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Dyslipidemia Induces Opposing Effects on Intrapulmonary and Extrapulmonary Host Defense through Divergent TLR Response Phenotypes

Jennifer H. Madenspacher,* David W. Draper,* Kathleen A. Smoak,† Haitao Li,‡ Gary L. Griffiths,† Benjamin T. Suratt,‡ Martha D. Wilson,§ Lawrence L. Rudel,§ and Michael B. Fessler*

Dyslipidemia influences innate immune responses in the bloodstream, but whether and how pulmonary innate immunity is sensitive to circulating lipoproteins is largely unknown. To define whether dyslipidemia impacts responses to bacteria in the airspace and, if so, whether differentially from its effects in other tissues, airspace, bloodstream, and i.p. responses to LPS and Klebsiella pneumoniae were investigated using murine models of dyslipidemia. Dyslipidemia reduced neutrophil (PMN) recruitment to the airspace in response to LPS and K. pneumoniae by impairing both chemokine induction in the airspace and PMN chemotaxis, thereby compromising pulmonary bacterial clearance. Paradoxically, bacteria were cleared more effectively from the bloodstream during dyslipidemia. This enhanced systemic response was due, at least in part, to basal circulating neutrophilia and basal TLR4/MyD88-dependent serum cytokine induction and enhanced serum cytokine responses to systemically administered TLR ligands. Dyslipidemia did not globally impair PMN transvascular trafficking to, and host defense within all loci, because neutrophilia, cytokine induction, and bacterial clearance were enhanced within the infected peritoneum. Peritoneal macrophages from dyslipemic animals were primed for more robust TLR responses, reflecting increased lipid rafts and increased TLR4 expression, whereas macrophages from the airspace, in which cholesterol was maintained constant during dyslipidemia, had normal responses and rafts. Dyslipidemia thus imparts opposing effects upon intra- and extrapulmonary host defense by inducing tissue-divergent TLR response phenotypes and dysregulating airspace/blood compartmental levels of PMNs and cytokines. We propose that the airspace is a “privileged” site, thereby uniquely sensitive to dyslipidemia. The Journal of Immunology, 2010, 185: 000–000.

Serum lipoproteins, such as low-density lipoprotein (LDL), exert complex effects upon intravascular innate immunity. Lipoproteins neutralize bacterial LPS (1). Oxidation of LDL, which may occur during dyslipidemia and infection (2, 3), also generates lipoprotein species with activity upon TLR4, the LPS receptor. Stimulation of TLR4 by minimally oxidized LDL (oxLDL) (4) is proposed to underlie TLR4-dependent vascular inflammation induced by dyslipidemia (5). In contrast, more completely oxLDL inhibits TLR responses (3, 6), attenuating antimicrobial functions in dendritic cells during dyslipidemia (3). Perhaps reflecting the complexity of cross-talk between lipoproteins and innate immunity, varying effects of dyslipidemia upon host defense have also been reported. Clearance of some pathogens (e.g., Leishmania) is compromised during dyslipidemia (3, 7, 8), whereas for others (e.g., Klebsiella pneumoniae), both enhanced and impaired host defense are reported (9, 10). Most reports have used models of bloodstream infection, and several are complicated by the use of animals deficient in apolipoprotein E, itself a direct regulator of inflammation and LPS (1, 11). Given the high prevalence of dyslipidemia in humans, an improved understanding of the effects of dyslipidemia on host defense at the initial extravascular sites of pathogen encounter may bear important implications.

Pulmonary host defense is critically important as acute lower respiratory tract infections impose the greatest burden of morbidity and mortality of all infections in the United States (12). Serum lipoproteins are taken up by pulmonary endothelium (13) and modify the function of pulmonary cells in vitro (14–16). Moreover, recent studies of ATP binding cassette transporter G1 null mice indicate that cholesterol trafficking and inflammation may be coupled uniquely within the lung (17, 18). Despite this, there have been very few reports of the effect of dyslipidemia on pulmonary innate immunity; indeed, regulation of airspace lipids during dyslipidemia and the effects of dyslipidemia upon lung biology in general are both poorly understood. The lung may, in fact, have unique challenges, given its anatomical positioning immediately downstream of return of lipoprotein-rich first-pass thoracic duct lymph and portal blood and the sensitivity of surfactant function to cholesterol (19). Moreover, as recruitment of neutrophils (PMNs) to the airspace entails several microvascular events distinct from those in other tissues (20), PMN-dependent...
intra-alveolar host defense may have distinct sensitivity to circulating lipoprotein levels.

