NF-κB Inhibitors that Prevent Foam Cell Formation and Atherosclerotic Plaque Accumulation

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Abstract:

The transformation of monocyte-derived macrophages into lipid-laden foam cells is one inflammatory process underlying atherosclerotic disease. Previous studies have demonstrated that fullerene derivatives (FDs) have inflammation-blunting properties. Thus, it was hypothesized that FD could inhibit the transformation process underlying foam cell formation. Fullerene derivatives inhibited the phorbol myristic acid/oxidized low-density lipoprotein-induced differentiation of macrophages into foam cells as determined by lipid staining and morphology. Lipoprotein-induced generation of TNF-α, C5a-induced MC activation, ICAM-1 driven adhesion, and CD36 expression were significantly inhibited in FD treated cells compared to non-treated cells. Inhibition appeared to be mediated through the NF-κB pathway as FD reduced expression of NF-κB and atherosclerosis-associated genes. Compared to controls, FD dramatically inhibited plaque formation in arteries of apolipoprotein E null mice. Thus, FD may be an unrecognized therapy to prevent atherosclerotic lesions via inhibition of foam cell formation and MC stabilization.

Keywords: Atherosclerosis | NF-κB | Foam cells | Fullerenes | Low-density lipoprotein

Article:

Atherosclerotic disease begins when monocytes are exposed to oxidized low-density lipoprotein (ox-LDL) which form macrophage-derived foam cells that initiate plaque formation on blood vessels.[1] The plaque can suddenly rupture and cause thrombosis; leading to myocardial infarction or stroke. Given the severe implications of this process, new strategies that inhibit this progression before it occurs would reduce patient mortality and morbidity.

Mast cells (MC) are ubiquitously expressed in tissue and have been shown to be involved in the pathogenesis of atherosclerotic plaque buildup, erosion and rupture.[2, 3] These cells are

uniquely positioned to release their vast content of inflammatory mediators in and around affected vessels and into the circulation. Mast cells accumulate in human atherosclerotic lesions[4] and chronic activation of MC in the atherosclerotic lesions may be a pre-disposing stimulus leading to plaque rupture.[2]

The mechanisms leading to the transformation of monocytes into foam cells is complex, involving MC activation, oxidative stress, NF-κB activation, localized cytokine secretion, and the up-regulation of scavengerand cell adhesion receptors/ligands.[5, 6] Thus, a therapy that would block macrophage-to-foam cell transformation, stabilize MC activation, and reduce MC noxious mediator secretion may serve as a platform for new treatment strategies aimed at preventing atherosclerosis.

Fullerenes are carbon spheres that have a wide range of potentially beneficial biological properties. We recently showed that water soluble FDs are non-toxic[7] and inhibit allergic,[8, 9] pulmonary, and dermatological inflammation.[10] Previous studies examining the biodistribution of FD have shown them to be efficiently excreted from the body with negligible accumulation in major organs.[11, 12, 13, 14, 15, 16] Our studies suggest that the anti-inflammatory effects of FD may be due to their ability to interfere with several molecules involved in NF-κB-mediated activation, cytokine secretion, and oxidative stress responses.[9] Given the link between inflammation, oxidative stress, NF-κB activation and atherosclerosis, we hypothesized that FD could inhibit the underlying mechanisms of atherosclerosis and foam cell formation. We show that certain FDs inhibit foam cell formation and inflammatory mediator release associated with atherosclerosis through an NF-κB-dependent mechanism *in vitro* and can prevent the morbidity and mortality associated with a high fat diet in plaque-susceptible mice *in vivo*.

Methods

Fullerene derivatives

A panel of approximately 30 anti-inflammatory FDs[9] were initially tested for their ability to inhibit foam cell formation *in vitro*. From this initial screen both amphiphilic liposomal malonylfullerene (ALM) and tris-malonate fullerene (C_3) were chosen for further analysis. The FDs were bought from Luna Innovations and their formulation is described elsewhere.[12, 17]

Generation of monocyte-derived macrophage foam cells

Low-density lipoproteins from human plasma were oxidized as previously described to produce ox-LDL.[18] To obtain macrophage-derived foam cells U937 monocytic cells (0.5-1.0 × 10^6 cells/mL) were treated with 100 ng/mL phorbol myristilic acid (PMA) for 24 h and washed 1 time with media, and ox-LDL (10 μg/mL) was added for 48 h as described previously.[19] Confirmation of macrophage and foam cell development was determined using surface markers to CD68, CD36, and oil red O staining (ORO), respectively (data not shown) as described.[9, 20, 21] Tumor necrosis factor α (TNF-α) levels were measured using a kit from Sigma.

Scanning electron microscopy

U937 cells were challenged with PMA as above, followed by FD (5 μg/mL) for 24 h before the addition of ox-LDL. Following treatment cells were fixed on an aluminum foil substrate, and dehydrated using a gradient of ethanol with increasing percentages of 60%, 70%, 80%, 90% and 100% for 10 min, each. After air-drying, the foil substrate was mounted on standard Hitachi 15mm aluminum specimen mount with standard double sided carbon tape. Images were obtained using a Hitachi S-4800 FESEM with a beam setting of 1.0 kV and 5.0 μA.

Effect of FD on ICAM-1 and CD36 expression and NF-κB signaling intermediates

U937 cells were treated with PMA and ox-LDL as above followed by various concentrations of FD for 24 h before the ox-LDL was added. Treated cells were subjected to FACs analysis with ICAM-1 and CD36 specific mouse antibodies (Santa Cruz) and FITC-conjugated anti-mouse antibodies. For Western blotting to examine concentration and time-dependent effects on macrophages signaling molecules, cells with or without FD treatment were lysed, subjected to SDS-PAGE, and probed with the indicated mouse anti-human antibodies as described previously.[9, 20] As a negative control a non-specific mouse antibody was used in all experiments.

Analysis of NF-κB pathway and related molecules by qPCR

U937 cells were treated with PMA and ox-LDL as above followed by various concentrations of FD for 24 h before the ox-LDL was added. Total cellular RNA was extracted from FD treated or untreated cells as per manufacturer's protocol using the RNeasy® mini kit (Qiagen, Valencia, CA). Gene expression was determined using Applied Biosystems Human NF-κB TaqMan® Array Gene Signature Plates and TaqMan® RNA-to-CT™ 1-Step Kit (Life Technologies, Carlsbad, CA) using an Applied Biosystems 7500 Fast Real-Time PCR System. In addition, custom primers for ICAM-1, CCL2, NF-κB1, TRAF2, IKK-β and IL-8 were designed and purchased from Integrated DNA Technologies.[6, 22] Experiments with the custom primer sets were performed using the AMV First Strand Synthesis Kit (New England Biolabs, Ipswich, MA) to produce cDNA followed by the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) for the qPCR. Fold change in expression was obtained by the ΔΔCt method which gives a comparative analysis of gene expression between two samples. Briefly, to obtain the up- or down-regulation of those genes affected by FD, control cell RNA (no FD treatment) was probed with housekeeping and target-specific primers to establish a baseline expression. The RNA expression from cells treated with FD was then normalized to the controls in the non-treated cells. Data are presented as the fold change of expression between these two conditions.

