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Effects of acute and chronic low density lipoprotein exposure on neutrophil function

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Abstract

Mounting evidence suggests that obesity and the metabolic syndrome have significant but often divergent effects on the innate immune system. These effects have been best established in monocytes and macrophages, particularly as a consequence of the hypercholesterolemic state. We have recently described defects in neutrophil function in the setting of both obesity and hypercholesterolemia, and hypothesized that exposure to elevated levels of lipoproteins, particularly LDL its oxidized forms, contributed to these defects. As a model of chronic cholesterol exposure, we examined functional responses of bone marrow neutrophils isolated from nonobese mice with diet-induced hypercholesterolemia compared to normal cholesterol controls. Chemotaxis, calcium flux, CD11b display, and F-actin polymerization were assayed in response to several chemoattractants, while neutrophil cytokine transcriptional response was determined to LPS. Following this, the acute effects of isolated LDL and its oxidized forms on normal neutrophils was assayed using the same functional assays. We found that neutrophils from nonobese hypercholesterolemic mice had blunted chemotaxis, altered calcium flux, and normal to augmented CD11b display with prolonged actin polymerization in response to stimuli. In response to acute exposure to lipoproteins, neutrophils showed chemotaxis to LDL which increased with the degree of LDL oxidation. Paradoxically, LDL oxidation yielded the opposite effect on LDL-induced CD11b display and actin polymerization, and both native and oxidized LDL were found to induce neutrophil transcription of the monocyte chemoattractant MCP-1. Together these findings suggest that chronic hypercholesterolemia impairs neutrophil functional responses, and these defects may be in part due to protracted signaling responses to LDL and its oxidized forms.

Keywords

Neutrophil; Cholesterol; Dyslipdemia; Chemotaxis; Cytokines; Immunity; Innate

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1. Introduction

Though obesity is known to be associated with increased health risks, there is a growing body of evidence that in the Acute Respiratory Distress Syndrome (ARDS), increasing BMI appears to be associated with a protective effect [1-6]. This, paradoxically, occurs despite the well-described systemic *pro-inflammatory* effects of obesity [7], and suggests that obesity's effects on inflammatory response may be inhibitory in some contexts. Such an effect is supported by recent findings in ARDS patients demonstrating that circulating levels of the inflammatory cytokines IL-6 and IL-8 *decrease* with rising BMI [8]. In further support of this, recent animal studies demonstrate that obesity, and in particular the dyslipidemic state that often accompanies it, may have inhibitory effects on innate immune function and the acute inflammatory response in acute lung injury [9-13]. Neutrophils have been shown to express receptors binding Low Density Lipoprotein (LDL) [14, 15], however most studies of the effects of LDL on leukocyte function to date have focused on monocyte and macrophage responses, particularly in the context of atherogenesis [16-18]. Far less is known about LDL effects on polymorphonuclear leukocytes, which are also critical effectors of innate immunity and the acute response to infection or injury in the lung.

Similar to monocytes and macrophages, neutrophils express functional LDL receptor (LDLR) and multiple scavenger receptors for modified (i.e. oxidized) LDL, although the expression pattern of these receptors appears to differ substantially from that of the monocytic lineage [14, 15]. Modification of native serum LDL (nLDL) by a variety of oxidative stresses is a consequence of the dyslipidemic state, and such oxidized LDL has been implicated in the pathogenesis of atherosclerosis through multiple effects on monocytes and macrophages [19].

When present, the degree of oxidation is typically categorized as minimal (mmLDL) or extensive (ox-LDL), and these distinctions dictate receptor interactions: ox-LDL appears to bind primarily to scavenger receptors, (e.g. CD36) while mmLDL has been shown to bind LDLR and, growing evidence suggests, also interact with the CD14/TLR4 complex [20-22]. Although few studies have examined the effects of nLDL or its oxidized forms on neutrophils, these have been shown to induce neutrophil calcium flux, oxidant release, integrin expression, and chemotaxis through one or more of the cholesterol receptors, perhaps proportionately to the degree of LDL oxidation [15, 23-25]. The effects of *protracted* neutrophil exposure to such LDL species, as would occur in the dyslipidemic circulation and bone marrow, are even less well defined.