We hypothesized that dyslipidemia would modify pulmonary innate immunity. In this paper, we report that dyslipidemia attenuates PMN trafficking to the airspace triggered by both LPS and *K. pneumoniae*, compromising pulmonary clearance of the latter. The reduced influx of PMNs stems from deficient induction of air-space chemokines associated with reduced pulmonary NF-κB activation as well as impaired PMN chemotaxis, indicating that dyslipidemia impacts airway responses by modulating both lung-resident and circulating cells. Paradoxically, bacteria are cleared more much more effectively from the bloodstream during dyslipidemia, reflecting basal circulating neutrophilia and activation of systemic innate immunity as well as hypersensitivity to TLR ligands in the serum. Dyslipidemia does not globally impair PMN egress into, and TLR responses within, all peripheral loci, because PMN influx and TLR responses are instead enhanced within the infected peritoneum. Lipid rafts and cell surface TLR4 are increased in peritoneal macrophages during dyslipidemia, whereas rafts are unchanged in alveolar macrophages. Dyslipidemia thus imparts opposing effects upon intra- and extrapulmonary host defense by dysregulating compartmental TLR responses and airspace/blood compartmentalization of PMNs and cytokines. We propose that the airspace may be a “privileged” site, thereby uniquely sensitive to dyslipidemia.

Materials and Methods

**Reagents**

*Escherichia coli* 0111:B4 LPS, penicillin, streptomycin, and methyl-β-cyclodextrin-cholesterol were from Sigma-Aldrich (St. Louis, MO). For the latter, concentrations were based on cyclodextrin formula weight. *K. pneumoniae* 43816 (serotype 2), DMEM, FBS, and RAW 264.7 cells were from American Type Culture Collection (Manassas, VA). Keratinoxy-certified chemokine (KC), MIP-2, and IL-17 were from R&D Systems (Minneapolis, MN).

**Mice and diets**

Female mice (7–14 wk old, weighing 18–22 g) were used in all experiments. C57BL/6 and LDL receptor (*ldlr*) null (backcrossed 10 generations onto C57BL/6) mice were from The Jackson Laboratory (Bar Harbor, ME). Myd88 and Tlr4 null mice backcrossed less than eight generations onto C57BL/6 (21, 22) were provided by S. Akira (Osaka University, Osaka, Japan). All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences. Mice were placed for 2–5 wk on either a high-cholesterol diet (HCD) (15.8% fat, 1.25% cholesterol, and 0.5% cholate [TD.90221; Harlan Laboratories, Indianapolis, IN]) or a normal diet (ND), which was identical, except for omission of cocoa butter, cholesterol, and cholate (TD.95138). In other studies, mice fed TD.95138 were compared with those fed a matching control diet supplemented with 0.5% sodium cholate (TD.98244) or were fed a cholate-free Western diet (TD.88137, 4 wk).

**In vivo exposures**

Exposure to aerosolized LPS (300 μg/ml, 20 min) was described previously (24). KC (0.5 μg in 60 μl), IL-17 (3 μg in 60 μl), and *K. pneumoniae* (1500 or 2000 CFU in 60 μl) were delivered intratracheally (i.t.) by oropharyngeal aspiration during isoflurane anesthesia. In other experiments, *K. pneumoniae* was injected i.v. (7 × 10⁶ CFU) into the retro-orbital venous plexus or i.p. (1 × 10⁶ CFU), or LPS (2 mg/kg) was injected i.p. PBS (1×), pH 7.4), or native LDL (nLDL) or oxLDL (200 μg) was delivered i.v. into the retro-orbital venous plexus 2 h before and 2 h following exposure to aerosolized LPS.

**Bronchoalveolar lavage fluid collection and analysis**

Bronchoalveolar lavage fluid (BALF) was collected immediately following sacrifice, and total leukocyte and differential counts were performed, as described previously (23). Total protein was quantified by the method of Bradford.