Human mast cell preparation and mediator release

Mast cells from human skin and lung[20, 23] were incubated with or without FD for 16 h at 37°C (optimal for cellular uptake).[9, 24] The next day, cells were stimulated with 500 μg/mL C5a for 30 min (degranulation) or overnight (cytokine production). TNF-α levels were measured as described above and degranulation was measured as previously described.[9]

In vivo assessment of plaque formation

Homozygous male B6.129P2 apolipoprotein E knockout (ApoE−/−) and wild-type C57BL/6J mice (n = 6/group) were treated by i.p. with ALM or C_3(i.p. 5mg/kg/100 μL per injection) or fed *ad libitum* (2.5 μg/mL) in drinking water. There was no observed difference in the amount of water intake by the mice given control water (no FD) and mice given FD. Given an average water intake of 5.8mL/day, each mouse consumed approximately 14.5 μg (0.2 μg/g body weight) of FD each day. At day eight (after four injections) mice were fed a Teklad custom atherogenic rodent high fat diet (HFD; 02028) for up to nine weeks with FD or PBS injected every other day. Mice were maintained in a temperature and humidity-controlled room with a 12 hlight/dark cycle and were given free access to food and water. At week 10 mice were sacrificed and tissue sample was prepared as described[10] and serum cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and lactate dehydrogenase (LDH) levels were evaluated using kits from Cayman Chemical. All studies were approved by the UNCG Animal Welfare Committee.

Results

Fullerene derivatives

The size distribution profile of ALM and C_3 was 0.251 μm and 0.117 μm, respectively while the zeta potentials were −59.02 mV and −95.50 mV, suggesting stability of FD in solution. The size distribution and zeta potential did not significantly change after vigorous vortexing or sonication. Nanosight nanoparticle tracking of ALM and C_3 revealed a mean particle size of 0.142 μm for ALM and 0.082 μm for C_3. The smaller mean size from the Nanosight is a result of the individualized nature of particle size measurement. No cellular (U937 and THP-1) toxicity was observed with either FD when using up to 100 μg/mL (data not shown).

Fullerene derivatives prevent morphological changes in PMA/ox-LDL-challenged cells

Treating monocytes with PMA and ox-LDL induces their transformation into lipid-laden foam cells.[25] SEM was used to examine the morphological changes associated with foam cell formation under these conditions with or without FD pre-incubation. As seen in Figure 1, *A* and *B*, untreated U937 cells have a relatively smooth plasma membrane with minor areas of surface irregularity and are spherical in shape. The addition of PMA/ox-LDL induces a drastic phenotypic change characterized by an amorphous shape, cellular elongation, and ruffling of the plasma membrane (Figure 1, *C* and *D*) as described previously.[19] However, the addition of ALM prevented this phenotypic change in the U937 cells (Figure 1, *E* and *F*). Similar results were observed with C_3 (data not shown).

Fullerene derivative pre-incubation prevents cytokine release, foam cell formation, and cell adhesion

Previous studies have shown that TNF-α release from activated monocytes induces foam cell formation.[26] Given that FDs have been shown to prevent TNF-α release in other cell types,[8, 9, 27] we hypothesized that FD would also inhibit the production of TNF-α from monocytes/macrophages. To test this hypothesis, we examined the effects of ALM on TNF-α release from PMA activated U937 monocytes. As shown in Figure 2, *A*, cells were treated for

24 h with PMA and ox-LDL, and those untreated with FD showed a significantly higher amount of TNF-α than FD treated U937 cells. However, when cells were pretreated with 5.0 μg/mL of FD for 24 h there was a statistically significant inhibition of TNF-α release, ranging from 58% to 77%. Similar statistically significant inhibition of TNF-α release was seen with ALM and C_3 when levels were monitored at 12 h (data not shown). Thus, FD may inhibit foam cell formation through inhibition of TNF-α release.

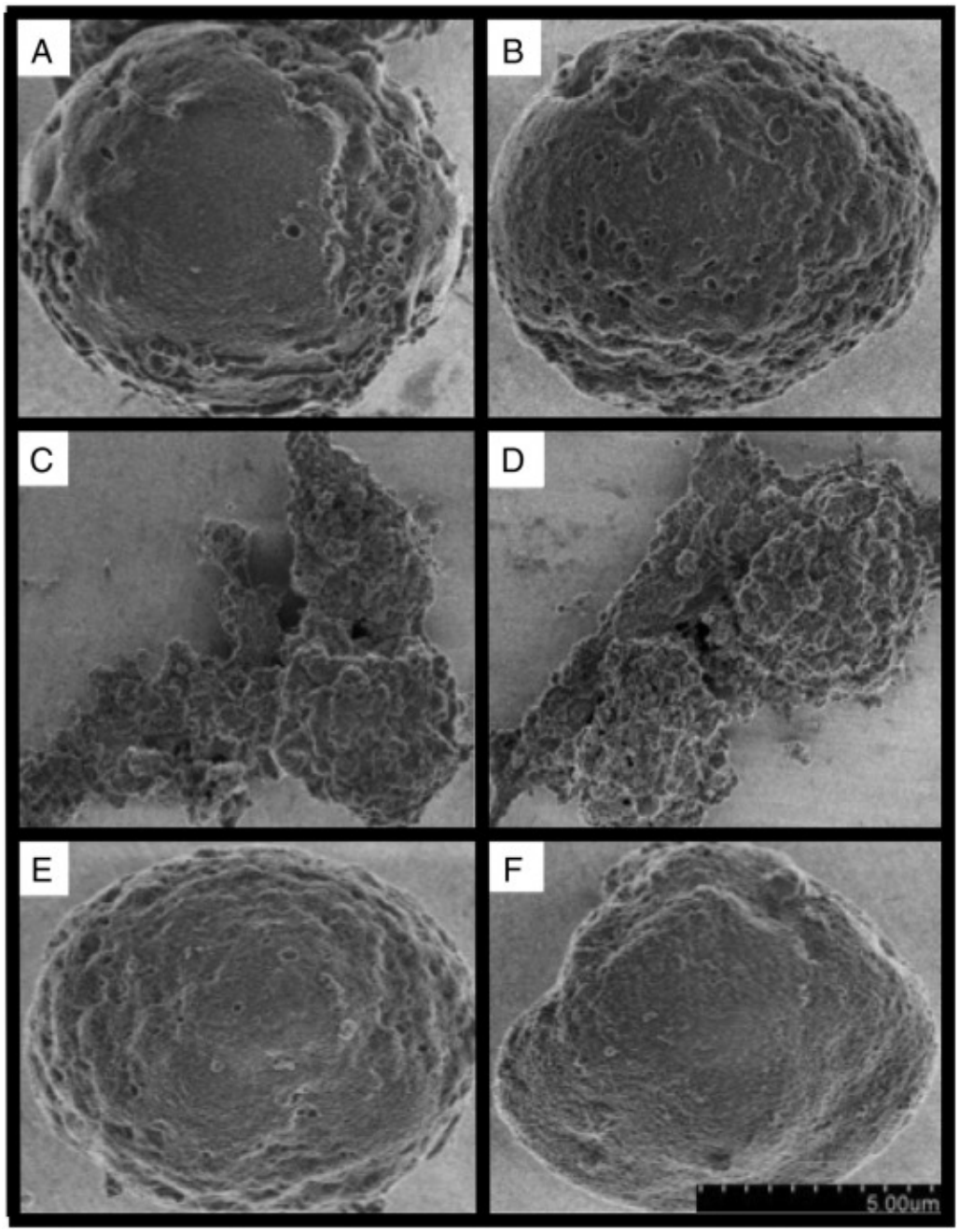
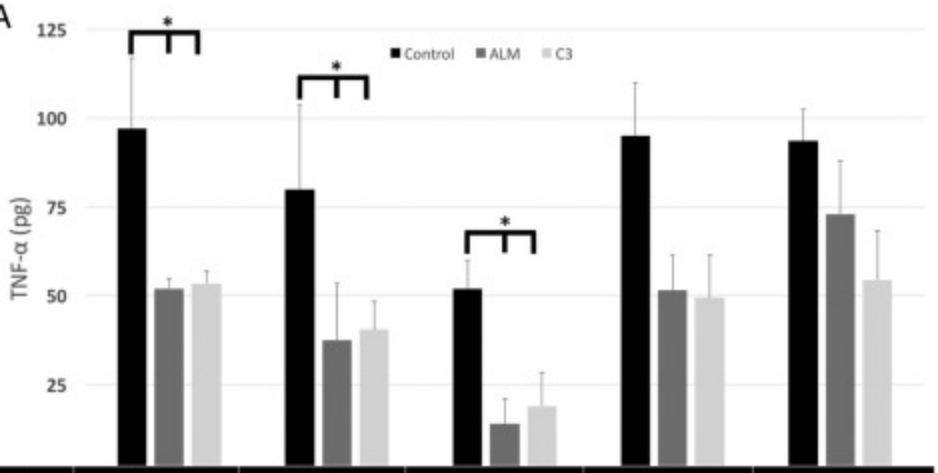