Neutrophils isolated from hypercholesterolemic patients appear to have blunted calcium flux and release of neutrophil elastase in response to exposure to formyl-Met-Leu-Phe (fMLP) or IL-8 [26]. Using a mouse model of diet-induced hypercholesterolemia without obesity [27], we recently demonstrated hypercholesterolemia-associated impairment of neutrophil migration and trafficking to the lung following inhaled LPS exposure, and have linked this to reduced surface expression of CXCR2, the receptor for the IL-8 homologue KC.

We hypothesize that continuous neutrophil signaling in response to high levels of LDL in the blood and marrow may in part be responsible for the findings of neutrophil dysfunction in chronic dyslipidemia. The aim of the present report is to further describe in detail the effects of chronic exposure of dyslipidemia and oxidized LDL on neutrophil function using this mouse model, and examine how this model may relate to the acute effects of LDL on neutrophils .

2. Methods

2.1. Mice

In a genetic model of obesity, homozygous B6 db/db mice (Jackson Labs, Bar Harbor, ME) and their lean heterozygous littermates were examined at 6-8wks of age. For non-obese hypercholesterolemia experiments, C57BL/6 mice (Harlan, Indianapolis, IN) were fed high-cholesterol diet (Harlan Teklad TD.90221) or a normal diet (TD.95138) [27], *ad libitum* for 4-5 wks. Experiments were performed in accordance with the Animal Welfare Act and the USPHS Policy on Humane Care and Use of Laboratory Animals, after review by the Animal Care and Use Committee of the University of Vermont. Additional details may be found in the Supplementary Material.

2.2. Preparation of Native and Oxidized LDL

Human nLDL (Intracel, Frederick, MD) was dialyzed into 1× PBS to remove EDTA using 3cc dialysis cassettes (MWCO 7kDa, Thermo, Rockford, IL). Oxidized LDL was prepared by incubating nLDL in dialysis cassettes with either 5mM (for minimal oxidation; mmLDL) or 10mM (for moderate oxidation; ox-LDL) CuSO₄ in PBS overnight at 4°C, followed by dialysis back in to 1× PBS. In all samples, LDL concentration was determined using micro-BCA assay (Thermo), and the degree of oxidation was determined by TBARS (thiobarbiturate acid reactive substances) assay (Oxitech, Zeptometrix, Buffalo, NY), which was then normalized to protein concentration. All LDL preparations were stored under nitrogen in the dark at 4°C.

2.3. Determination of Plasma Cholesterol Levels and Oxidation State of LDL

Mouse plasma LDL cholesterol levels were assayed using an Advia Chemistry System (Siemens, Tarrytown, NY). The level of plasma LDL oxidation was determined using heparin/manganese precipitation [28] followed by protein quantification and TBARS, as above.

2.4. Preparation of Morphologically-Mature Murine Bone Marrow Neutrophils

Femurs/tibias of euthanized mice were dissected, marrow flushed with HBSS, and layered on a 3-step Percoll (GE Healthcare, Piscataway, NJ) gradient (72%, 64%, and 52%) which was centrifuged at 1060*g* for 30min, as previously described [29, 30]. Samples of the 72:64% interface revealed >95% morphologically mature-appearing neutrophils.

2.5. Neutrophil Function Assays

Chemotaxis of marrow neutrophils was measured using a modified Boyden Chamber, as described [31]. Calcium flux was measured using Indo-1/AM (Molecular Probes, Carlsbad, CA), as described [30]. Neutrophil surface display of CD11b and cytosolic F-actin polymerization were assessed by flow cytometry as described [29]. Additional details may be found in the Supplementary Material.