**In vitro chemotaxis**

Bone marrow-isolated PMNs (24) (1 × 10⁶, 0.1 ml) were seeded into the upper chamber of a Transwell system (polycarbonate membrane, 3.0 μm pore) (Corning, Corning, NY). Medium (0.4 ml) with or without MIP-2 (5 ng/ml) or KC (25 ng/ml) (PeproTech, Rocky Hill, NJ) was added to the lower chamber. Cells were counted in the lower chamber in triplicate after 1 h (37°C).

**Bactericidal assays**

Lung, spleen, and liver were excised after sacrifice and homogenized in PBS, and serial dilutions were plated on tryptic soy agar for bacterial quantification, as described previously (23). Blood was collected from the inferior vena cava and plated after serial dilution. Intracellular killing capacity of PMNs against i.p.-injected *K. pneumoniae* was quantified as reported previously (25).

**Synthesis of PEG_{10000}-Chol**

To polyethylene glycol (PEG),_0000-bis(amine) (1 g, 0.1 mmol) in dichloromethane (DCM) (75 ml) was added triethylamine (41 μl, 0.3 mmol). Cholesteryl chlororomate (54 mg, 0.12 mmol) dissolved in DCM (5 ml) was then added dropwise and stirred (room temperature, overnight). TLC analysis indicated formation of dihydroxyle and monocholesteryl products. The reaction mixture was diluted with DCM (425 ml), washed with 5% potassium hydrogen sulfate, brine, and 5% sodium bicarbonate, dried over sodium sulfate, and evaporated to dryness. Column chromatography using a gradient of methanol (0–20%) in DCM afforded monocholesteryl PEG (Chol-PEG_{10000}-NH₂) as a white solid (574 mg, 55%), confirmed by MALDI-mass spectroscopy and ¹H NMR. To Chol-PEG_{10000}-NH₂ (135 mg, 0.012 mmol) in 0.1 M sodium bicarbonate (13 ml) was added FITC isomer I, and the reaction mixture was stirred (72 h), after which all Chol-PEG_{10000}-NH₂ was consumed by HPLC analysis. The reaction mixture was concentrated (Millipore Centriprep, YM-3) over eight rounds, after which HPLC analysis of the retentate showed only one peak. This was lyophilized, loaded onto a PD-10 column, and eluted with water. The desired m.w. fractions from the PD-10 column were pooled and lyophilized to give the final product as an orange solid (108 mg, 78%), which was confirmed by HPLC, MALDI-mass spectroscopy, and ¹H NMR analysis.

**PMN and macrophage harvests and culture**

Mature murine bone marrow PMNs were isolated from mouse femurs and tibias by discontinuous Percoll gradient centrifugation, as described previously (24). This preparation yields cell populations that are >95% PMNs (data not shown). Peritoneal exudate macrophages (PEMs) were harvested by peritoneal lavage 96 h after i.p. injection of Brewer’s thioglycollate (2 ml, 4% solution). Alveolar macrophages were harvested by BAL of restitting, unexposed animals (macrophages represent >98% of airspace cell pellet). Both macrophage types were plated in DMEM supplemented with 10% FBS, penicillin, and streptomycin. RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS, 2 mM t-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin under a humidified 5% CO₂ atmosphere at 37°C. For in vitro cell exposures, highly purified *E. coli* 0111:B4 LPS (product number 201; List Biological, Campbell, CA) and endotoxin-free PAM_CSK₄ (InvivoGen, San Diego, CA) were used.

**Western blotting**

Plated macrophages were lysed by 1× Laemmli/20 mM DTT. Protein was resolved by 10% SDS-PAGE, transferred to nitrocellulose (Bio-Rad, Hercules, CA), and probed with primary Abs. Rabbit anti–PO4-P38p38 (1/1000), rabbit anti–PO4-JNK, and rabbit anti-JNK were from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were then washed in Tween 20 and Tris-buffered saline and exposed for 60 min to a 1/5000 dilution of species-specific, HRP-conjugated secondary Ab (GE Healthcare, Piscataway, NJ) in 5% milk/Tween 20 and Tris-buffered saline. Following further washes, the signal was detected with ECL Western Blot Detection Reagents (GE Healthcare), followed by film exposure (GE Healthcare).