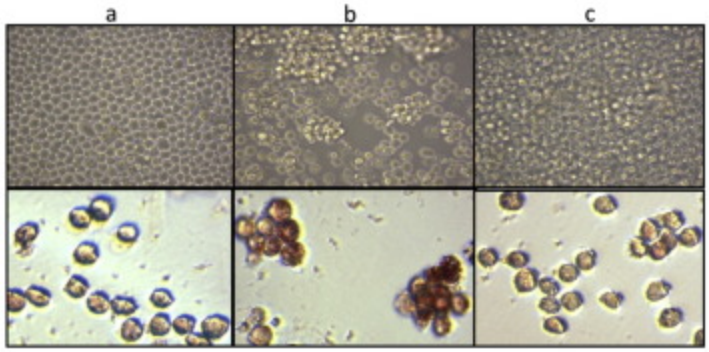
Figure 1. FDs prevent morphological changes associated with foam cell formation. U937 cells not treated with ALM (**A**, **B**) show the characteristic smooth plasma membrane and spherical shape of a monocyte. The PMA/ox-LDL-challenged U937 cells transformed to foam cells exhibit amorphous shape, elongation, and roughing of the plasma membrane (**C**, **D**). However, when the PMA/ox-LDL-challenged U937 cells were treated with ALM (5 μg/mL), the phenotypic changes were prevented (**E**, **F**).

A

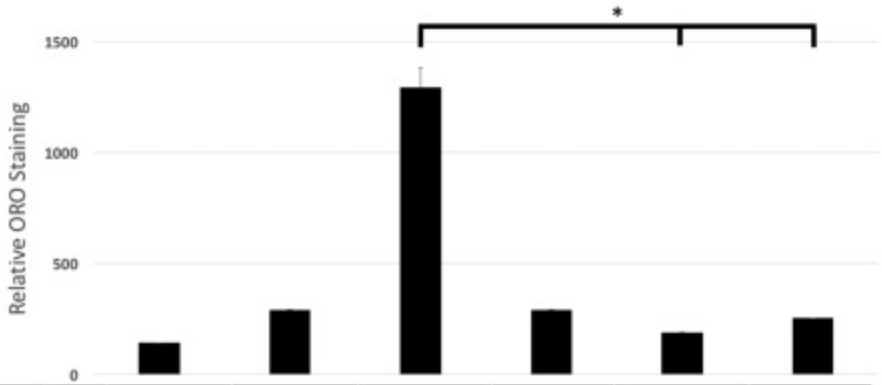


PMA (ng/mL)	100	100	100	100	100
ox-LDL (µg/mL)	0.5	1	5	8	10

B



C



PMA (ng/mL)	-	-	100	100	100	100
ox-LDL (µg/mL)	-	10	10	-	10	10
ALM (µg/mL)	-	-	-	-	10	-
C_3 (µg/mL)	-	-	-	-	-	10