2.6. Determination of Neutrophil mRNA Transcription Levels

To assess the effects of chronic exposure to hypercholesterolemia, isolated marrow neutrophils from mice fed normal or high cholesterol diets were incubated for four hours in RPMI/1% heat-inactivated mouse serum with either lipopolysaccharide (LPS, 100ng/mL; *E. coli* 0111:B4, Sigma) or PBS control. Transcription of IL-6, IL-1β, TNF-α, and MCP-1 was assessed using a Bio-Rad quantitative PCR system, as described [31].

To determine the effects of acute LDL exposure on neutrophils, isolated marrow neutrophils from normal cholesterol diet fed mice were similarly incubated with LPS (100ng/mL),

nLDL (100 $\mu\text{g/mL}$), ox-LDL (100 $\mu\text{g/mL}$), or PBS control. Transcription of IL-6, IL-1 β , KC, TNF- α , and MCP-1 was assessed as above. Additional details may be found in the Supplementary Material.

2.7. Apoptosis Detection

Cultured lean mouse neutrophils were exposed to 100 $\mu\text{g/mL}$ nLDL or ox-LDL for 4h, before cytospin preparation and fixation with 4% PFA. Slides were immediately stained for cleaved caspase 3, as described [32] and scored by fluorescent microscopy.

2.8. Statistical Analysis

The relationship between the degree of LDL oxidation and its chemoattractant effect were analyzed by one-way ANOVA with test for trend using Prism 5 software (GraphPad, La Jolla, CA). All other data were analyzed with the Student's or Welch's t-test. Results are reported as mean values with SEM. *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. Obese mice have elevated serum cholesterol levels with evidence of LDL oxidation

To assess the degree of hypercholesterolemia present in a mouse model of obesity, we compared serum levels of total, LDL, and HDL cholesterol from resting obese (db/db) mice (weighing $40.0 \pm 1.6\text{g}$) to those of lean heterozygous mice (weighing $24.5 \pm 0.6\text{g}$, $p < 0.001$). Obese mice had significantly elevated serum total and LDL cholesterol levels (Fig. 1A) compared to lean mice, and the level of oxidized LDL in obese mice was markedly elevated compared to lean mice (Fig. 1B). We next examined a high cholesterol diet (HCD) mouse model of non-obese hypercholesterolemia [27] to determine whether serum cholesterol levels in this model might approximate those seen in obese mice. We found a similar pattern of hypercholesterolemia in the HCD mice compared to the obese mice (Fig. 1A) without evidence of weight gain (HCD, $23.1 \pm 1.2\text{g}$ vs. NCD, $24.4 \pm 1.6\text{g}$). Thus, this model allows examination of the effects of chronic hypercholesterolemia in the absence of obesity and the other confounding features of the metabolic syndrome.

3.2. Diet-induced hypercholesterolemia impairs murine neutrophil chemotaxis response to both KC and fMLP

As we have previously shown that hypercholesterolemia impairs neutrophil chemotaxis response to the CXC chemokine and IL-8 homologue KC [10], we next sought to expand our examination to other neutrophil chemoattractant molecules. To do so, we examined the chemoattractant response to the bacterial peptide and chemoattractant fMLP in neutrophils isolated from the high cholesterol diet model using modified Boyden chambers (Fig. 2). These results confirm our earlier findings of impaired neutrophil chemotaxis to KC (a CXCR2 ligand) in this model [10] and extend them to fMLP (an FPR1 ligand), suggesting that there may be a more widespread defect in chemotaxis in these cells, affecting multiple G-coupled protein receptors on neutrophils.

3.3. Neutrophils from hypercholesterolemic mice exhibit altered calcium flux in response to chemoattractants

To further dissect the witnessed defects in chemotaxis, we next examined calcium flux, the earliest signaling event following either KC or fMLP receptor binding. Neutrophils from HCD mice exhibited consistently blunted flux in response to both of these ligands compared to cells from NCD mice (Fig. 3).