**NF-κB activity assay**

Activation of p65 NF-κB in the nuclear fraction of lung homogenates (Nuclear Extract Kit, Active Motif, Carlsbad, CA) was quantified by ELISA (p65 TransAM kit; Active Motif) after normalizing nuclear protein input (Bradford assay).
Cytokine analysis

Cytokines were quantified by multiplex assay (Bio-Plex; Bio-Rad). LPS-induced CXC chemokine (LIX) was quantified by ELISA (ELISA Tech, Aurora, CO). The lower limits of detection were as follows: IL-17 (2.0 pg/ml), MIP-2 (2.0 pg/ml), G-CSF (2.8 pg/ml), TNF-α (3.0 pg/ml), KC (2.7 pg/ml), IL-6 (1.0 pg/ml), and LIX (8.0 pg/ml).

Peripheral blood leukocyte typing and enumeration

The blood samples were analyzed using the HEMAVET 1700 hematology analyzer (Drew Scientific, Waterbury, CT). Manual WBC differential counts were reported, and smear estimates were used to confirm values.

Serum lipoprotein cholesterol quantification

Plasma lipoprotein separation was achieved by size-exclusion chromatography (fast protein liquid chromatography). Cholesterol concentrations were measured by gas chromatography or enzymatically, as described previously (26).

Intracellular bacterial killing assay

A previously reported protocol was followed (25). Mice received 2.5 ml 4% thioglycollate i.p., followed 4 h later by 1 × 10^6 CFU K pneumoniae i.p. Intraperitoneal leukocytes were collected 30 min later by lavage (5 ml HBSS containing 100 µg/ml gentamicin) and then washed. Cells (1 × 10^6) were then incubated (37°C) for varying durations, followed by lysis (0.1% Triton X-100) and CFU quantification by plating of serial dilutions.

Flow cytometry

Anti-CXCR2 (PerCP/Cy5.5), -Gr-1 (FITC), -F4/80 (PE or allophycocyanin), -CD11c (PE-Cy5), and isotype control Abs were from BioLegend (San Diego, CA). Anti-CD14 (PE) and -TLR4 (PE) were from eBioscience (San Diego, CA). Cells were stained and fixed with 2% paraformaldehyde/PBS. For iPEG10000-chol staining, cells were fixed (2% paraformaldehyde), washed, and then exposed to iPEG10000-chol (10 µg/ml, 10 min, room temperature). Flow cytometry was performed using an LSR II (BD Biosciences, San Jose, CA) and analyzed using FlowJo (Tree Star, Ashland, OR) and FCS Express (De Novo Software, Los Angeles, CA) software.

Statistical analysis

Analysis was performed using GraphPad Prism statistical software (San Diego, CA). Data are represented as mean ± SD. Two-tailed Student’s t test was applied for comparisons of two groups and ANOVA for analyses of three or more groups. Survival was evaluated by log-rank test. For all tests, p < 0.05 was considered significant.

Results

Dyslipidemia induces circulating neutrophilia and TLR4/MyD88-dependent serum cytokines

To investigate the effects of dyslipidemia on responses in the airspace, we used a well-established model of HCD-induced dyslipidemia in wild-type (WT) (C57BL/6) mice (27, 28). The effects of dyslipidemia upon airspace/blood compartmental levels of cholesterol, leukocytes, and cytokines in the steady state were first defined. As expected, compared with ND-fed mice, HCD-fed mice developed significantly elevated serum total cholesterol, with increases in the LDL and very LDL fractions and a decrease in high-density lipoprotein cholesterol (Supplemental Fig. 1A). There was no difference in BALF cholesterol between mice fed the two diets (Supplemental Fig. 1B), suggesting that cholesterol levels are regulated by distinct mechanisms in the airspace and serum. By contrast, peritoneal lavage fluid cholesterol was increased in HCD-fed mice (Supplemental Fig. 1C), indicating that extravascular compartments differ in regulation of cholesterol levels during dyslipidemia.