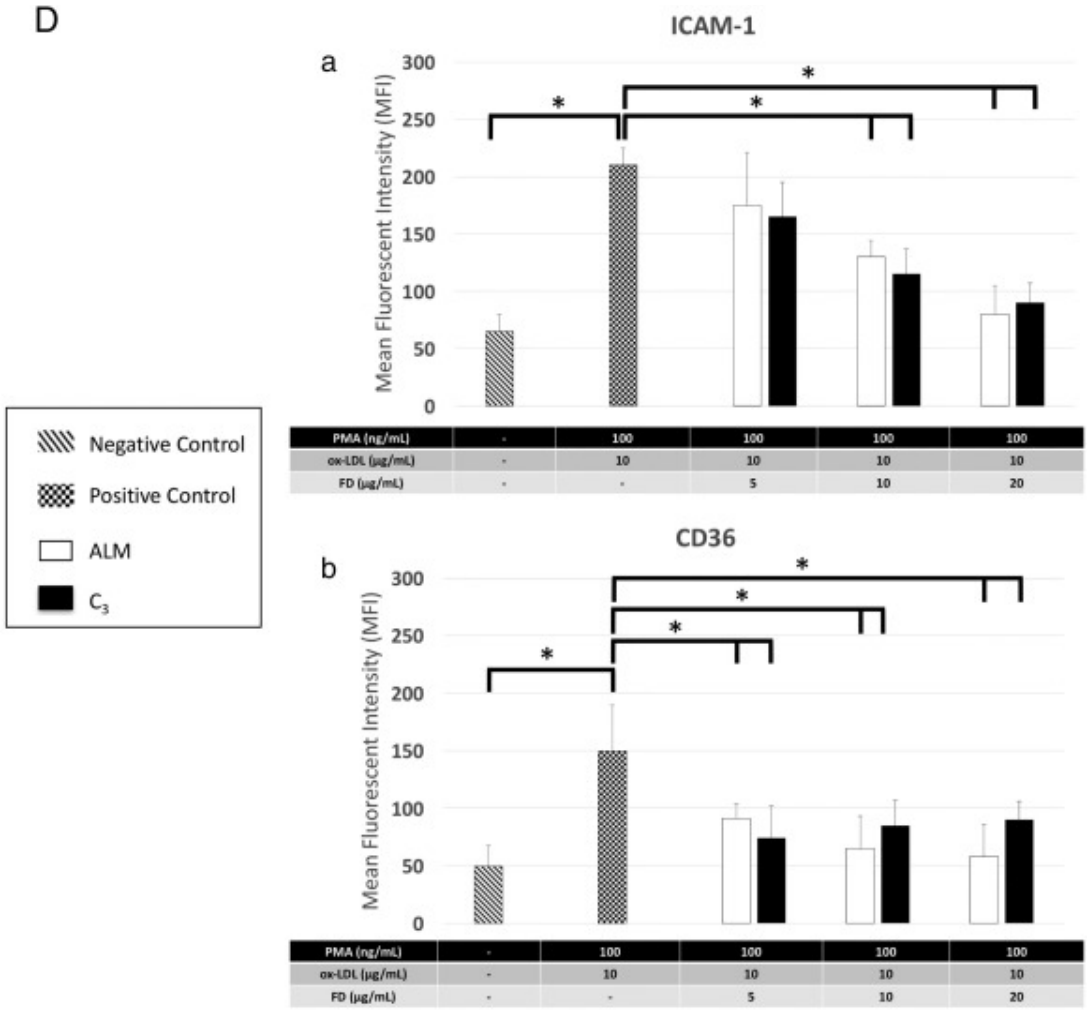


Figure 2. (A) TNF-α release from U937 cells is inhibited by FD. U937 cells were incubated with PMA, 5 µg/mL FD [ALM (dark gray bars), C_3 (light gray bars), or no FD (black bars)], and ox-LDL as described in Methods. Cells were centrifuged and supernatants assayed for TNF-α via ELISA. Significance ($P < 0.05$) indicated by an asterisk * was determined using the Student *t* test. Results are the average of three separate experiments, each done in triplicate. (**B** and **C**) Fullerene derivatives inhibit cell adhesion and lipid uptake in U937 cells. U937 cells were challenged with PMA (**b**, **c**) or without (**a**; negative control). PBS (**b**, vehicle control), or ALM (**c**; 10 µg/mL) was added followed by ox-LDL as described above. In the top panels cells in suspension were photographed for clumping while the lower panel cells were stained with ORO. Pictures of the cells in culture or after ORO staining were taken using an inverted microscope at 10× (top) or 40× (bottom) magnification. The graph represents the quantification of ORO staining. Results are representative of three separate experiments using both ALM and C_3. All values with an * are significantly different compared to non-FD cells ($P < 0.05$). (**D**) Fullerene treatment prevents the up-regulation of ICAM-1 and CD36. U973 Cells (in duplicate) were treated with PMA, ox-LDL, followed by various concentrations of FD. Treated cells were assayed via FACS using antibodies against ICAM-1 (**a**) and CD36 (**b**). The graph shows the average mean fluorescent intensity (MFI) of the signal (±SD) from three separate experiments. As a control MOPC was substituted for the primary antibodies and demonstrated no staining (not shown). All values with an * are significantly different compared to non-FD cells ($P < 0.05$).

TNF-α activated monocytes initiate cellular clumping and lipid uptake as part of the foam cell formation process.[28] PMA-treated cells showed significant clumping and ORO staining (Figure 2, *B*; middle) consistent with previous studies.[28] However, the ALM-treated cells showed no significant clumping or ORO staining (Figure 2, *B*; right) compared to non-treated U937 cells (Figure 2, *B*; left). In order to quantify the observations in Figure 1, Figure 2, *B*, the uptake of

lipids was assessed. Quantification of the ORO staining demonstrated significant inhibition of lipid uptake in FD treated vs. untreated cells (Figure 1, *C*).

The clumping induced by activated monocytes in atherogenesis is mediated through the up-regulation of ICAM-1.[28] We hypothesized that FD inhibited the clumping of activated monocytes through ICAM-1 inhibition. To test this hypothesis, we treated cells with PMA (100 ng/mL), ox-LDL (10 μg/mL), and varying concentrations of FD and examined for the up-regulation of ICAM-1 using flow cytometry. As seen in Figure 2, *D*, the same FD treatment conditions that prevented cell clumping also prevented the up-regulation of ICAM-1, in a dose-dependent manner. Although a trend can be observed, the data were not statistically significant. Thus, FD prevents activation-induced monocytic cell–cell adhesion possibly through the inhibition of ICAM-1.

Up-regulation of CD36 scavenger receptors on foam cells is another key event in ox-LDL uptake and foam cell formation during atherosclerosis. We investigated the effect of ALM and C_3 on CD36 receptor expression. As seen in Figure 2, *D*, CD36 receptor expression was downregulated with varying concentrations of both FD. Taken together, FDs inhibit several foam cell specific functional and phenotypic characteristics.

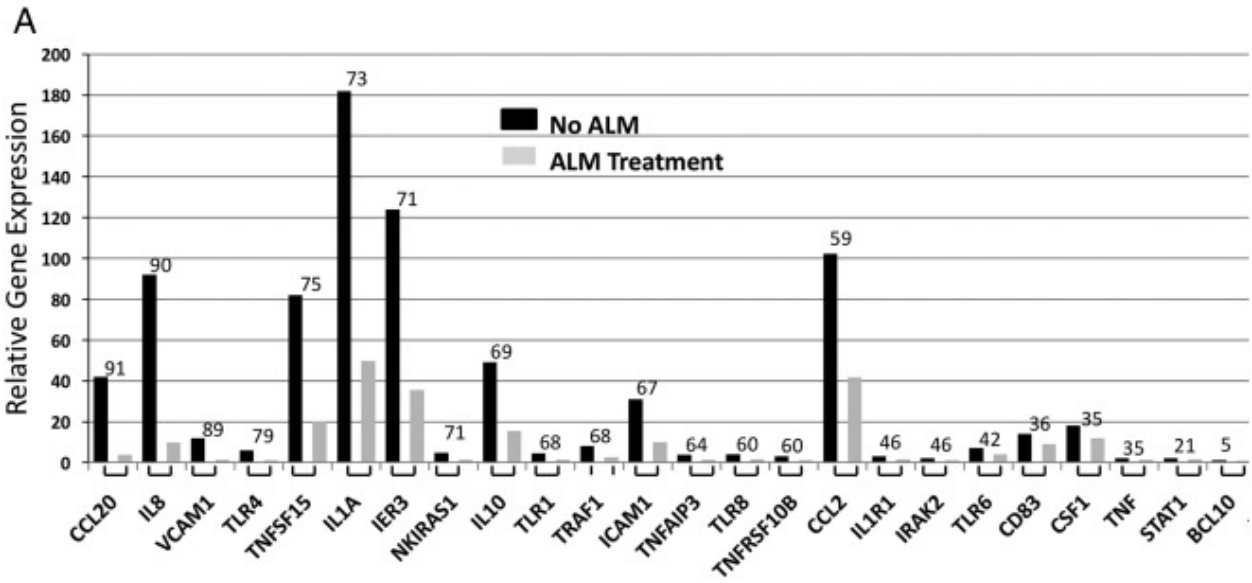
Fullerene derivatives effects on atherosclerosis-associated RNA transcripts, protein expression, and NF-κB-signaling intermediates in foam cells