3.4. Hypercholesterolemia does not alter neutrophil CD11b expression but may impair resolution of F-actin polymerization in response to stimuli

As integrin expression and F-actin polymerization are both critical to neutrophil adhesion and movement in chemotaxis, both neutrophil functions were examined in HCD vs. NCD mouse neutrophils. Cell surface-display of CD11b in response to phorbol myristate acetate (PMA, 1 μ M) although elevated in neutrophils from hypercholesterolemic mice failed to reach significance ($p=0.06$) (Fig. 4A). These cells also demonstrated rapid early F-actin polymerization (Fig. 4B), similar to neutrophils from NCD mice, but then appeared to have a markedly slowed depolymerization over time, suggesting initial cell stiffening without the subsequent actin reorganization necessary for locomotion.

3.5. Neutrophil transcription of inflammatory cytokines in response to LPS exposure is diminished in hypercholesterolemic mice

To assess the broader effects of chronic hypercholesterolemia on neutrophil function and response to bacteria-derived ligands, we next examined neutrophil cytokine transcription in response to LPS stimulation. We observed blunted transcriptional response in HCD neutrophils: although NCD neutrophils responded with significant induction of IL-1 β , IL-6, MCP-1, and TNF- α in response to LPS ($p<0.05$), only TNF- α significantly induced following LPS exposure in HCD neutrophils ($p<0.05$) (Supplementary Fig. S1).

3.6. LDL induces neutrophil chemotaxis response and LDL oxidation augments this effect

To determine the effect of acute exposure to increased levels of LDL and its oxidized species we isolated neutrophils from NCD mice and examined the chemoattractant response using modified Boyden chambers. LDL was found to induce significant migration from upper to lower chambers (Fig. 5). This chemoattractant response increased with increasing concentration (data not shown) and level of oxidation of LDL.

3.7. Native LDL but not oxidized LDL induces neutrophil calcium flux

In efforts to initially dissect the mechanisms of neutrophil chemotaxis to ox-LDL, we next examine neutrophil calcium flux response to nLDL and ox-LDL. Paradoxically, we found minimal if any flux to ox-LDL but significant flux to nLDL (Fig. 6), suggesting that ox-LDL induced chemotaxis is via a calcium independent pathway, while the flux induced by nLDL is not accompanied by migration.

3.8. LDL induces neutrophil CD11b expression and F-actin polymerization and LDL oxidation abrogates these effects

Given the paradox between our chemotaxis and calcium flux findings with nLDL and ox-LDL, we examined other processes underlying the chemotaxis response in neutrophils. Stimulation of neutrophils with nLDL led to increased cell surface expression of CD11b (Fig. 7A) and induced F-actin polymerization (Fig. 7B) compared with controls. However, There was no increase in CD11b expression and less F-actin polymerization seen with ox-LDL stimulation compared to nLDL .

3.9. LDL and its oxidized species induce transcription of MCP-1

To assess the effects of acute exposure to nLDL and ox-LDL on inflammatory cytokine gene transcription in neutrophils, we exposed cultured NCD mouse neutrophils to LPS, nLDL, ox-LDL or PBS control for four hours and assessed mRNA transcription by quantitative PCR. As expected, LPS exposure induced significant transcription, yet response to nLDL and oxidized LDL was variable and only reached significance with MCP-1 ($p<0.05$) (Supplementary Fig. S2). Cell death was noted particularly in the cells cultured with ox-LDL.

3.10. Exposure to LDL and particularly its oxidized form induces neutrophil apoptosis

To further investigate our findings of increased spontaneous cell death seen in the 4h neutrophil exposures to nLDL and oxLDL, we examined neutrophil apoptosis after nLDL and ox-LDL exposure (4h) and found ox-LDL induced significant apoptosis, yet nLDL had little effect on this (Fig. 8).