Whereas no significant difference was noted between ND- and HCD-fed mice in steady-state BAL total leukocyte count/differential (data not shown), HCD-fed mice displayed increased circulating PMNs and monocytes (Table I). Dyslipidemia is reported to induce endovascular inflammation through TLR4 and its intracellular adaptor, MyD88 (29, 30). Nonetheless, we found that HCD induction of circulating PMNs and monocytes were both MyD88 independent, because they occurred equivalently in WT and Myd88 null mice (Table I). Whereas the circulating lymphocyte count was unchanged by HCD in WT mice, it was significantly increased by HCD in Myd88 null mice (Table I), suggesting that MyD88-dependent signals may suppress HCD-induced lymphocytosis. HCD did not impact peripheral blood hemoglobin, erythrocyte count, or platelet count (data not shown), suggesting a selective effect upon hematopoietic lineages. IL-17 reportedly regulates granulopoiesis through its target gene G-CSF (31, 32), although we are unaware of any prior report of IL-17 regulation during dyslipidemia. We found that HCD modestly upregulated IL-17 and its target gene MIP-2 in the serum (Fig. 1A, 1B); although, HCD did not upregulate G-CSF or TNF-α (Fig. 1C, 1D). HCD-associated induction of IL-17 and MIP-2 were noted to occur in a MyD88- and TLR4-dependent fashion, as animals deficient in these genes, unlike WT animals, had no HCD induction of IL-17 and blunted induction of MIP-2 (Fig. 1A, 1B). Unlike the serum compartment, there was no induction by HCD of basal BALF levels of MIP-2 (data not shown).

Taken together, these findings indicate that 1) cholesterol is regulated differentially between serum and airspace during dyslipidemia, yielding divergent lipid environments; 2) dyslipidemia impacts steady-state compartmental leukocyte and cytokine levels differentially between airspace and serum; and 3) TLR4/MyD88 regulates dyslipidemia-associated induction of serum cytokines but not expansion of PMNs and monocytes in the bloodstream.

Dyslipidemia attenuates induced trafficking of PMNs to the inflamed airspace

To determine the effect of dyslipidemia upon induced trafficking of leukocytes to the airspace, we next exposed mice to aerosolized LPS, using an established model (23). Despite their relative circulating neutrophilia compared with ND-fed mice, HCD-fed mice had significantly reduced influx of total leukocytes (WBCs) into the BALF following LPS inhalation (Fig. 2A). The reduction in WBCs was accounted for by a reduction in BALF PMNs (Fig. 2A), because BALF macrophage and lymphocyte numbers were differentially regulated in Myd88+/+ and Myd88−/− mice fed ND or HCD

<table>
<thead>
<tr>
<th>Myd88+/+</th>
<th>Myd88−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>wbc</td>
<td>pmns</td>
</tr>
</tbody>
</table>

Table I. Blood leukocyte count and differential in Myd88−/− and Myd88−/− mice fed ND or HCD

<table>
<thead>
<tr>
<th>Cell Count (×10^6/ml)</th>
<th>ND (n = 13)</th>
<th>HCD (n = 13)</th>
<th>ND (n = 10)</th>
<th>HCD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wbc</td>
<td>2.35 ± 0.71</td>
<td>2.68 ± 1.03</td>
<td>1.95 ± 0.46</td>
<td>3.34 ± 0.79*</td>
</tr>
<tr>
<td>pmns</td>
<td>0.24 ± 0.1</td>
<td>0.39 ± 0.24</td>
<td>0.21 ± 0.12</td>
<td>0.45 ± 0.24†</td>
</tr>
<tr>
<td>monocytes</td>
<td>0.09 ± 0.05</td>
<td>0.14 ± 0.06†</td>
<td>0.08 ± 0.03</td>
<td>0.12 ± 0.04†</td>
</tr>
<tr>
<td>lymphocytes</td>
<td>1.93 ± 0.63</td>
<td>2.05 ± 0.7</td>
<td>1.49 ± 0.43</td>
<td>2.67 ± 0.64*</td>
</tr>
<tr>
<td>eosinophils</td>
<td>0.03 ± 0.02</td>
<td>0.05 ± 0.06</td>
<td>0.04 ± 0.03</td>
<td>0.04 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