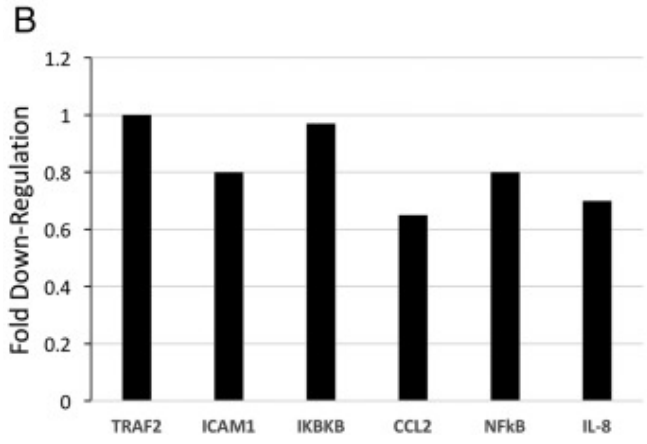
Given the observation that TNF-α release was significantly inhibited by FD we focused our efforts on the NF-κB pathway using qPCR. As seen in Figure 3, *A*, ALM caused a significant reduction in several atherosclerosis-related intermediates including CCL2 (91% reduction), IL-8 (90% reduction), VCAM (89% reduction), IL-1 (73% reduction), and TNFS15 (75% reduction). To further validate how FD affected these gene products, we performed more focused studies on several genes including TRAF2, ICAM1, IKBKB, CCL2, IL8, and NF-κB. As seen in Figure 3, *B* those genes that were observed to be downregulated by FD were again observed to be downregulated using separate primers thus validating those observations in Figure 3, *A*.

NF-κB is involved in TNF-α induced foam cell formation,[29] which involves the signal transducer TRAF2[30] and IκB kinase (IKK).[31] Since both C_3 and ALM appear to similarly inhibit foam cell formation, we chose ALM for the signaling studies. We hypothesized that ALM was inhibiting foam cell formation through the inhibition of the NF-κB pathway through RIP, IKK, TRAF, or Nf-κB1 intermediates. As seen in Figure 3, *C*/*D*, ALM/PMA/ox-LDL-treated U937 cells that were pretreated with ALM had a significant reduction of NF-κB1 and TRAF2 expression at the protein level. This effect was dose (C) and time (D) dependent. The ALM-mediated inhibition of NF-κB was maximal at two hours after ox-LDL challenge and total levels remained significantly lower at 16 h compared to non-ALM treated cells. Similar results were observed with TRAF2, however little expression or change was observed with other signaling intermediates involved in the NF-κB signaling pathway. Antibodies to detect phospho-IKK-α/β and IKK-α revealed a slight reduction in expression after two hours of ox-LDL treatment in ALM treated cells. Thus, ALM apparently inhibits the ox-LDL-induced increases in foam cell formation by affecting NF-κB1, TRAF2, and IKK proteins.

Stabilization of human connective tissue mast cells by FD

The complement system is activated in atherosclerosis[32, 33] and patients with the disease have elevated serum levels of complement activationproducts (e.g., C5a).[34] Mast cells are activated by complement byproductssuch as C5a,[35] which may be one initiating or amplifying mechanism of atherosclerotic disease. As seen in Figure 4, human connective tissue MC pre-treated with ALM showed significant inhibition of C5a-induced degranulation (A) and cytokine production (B).





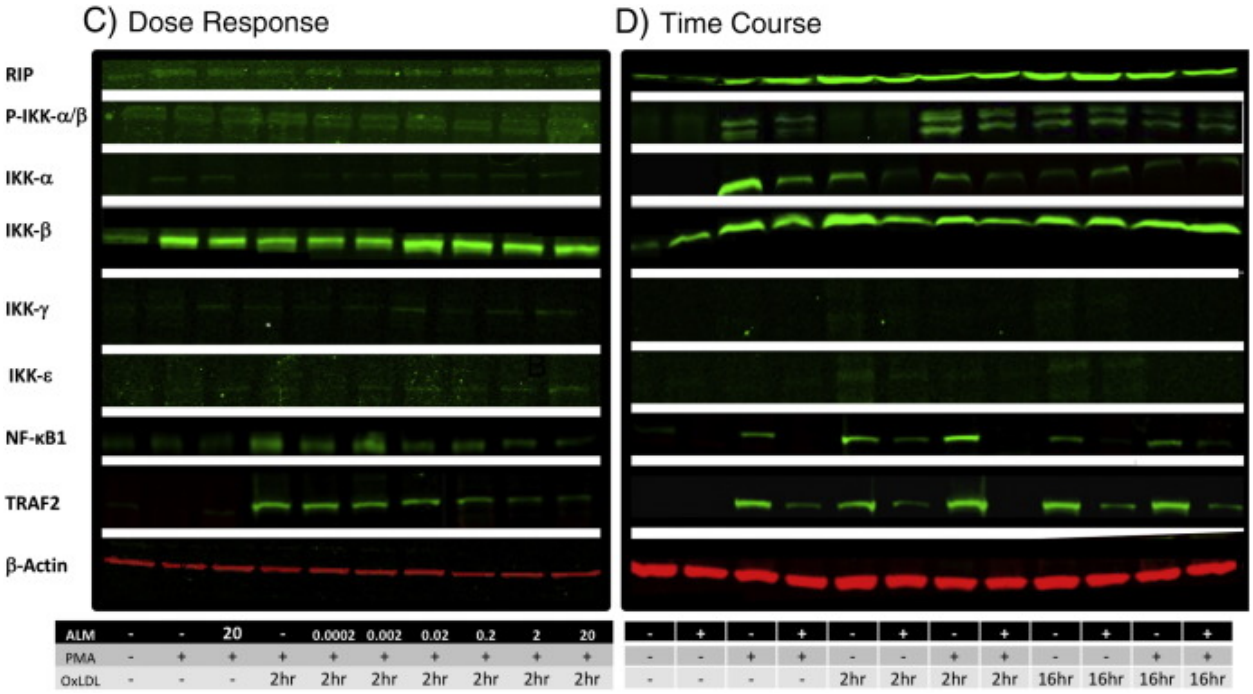


Figure 3. Fullerene derivatives effects on atherosclerosis-related mRNA transcripts in macrophages (**A** and **B**). U937 cells (in duplicate) were treated as above with PMA and ox-LDL with or without ALM (5 μg/mL). RNA was extracted via the RNeasy mini kit (Qiagen, Valencia CA) and qPCR was preformed using the Applied BiosystemsHuman NF-κB TaqMan® Array Gene Signature Plates per manufacturer's instructions. Values above the bars represent the percent downregulation of each gene as compared to non-treated cells and FD treated cells. Fullerene derivatives differentially affect NF-κB associated signaling intermediate proteins in a dose (**C**) and time (**D**) dependent manner. In panel (**C**) U937 cells treated with PMA followed by treatment with or without varying concentrations of FD and ox-LDL for 2 h. In panel (**D**) U937 cells treated with or without PMA followed by FD treatment and ox-LDL for 2 h or 16 h. Cells were lysed, subjected to SDS-PAGE, and Western blotted using the indicated antibodies as described previously.[14]