4. Discussion

Using a lean mouse model of diet-induced hypercholesterolemia we were able to produce elevated levels of LDL and its oxidized species similar to those seen in obese mice. We used this model to examine the effects of chronic exposure to hyperlipidemia on neutrophil function and found that neutrophils from mice fed a high cholesterol diet exhibited abnormal function in response to inflammatory stimuli. Our *in vitro* assessments showed diminished or abnormal neutrophil response to acute inflammation following chronic exposure to LDL and its oxidized species. Conversely, we also found that acute exposure to LDL and its oxidized forms appeared to activate many of the same neutrophil functions.

Previous work with dyslipidemic human neutrophils has shown that resting levels of intracellular calcium are higher in these cells, as are levels of membrane-bound protein kinase C, but calcium signaling in response to formyl-Met-Leu-Phe (fMLP) is decreased [33]. Similarly, hypercholesterolemic patients exhibit higher circulating levels of neutrophil elastase and myeloperoxidase, but demonstrate decreased release of these substances in response to stimulation with IL-8 or fMLP compared to normal cholesterol controls [26]. These findings may suggest a baseline increase in neutrophil activation but a blunted response to acute stimuli in the neutrophils of dyslipidemic patients. We have previously demonstrated a similar defect in the hypercholesterolemic mouse model in which neutrophil recruitment to the airspace in response to LPS and *K. pneumoniae* is attenuated. Further investigation showed that this decreased recruitment is associated with decreased neutrophil chemotaxis to the CXC chemokine KC [10]. Interestingly, we have recently described similar defects in both diet-induced and genetic mouse models of obesity, which we also find to be hypercholesterolemic [9].

Our current studies show that neutrophils from hypercholesterolemic mice exhibit impaired chemotaxis in response not only to KC but to the bacterial peptide fMLP, as well. Although we observed decreased chemoattractant-induced calcium flux in neutrophils from hypercholesterolemic mice, suggesting that initial signaling to chemoattractants may be impaired, such effects were subtle. In the case of KC, this is accordance with our previous finding that neutrophil surface CXCR2 display is mildly depressed in mice fed a high cholesterol diet [10]. Yet, this also suggests that hypercholesterolemia-associated defects in neutrophil chemotaxis may involve more widespread alterations in the mechanisms underlying this function.

To preliminarily investigate this possibility, we examined two critical elements of neutrophil chemotaxis, CD11b expression and F-actin polymerization. We find that HCD neutrophil surface CD11b display is normal to elevated in response to stimuli, and that actin polymerization is enhanced/sustained (as suggested by the persistence of actin polymerization over time in mice fed a high cholesterol diet). Such exaggerated CD11b expression and actin polymerization might impair proper chemotaxis, as this process requires dynamic control over these elements. Thus, these findings suggest that hypercholesterolemia-associated defects in chemotaxis may in fact be multifactorial, and reflect elements of tonic neutrophil activation.

Our current findings also demonstrate that neutrophil inflammatory cytokine transcription is abnormal in mice fed a high cholesterol diet. Although inflammatory cytokine transcription was increased in neutrophils from all mice following exposure to LPS, this was blunted in HCD mouse neutrophils. This is consistent with our overall findings of widespread alterations or impairments of the inflammatory response in neutrophils from non-obese hypercholesterolemic mice.

We hypothesized that some, if not all of the effects of a high cholesterol diet on neutrophil function may be the result of chronic exposure to LDL and its oxidized species *in vivo*. In contrast to the diminished inflammatory response we noted following chronic exposure to hypercholesterolemia, we further hypothesized that acute exposure to LDL and oxidized LDL may have stimulatory effects on the neutrophil inflammatory response.

