobalt, which is indic-
ative of intracellular ROS production (Fig. 4A). We observed
that basal ROS production in BMMo was negligible, whereas oxLDL stimulation induced dose-dependent ROS production (Fig. 4B). Native LDL did not induce detectable ROS (data not shown), indicating that only the modified lipoprotein exerts a pro-oxidative effect in macrophages. It is noteworthy that 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 (Fig. 4C). Collectively, these experiments are the first demonstration that ROS production triggered by oxLDL in macrophages is modulated by quercetin.

**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 sublethal concentrations of oxLDL did not induce TNFα, IL-1β, IL-10, IL-12p40, IL-12p70, MIP-1α, or MCP-1 (data not shown). In contrast, IL-6 was readily induced in a dose-dependent manner (Fig. 5A, top). It is noteworthy that IL-6 secretion was significantly inhibited when quercetin was present in oxLDL-stimulated BMMo cultures (Fig. 5A, bottom). As expected (Comalada et al., 2006), quercetin also inhibited IL-1β, TNFα, IL-12p70, and MCP-1 secretion in LPS-stimulated BMMo (Fig. 5B). A novel proinflammatory pathway triggered by cholesterol crystals in macrophages that leads to NLRP3 inflammasome activation and IL-1β maturation/release was 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 the 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 (Fig. 5C, left). Under those conditions, quercetin exerted a potent inhibitory effect, reducing the secreted amount of active IL-1β (Fig. 5C, right). This suggested a potential inhibitory activity of quercetin on cholesterol crystal-induced NLRP3 inflammasome activation. Collectively, these results demonstrate that quercetin targets multiple innate inflammatory pathways that are known to operate in macrophages during atherosclerosis.

**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 quercetin, a route of administration that is expected to improve bioavailability and systemic exposure to active aglycone (Russo et al., 2012). As shown in Fig. 6A, mice treated with quercetin had smaller atheromatous plaques of intraperitoneal quercetin, a route of administration that is expected to improve bioavailability and systemic exposure to active aglycone (Russo et al., 2012). As shown in Fig. 6A, mice treated with quercetin had smaller atheromatous plaques compared with vehicle-treated mice. It is noteworthy that quercetin-treated mice also had significantly lower plasma cholesterol levels than vehicle-treated mice (Fig. 6B). Moreover, MDA serum levels were also reduced (Fig. 6C), indicating that quercetin-treated mice were protected from dyslipidemia-induced oxidative stress. It is noteworthy that these
atheroprotective effects were observed in the absence of any sign of toxicity, because no animal presented weight loss, piloerection, dehydration, secretions, changes in mucosal surfaces, or abnormal behavior, locomotion, or activity during or after the experiment (data not shown). In addition, no evidence of nephrotoxicity, hepatotoxicity, or pancreatic toxicity was found with serum clinical biochemical tests (Supplemental Fig. 2). These results are in accordance with the in vitro effects of quercetin described in 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 pro-oxidant/proinflammatory activity (Supplemental Table 1). Our results provide new insights into the atheroprotective mechanism of this flavonoid.

Nonregulated accumulation of lipoproteins in macrophages is a constant process during atherosclerosis. oxLDL are internalized via scavenger receptors, 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 (Fig. 1), and this effect paralleled a reduced surface CD36 expression (Fig. 2). Accordingly, apoE(−/−) mice treated with quercetin had smaller atherosclerotic lesions with less lipid deposition (Fig. 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 messen-
ger level by reverse transcription-polymerase chain reaction 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 seem 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 that work 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 antioxidant flavonoids.

A TLR-dependent, CD36-independent pathway that mediates lipid body formation via alterations in macrophage lipid metabolism has been proposed as an alternative route for foam cell formation during atherosclerosis (Nicolaou and Erridge, 2010). We found that LPS-induced lipid droplet forma-

tion was significantly reduced by quercetin (Fig. 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 an article published during the revision of this manuscript, researchers elegantly demonstrated that quercetin stimulated cholesterol efflux in macrophages, via p38 mitogen-activated protein kinase-mediated up-regulation 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.

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 produc-
tion (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 other cytokines/chemokines (Fig. 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). It is noteworthy that we found a significant modulation of oxLDL-induced ROS production and IL-6 secretion by quercetin in BMMo (Figs. 4 and 5), suggesting that this flavonoid could affect the vicious circle of LDL oxidation and inflammation observed during atherosclerosis progression. In line with this, apoE\textsuperscript{−/−} mice treated with quercetin had lower plasma levels of MDA and less aortic inflammatory infiltrate (Fig. 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; Fig. 5B), indicating that these effects are not restricted to oxLDL stimulation. This is in accordance with the broad spectrum of anti-inflammatory diseases that have been ameliorated by quercetin administration in animal models. ROS production is implicated in macrophage apoptosis, secondary necrosis, and defective effectorcytosis, 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 important role in local and systemic inflammation during atherosclerosis and together with C-reactive protein are well established CVD risk factors (Hansson, 2005). Our results (Fig. 5) and other results (Kleemann et al., 2011) 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 peripheral blood mononuclear cells, including IL-6 cytokine secretion via interference with NF-κB signaling (Bhaskar et al., 2011). It is noteworthy that 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 (Fig. 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 (Fig. 5B), we cannot rule out the possibility that the results are caused by 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\textsuperscript{−/−} mice chronically treated with nontoxic doses of intraperitoneal quercetin presented significant reductions in all indicators of atherosclerosis progression, including lesion size, foam cell accumulation, oxidative stress, and inflammatory response in situ (Fig. 6). These results strikingly mirrored those obtained in vitro (Figs. 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; Jużwiak 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 after intraperitoneal chronic administration. Furthermore, we demonstrated reduced inflammatory macrophage and T cell infiltrate in the atheromatous plaque (Fig. 6), which could have been a consequence of the antioxidant/anti-inflammatory activity of quercetin on macrophages (Figs. 4 and 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 (Fig. 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 attenuating atherosclerosis progression, is able to reverse or cure advanced lesions or 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 us to address these questions in the future.

Despite the clear cardioprotective effects of quercetin in vivo, no or very little quercetin aglycone circulates in the 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 deconjugated before cell uptake (Perez-Vizcaíno et al., 2012). Because absorption after intraperitoneal 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 deconjugated 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-Vizcaíno 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 depend on its transformation to phenolic acids (PA) by colonic microorganisms (Vissiennon et
al., 2012). Several PA have been shown to inhibit CD36 expression, oxLDL-induced foam cell formation, and LPS-induced proinflammatory cytokine production and 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 it is plausible that atheroprotective effects of this flavonoid are 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.

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Made substantial contributions to the writing of the manuscript: Lara-Guzman and Ramírez-Pineda.

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