Effect of FD derivatives on atherogenesis *in vivo*

Given that FDs inhibit several underlying mechanisms leading to atherosclerosis *in vitro*, we hypothesized that they could prevent atherosclerosis *in vivo*. As seen in Figure 5, *A*, eighty percent of ApoE−/− mice fed a high HFD for ten weeks and injected with PBS did not survive. In contrast, the FD-treated mice appeared healthy, had no obvious abnormalities, and had weights similar to the age-matched WT mice (Figure 5, *A*). Upon further examination of the aortic arches, both ALM and C_3 significantly reduced the size of the vessel-clogging plaque lesions (Figure 5, *B*). The non-treated, ApoE−/− mice had extremely large, vessel-blocking lesions in the aortic arch (Figure 5, *C*/*D*-arrows) that were virtually absent when mice were treated with FD (Figure 5, *E*-*H*) over the nine weeks. Thus, FDs prevent the plaque build-up associated with atherosclerosis in mice fed an HFD.

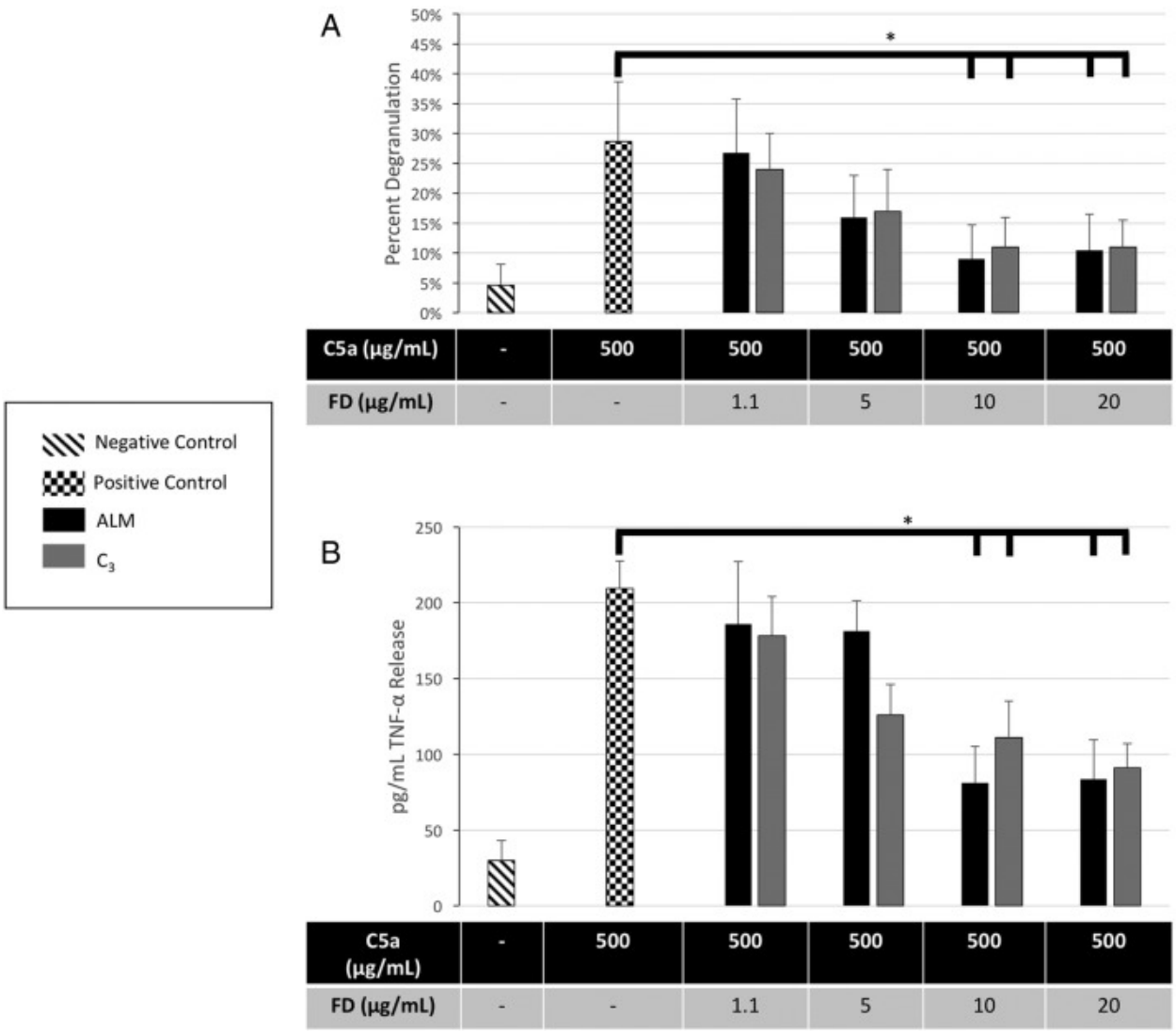


Figure 4. Fullerenes reduce C5a-induced noxious mediator release from connective tissue mast cells. Mast cells were cultured with ALM or C_3 at various concentrations, then washed and stimulated with C5a (500 µg/mL) for 30 min for degranulation (**A**) or 16 h for TNF-α release (**B**). Cells were centrifuged and β-hexosaminidase release or TNF-α production was determined via ELISA. Data shown are means ± SE of triplicate samples that are representative of three separate experiments with separate MC cultures. All values with an * are significantly different compared to non-FD cells ($P < 0.05$).

Fullerene derivatives prevent liver toxicity associated with HFD and do not affect kidney function

Several toxicological markers in ALM and C_3 treated mice were examined following administration via i.p. or oral administration. There were no morphological changes in the livers of ALM or C_3-treated mice compared to ApoE−/− mice on HFD (data not shown). Serum cholesterol levels were significantly elevated in the non-FD treated animals fed an HFD compared to controls ($P < 0.05$). ALM and C_3 did not affect total cholesterol, LDH, or AST levels (Figure 6, *A*, *B*, and *E*), but did reduce the HFD-induced elevations in creatinine and ALT levels (Figure 6, *C* and *D*). Thus, FDs do not induce toxicity in animals challenged for up to 10 weeks.