Previous studies of human neutrophils have reported that acute exposure to LDL induces calcium flux and subsequent oxidative burst [23], neutrophil degranulation [34], and chemotaxis [10, 15]. These reports further show these effects to be increased by or dependent upon LDL oxidation. In the present study we find that native LDL induces minimal chemotaxis in murine neutrophils but its oxidized forms yield graded increase in chemoattractant effect proportionate to the degree of LDL oxidation. Counter intuitively, when examining the initial step of G-protein couple receptor (GPCR) signaling in response to both KC (CXCR2) and fMLP (FPR1), we find that, in contrast to what our chemotaxis results would suggest, nLDL induces calcium flux in murine neutrophils, yet ox-LDL does not. This would imply that the chemoattractant effect of ox-LDL may be mediated by a non-GCP receptor, such as one of the toll-like receptors. Similarly, the preferential induction of neutrophil calcium flux, surface CD11b display, and F-actin polymerization by nLDL over ox-LDL in our studies indicates a dichotomy exists in response to these two forms of LDL, and implies that different receptor and or signaling pathways are induced based on the degree of LDL oxidation. These findings may also suggest that the failure of LDL to induce chemotaxis, despite its ability to induce calcium flux, actin polymerization, and CD11b expression, is not related to initial signal transduction and might occur during the dynamic phase of actin disassembly, perhaps, as we suggest in the case of HCD neutrophils, through some form of ‘paralytic hyperresponsiveness’. Thus, the lesser effects of ox-LDL to induce these specific effects may in fact represent functioning mechanisms of chemotaxis.

Though chronic exposure of neutrophils to dyslipidemia appears to result in decreased cytokine transcription in response to inflammatory stimuli, we observed evidence of gene transcription in neutrophils following acute exposure to LDL and oxidized LDL. The combination of neutrophil activation and inflammatory cytokine transcription in response to LDL may suggest that neutrophil signaling response contributes to the systemic inflammation seen in dyslipidemia. Although speculative at this time, these data may further suggest that the impairment of neutrophil cytokine transcription in chronic hypercholesterolemia may represent a form of desensitization as has been shown to occur for example in chronic TLR4 signaling [35].

Finally, we observed that following exposure to oxidized species of LDL there was an increase in neutrophil apoptosis. This was not observed following exposure to native LDL. This may suggest further alterations in neutrophil signaling, in this case leading to cell death, though the mechanisms of apoptosis induction were not elucidated in the current studies.

5. Conclusion

Utilizing a mouse model of diet induced dyslipidemia that produced levels of serum LDL and its oxidized species similar to those seen in obesity, we demonstrated alterations and impairments in neutrophil function that are consistent with multiple prior investigations. Furthermore, we demonstrated that acute exposure to LDL and oxidized LDL result in activation of the inflammatory response in neutrophils which parallels the very functions impaired in chronic hypercholesterolemia, and which over time may result in this impairment due to desensitization from protracted signaling. We have yet to elucidate the receptors and intracellular signaling pathways responsible for the effects, and further investigation is indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Serum levels of LDL cholesterol are similarly elevated in obese mice compared to lean mice on high cholesterol diet

(A) Serum levels of total, LDL and HDL cholesterol in lean and obese db/db mice compared to wild type mice fed a normal or high cholesterol diet. (B) Oxidation of serum LDL in lean and obese db/db mice was assayed by heparin/manganese precipitation followed by TBARS and normalized to LDL content. n = 3-5 mice/group. * p < 0.05, ** p < 0.01 compared to control (lean or normal cholesterol). Cholesterol levels in high cholesterol diet mice are not significantly different from the corresponding levels found in obese mice.

Figure 2. Chronic hypercholesterolemia impairs neutrophil chemotaxis

Chemotaxis of density centrifugation-isolated mature bone marrow neutrophils from lean hypercholesterolemic mice was compared to normal cholesterol controls using a modified Boyden chamber with KC (50ng/ml) and formyl-Met-Leu-Phe (fMLP, 1 μ M). Four separate experiments were performed. ns = not significant, * $p < 0.05$, ** $p < 0.01$. All fMLP and KC conditions were significantly higher than respective control ($p < 0.01$).