*p < 0.001; †p < 0.05; ‡p = 0.05 for HCD versus ND comparison of cell type count within genotype.
not different between mice on the two diets (data not shown). The reduction of LPS-induced airspace PMNs was observed following as little as 2 wk of HCD feeding (data not shown). By contrast, there was no difference in LPS-induced accumulation of airspace PMNs between mice on ND and mice on ND supplemented with cholate (Fig. 2B), indicating that the cholate component of HCD, previously reported to regulate proinflammatory genes in the liver via farnesoid X receptor (33), does not itself modulate the pulmonary innate immune response. Moreover, idlr null mice on a (cholate-free) Western diet, an alternate commonly used mouse model of dyslipidemia, also had significantly fewer total leukocytes/PMNs recruited to the airspace after LPS than Western diet-fed WT mice (Fig. 2C). oxLDL, like nLDL, is increased in the serum by HCD and mediates the effects of dyslipidemia upon vascular inflammation and innate immunity (3, 4, 6). Consistent with possible roles for nLDL and oxLDL in our model, naive (chow-fed) WT mice subjected to pharmacologic dyslipidemia by i.v. injection of either oxLDL or nLDL had lesser WBC influx into the airspace after inhaled LPS than did mice treated with i.v. PBS (vehicle) (Fig. 2D). Taken together, these data indicate that dyslipidemia, modeled by several independent approaches, dysregulates systemic versus pulmonary compartmentalization of PMNs during an inflammatory stimulus.

**Dyslipidemia modulates airspace cytokine induction by inhaled LPS**

Circulating PMNs migrate to the inflamed or infected airspace in response to locally expressed chemokines. In pursuit of mechanisms underlying the reduced recruitment of PMNs to the LPS-exposed lung during dyslipidemia, we thus first measured LPS-induced cyto-/chemokine expression in BALF. As PMNs begin to accumulate in the airspace at ∼4–6 h post-LPS in this model (data not shown) (23), we assayed BALF 2 h post-LPS as a temporally relevant time point largely reflecting cytokine production by lung-resident cells. As shown in Fig. 3, BALF levels of MIP-2 and KC, two CXC chemokines that play critical roles in PMN recruitment to the LPS-exposed lung (23), were lower in HCD than in ND mice, whereas expression of the chemokine LIX (CXCL5) was equivalent between diets. TNF-α, a cytokine important in PMN recruitment to lung, was also lower in HCD mice, whereas BALF IL-6, a negative regulator of LPS-induced PMN recruitment to the lung (34), was modestly higher in HCD mice. Serum KC, MIP-2, and IL-6 were equivalent between ND- and HCD-fed mice 2 h post-LPS inhalation (data not shown). Taken together, these findings suggest that airspace cyto-/chemokine expression is differentially modulated during dyslipidemia. Reductions in BALF TNF-α and in airspace-serum chemokine gradients may possibly contribute to the observed reduction in LPS-induced airspace neutrophilia, as proposed in other models (35).

LPS induction of KC and MIP-2 in the lung is driven by the transcription factor NF-κB in lung structural cells (36). Notably, we found that DNA binding of p65 NF-κB in lung parenchymal nuclear isolates 2 h post-LPS was indeed lower in HCD-fed than in ND-fed mice (Fig. 3F), suggesting that dyslipidemia may modulate airspace cytokine production through effects upon NF-κB activation.

**Dyslipidemia impairs PMN migration**

Given the relatively modest reductions in LPS-induced BALF chemokines in dyslipidemic animals, we next investigated whether dyslipidemia also modifies PMN migration itself. To model PMN migration in response to airspace chemokines in vivo, we first instilled KC directly into the airspace (23) of ND- and HCD-fed mice. HCD-fed mice indeed had reduced airspace recruitment of PMNs in response to i.t. KC (Fig. 4A). To more directly examine effects of dyslipidemia on PMN migratory function, we next isolated...
mature PMNs from the bone marrow of ND- and HCD-fed mice and quantified their chemotaxis in vitro. Consistent with the in vivo model, PMNs isolated from HCD mice had impaired chemotaxis to KC as well as MIP-2 (Fig. 4B). Cell surface expression of CXCR2 was decreased in circulating PMNs from HCD-fed mice as compared with ND-fed mice (Fig. 4C). By contrast, CD18 cell surface expression was equivalent in PMNs between the two diets (data not shown). Thus, in addition to reduction of chemokine expression by lung-resident cells, the dyslipidemic environment also confers a chemotactic defect upon circulating PMNs that may reflect, at least in part, downregulated cell surface expression of chemokine receptors.