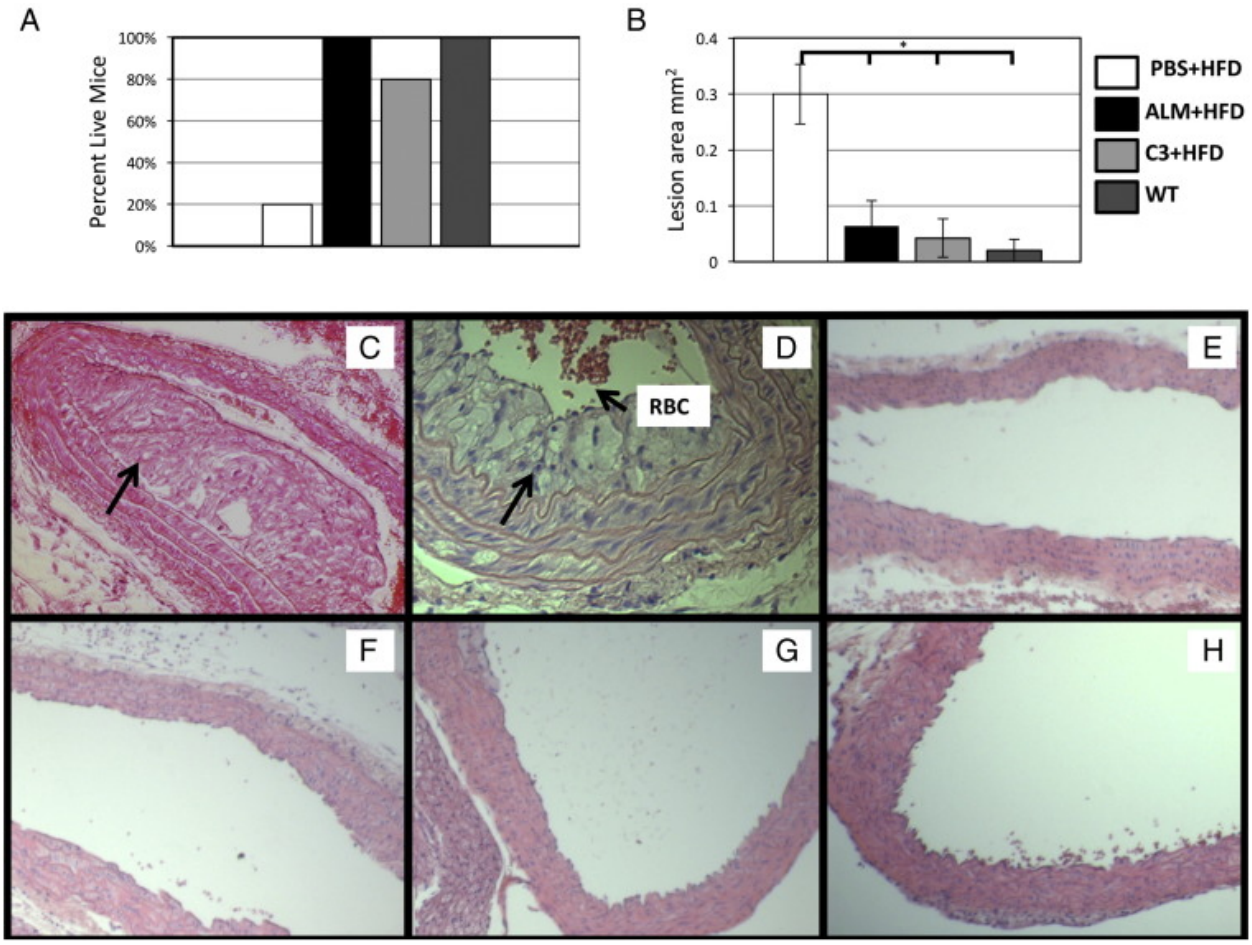


Figure 5. Fullerenes derivatives prevent atherosclerosis *in vivo*. Mice were treated orally with 2.5 μg/mL FD and i.p. with 5 mg/kg/100 μL. After 10 weeks the percent of ApoE−/− or WT mice living was assessed (**A**). The aortas from ApoE−/− treated with PBS and fed an HFD, ApoE−/− treated with ALM or C_3 plus HFD, were stained with hematoxylin and lesion sizes assessed as mean ± SD (**B**). Representative images of PBS treated (**C**, **D**), ALM oral (**E**, **F**), or C_3 oral (**G**, **H**) are shown in the panel image. Note the large fatty streak lesions (arrows) in the PBS treated mice that are absent in the ALM or C_3 treated mice. Pictures were taken using a 4× magnification. Pictures are representative of each of the six mice and were examined for each condition in a blinded fashion. All values with an * are significantly different compared to the PBS vehicle control group ($P < 0.05$).

Discussion

The aim of our study was to determine if FD could prevent the development of atherosclerosis based on the observation that certain FDs could attenuate other chronic inflammatory diseases.[11, 36, 37] After initial testing of several FD, two were chosen for further studies based on their ability to inhibit foam cell formation *in vitro*. Monocyte U937 cells treated with FD had a significant decrease in total ORO staining under conditions that transform them into foam cells compared to untreated cells. Second, SEM analysis clearly demonstrated that drastic morphological changes that occur in PMA/ox-LDL challenged U937 cells are prevented with FD pre-incubation. Third, the clumping process, which occurs upon the monocyte-to-foam cell transformation,[38] is prevented, possibly through the down-regulation of ICAM-1. Lastly,

inflammatory mediator release from macrophages (TNF-α and MC-derived) leading to atherosclerosis was inhibited by FD.