Figure 3. Chronic hypercholesterolemia attenuates neutrophil calcium flux in response to chemoattractants

Cellular calcium flux response to fMLP (1 μ M) was determined in mature bone marrow neutrophils isolated from lean hypercholesterolemic mice was compared to normal cholesterol controls using Indo-1AM cytosolic dye-loading and flow cytometry. Three separate experiments were performed on high cholesterol diet mouse isolated neutrophils and normal cholesterol control; representative runs are shown for each source.

Figure 4. Stimulated neutrophils from hypercholesterolemic mice show normal to elevated CD11b expression and F-actin polymerization, and actin polymerization is slow to reverse over time

(A) CD-11b expression by neutrophils from normal vs. high cholesterol diet mice was assessed by flow cytometry following stimulation with phorbol myristate acetate (PMA, 1 μ M) for 30min. Values are expressed as percent of CD-11b expressed in normal cholesterol diet mice. Three separate experiments were performed. (B) F-actin polymerization in neutrophils from normal vs. high cholesterol diet mice was assessed by phalloidin staining and flow cytometry following stimulation with fMLP (1 μ M) for 30s or 5min. Values are expressed as percent of F-actin polymerization at 30 seconds in normal cholesterol diet mice. Four separate experiments were performed. ** $p < 0.01$ lower compared to 30s, ns = not significantly different compared to 30s. NCD = normal cholesterol diet; HCD = high cholesterol diet.

Figure 5. LDL induces neutrophil chemotaxis, which increases with level of LDL oxidation
 Chemotaxis of density centrifugation-isolated mature bone marrow neutrophils from normal cholesterol diet mice was examined using a modified Boyden chamber with native LDL (nLDL), minimally oxidized LDL (mmLDL), moderately oxidized LDL (ox-LDL) (all 100 $\mu\text{g/mL}$), and the chemoattractants KC (50ng/ml) and fMLP (1 μM). Four separate experiments were performed. ** $p < 0.01$ by 1-way ANOVA with post-test for linear trend, *** $p < 0.001$ compared to control.

Figure 6. Native LDL but not oxidized LDL induces neutrophil calcium flux

Cellular calcium flux response to nLDL (100 μ g/mL), ox-LDL (100 μ g/mL), or fMLP (1 μ M) was determined in mature bone marrow neutrophils isolated from normal cholesterol diet mice using Indo-1AM cytosolic dye-loading and flow cytometry. Five separate experiments were performed; representative runs are shown for each stimulus.

Figure 7. Native LDL induces neutrophil CD11b surface display and intracellular F-actin polymerization, and is more effective than ox-LDL in this regard

(A) CD-11b expression by neutrophils from normal cholesterol diet mice was assessed following stimulation with PMA (1 μ M), nLDL (100 μ g/mL), or ox-LDL (100 μ g/mL) for 30min. (B) F-actin polymerization in neutrophils from normal cholesterol diet mice was assessed following stimulation with fMLP (1 μ M), nLDL (100 μ g/mL), or ox-LDL (100 μ g/mL) for 30s. Values are expressed as ratio mean fluorescence intensity normalized to buffer control in each experiment. Four separate experiments were performed for each assay. * $p < 0.05$ lower compared to control or indicated comparison, ** $p < 0.01$, ns = not significant.

Figure 8. Oxidized LDL induces neutrophil apoptosis

Neutrophils from mice fed normal cholesterol diet were treated with nLDL, ox-LDL (both 100µg/mL), or buffer control for 4h in culture, fixed and stained for caspase 3, and scored by fluorescent microscopy. The numbers of apoptotic neutrophils (per 100 counted) on low powered fields were determined on 5 fields and averaged for each experimental condition, and the experiment was repeated 3 times. * p<0.05. ns = not significant.