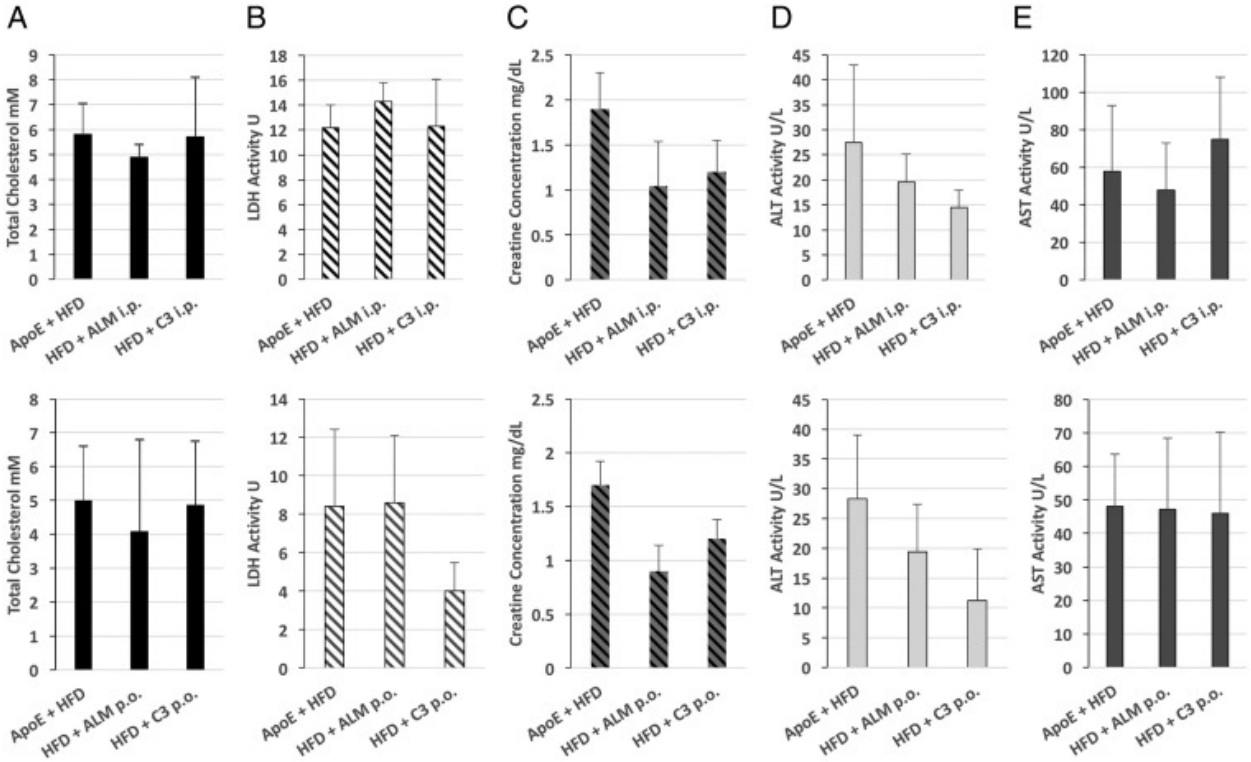


Figure 6. Fullerenes derivatives effect on *in vivo* toxicity. Mice (n = 6/group) were treated as described in Methods. After ten weeks, blood was collected from hearts of ApoE−/− mice fed an HFD and ApoE−/− treated via i.p. or oral (p.o.) administrations of ALM or C_3 plus HFD. The total serum cholesterol (**A**), lactate dehydrogenase (**B**), creatinine (**C**), ALT (**D**), and AST (**E**) levels were measured in triplicate.

Macrophages and MCs release a wide variety of inflammatory mediators involved in the initiation and progression of atherosclerosis including TNF-α which can upregulate ICAM-1.[39] Reducing serum levels of TNF-α improves patient outcomes.[40] Our findings show that FDs inhibit the production of TNF-α up to 72% compared to non-treated cells and reduce cellular adhesion in U937 cells through reduction of CD11 expression.

The CD36 receptor mediates the ox-LDL induction of atherogenic foam cells making it a therapeutic target for atherosclerosis.[41] For example, cholesterol lowering statins significantly reduce CD36 receptor expression.[42] Our results are similar as it is shown that FD can inhibit CD36 expression and thus interfere with a critical step involved in foam cells formation.

Several studies have demonstrated that the induction of foam cell formation in atherosclerosis is mediated in part through oxidative stress,[43]For example, CD36 expression in monocytes is increased by oxidative stress.[39, 44] Previous reports have proposed that membrane expression of CD36 involves redox signaling pathway via NADPH oxidase activation and the administration of antioxidants leads to a reduction in CD36 expression in monocytes derived from humans.[44] Given that certain FDs may possess anti-oxidant properties, these molecules may interfere in the progression of atherosclerotic disease at several levels.

Furthermore, it has been demonstrated that oxidative stress influences the expression of NF-κB.[45] NF-κB is a key regulator of cell survival and proliferation and previous studies have shown that NF-κB inhibition reduces foam cell formation.[46] TRAF2 is part of a group of adaptor proteins involved in signal transduction by most members of the TNF receptor family. TRAF2 has a key role in mediating TNFR1 induced activation of NF-κB.[47] As seen in Figure 3, ALM was capable of dramatic reductions in TRAF2 and NF-κB expression in cells activated with ox-LDL. The activation of NF-κB via TRAF2 has been shown to increase foam cell formation.[48]

Relatedly, we show for the first time that several atherosclerosis-related genes up-regulated in foam cells are dramatically inhibited in ALM-treated cells. For example, CCL2 is a chemokine found in the serum of hypercholesterolemic patients as well as atherosclerotic lesions.[49] We found that ox-LDL/PMA caused a 40-fold induction in its cellular expression, which dramatically decreased in FD-treated cells. Similarly, ICAM-1 expression was dramatically reduced at both the gene (Figure 3) and protein levels (Figure 2) in foam cells.

Mast cells have traditionally been associated with the initiation and proliferation of allergic responses, but a role for MC in atherosclerosis has now been clearly demonstrated.[3] Mast cell numbers are greatly increased in the intima at sites of arterial plaque rupture,[50] in advanced plaque lesions in the carotid artery,[51] and in vulnerable plaque.[52] Clinically, patients who died of acute myocardial infarction have an increased number of degranulated MCs at the site of plaque erosion or rupture.[53]Animal studies demonstrate that MC-deficient mice develop less atherosclerosis when fed an HFD compared to WT mice.[54] The complement system is extensively activated in human atherosclerosis and lesions.[32, 33] There also appears to be a positive correlation between serum levels of complement activation products (i.e., C5a) and human patients with atherosclerosis.[34] Similar to other studies examining FD, inhibition of MC mediator release through IgE and non-IgE pathways,[9, 37, 55] we show for the first time that human MC activation through C5a is also inhibited by FD. Thus, FD may not only affect foam cell formation but may also prevent atherosclerosis (via C5a stimulation) through the stabilization of MC and inhibition of the release of their noxious mediators.

ALM and C_3 demonstrated an almost complete inhibition of atherosclerotic plaque lesions in the aortic arches of ApoE−/− mice fed an HFD.The ALM and C_3 treated groups showed significant inhibition of plaque lesions when compared to the PBS vehicle group. This inhibition of plaque lesions by the FD most likely explains the striking difference in survival rates between the two groups; 80% of the non-treated mice did not survive at the 10 week HFD regimen. None of the ALM-treated mice died in these studies. In both treated groups body weights did not change when compared to controls. Thus, rationally designed FD may represent a novel way for the prevention of complications associated with the accumulation and rupture of atherosclerotic plaque.

The studies examining the toxicity of FD are still the subject of debate.[56, 57, 58] Our studies using highly purified and well characterized FD support studies demonstrating a lack of toxicity.[7, 59, 60] Here, it is demonstrated that the two anti-atherogenic FDs are not toxic to the liver and kidney under the conditions tested.

In conclusion, we have shown that certain FDs inhibit the formation of U937-derived foam cells possibly through the reduction of inflammatory cytokine release and adhesion molecule membrane expression through an NF-κB dependent mechanism. These compounds also significantly inhibit C5a-induced MC activation. The same FD also attenuated the accumulation of plaque lesions *in vivo*. These results further extend the utilization of FDs and suggest that they may be used as a platform for developing new therapeutics for the treatment of atherosclerosis.

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