**Dyslipidemia attenuates airspace neutrophilia and cytokines in response to K. pneumoniae**

Given the observed deficits in PMN migration and in recruitment of PMNs to the LPS-exposed lung, we predicted that PMN migration to the lung in response to intact Gram-negative bacteria would also be deficient during dyslipidemia. Indeed, compared with ND-fed mice, HCD-fed mice had fewer PMNs in the airspace following i.t. inoculation with K. pneumoniae (Fig. 5A). As with LPS, in response to K. pneumoniae, HCD-fed animals had markedly reduced BALF MIP-2 (Fig. 5B), a chemokine of established importance to PMN recruitment to the K. pneumoniae-infected lung (37). There was, however, no significant alteration in BALF IL-6 (Fig. 5C), LIX (Fig. 5D), or KC (data not shown) and a ~2-fold increase in G-CSF in HCD-fed mice (Fig. 5E).

IL-17 plays a critical role in secondary induction of PMN-attracting chemokines and cytokines including MIP-2, LIX, and G-CSF in the K. pneumoniae-infected lung (38). Given the varying effects of HCD upon MIP-2, LIX, KC, and G-CSF expression in the lung, we questioned whether dyslipidemic animals might have an underlying change in pulmonary IL-17 expression or activity. HCD-fed mice had significantly increased BALF IL-17 24 h after i.t. K. pneumoniae (Fig. 5F). BALF concentrations of MIP-2, LIX, KC, and G-CSF were equivalent between ND- and HCD-fed animals 2 h following i.t. delivery of rIL-17 (data not shown), suggesting that IL-17 responsiveness is not modified in lung-resident cells during dyslipidemia. Collectively, these findings suggest that reduced PMN recruitment to the airspace in response to i.t. K. pneumoniae during dyslipidemia reflects both impaired PMN chemotaxis and reduced airspace expression of chemokines and that the latter occurs at least in part through an IL-17-independent mechanism.

**Dyslipidemia exerts opposing effects on intra- and extrapulmonary antibacterial host defense**

Airspace-recruited PMNs play a critical role in the clearance of pulmonary infection with extracellular pathogens, such as K. pneumoniae. Thus, we predicted that intrapulmonary host defense against K. pneumoniae would be impaired in HCD-fed animals. Indeed, compared with ND-fed animals, HCD-fed animals had significantly increased K. pneumoniae CFUs in lung homogenates following i.t. inoculation (Fig. 6A), indicating impaired intrapulmonary host defense. As IL-17 induction in the infected lung parallels K. pneumoniae burden (38), increased microbial load may account for the increased BALF IL-17 in HCD-fed mice and, conversely, highlights the significance of the BALF MIP-2 deficit in these animals (Fig. 5B).

Dissemination of bacteria from the infected lung to the bloodstream and from there to peripheral organs generally tracks in parallel with intrapulmonary bacterial burden (23). Nonetheless, we found that HCD-fed animals had markedly reduced K. pneumoniae CFUs in spleen and liver following i.t. infection (Fig. 6B, 6C). This suggested the two possibilities that either: 1) the integrity of the alveolar-capillary barrier was enhanced during dyslipidemia, thus reducing...
Dyslipidemia primes for more robust extrapulmonary TLR responses

We next explored mechanisms in addition to neutrophilia and cytokine induction (Fig. 1, Table I) that might underlie the enhanced systemic host defense response during dyslipidemia. Consistent with dyslipidemia priming for a more robust systemic response to infection, HCD-fed mice had markedly enhanced (~20-fold greater than ND-fed mice) serum TNF-α and IL-1β (~3-fold greater) but not MIP-2 following i.p. LPS (Fig. 7A). Suggesting a contribution from dyslipidemia-primed extrapulmonary macrophages to this enhanced systemic response, cultured PEMs from HCD-fed mice also had enhanced TNF-α induction (Fig. 7B) and enhanced activation of p38, JNK, and NF-κB (Fig. 7C) following in vitro treatment with either LPS or the TLR2 ligand PAM3CSK4. By contrast, alveolar macrophages harvested from unexposed ND- and HCD-fed mice, although not strictly analogous to elicited peritoneal macrophages nonetheless informative of the airway macrophage response, produced equivalent amounts of TNF-α protein in response to LPS ex vivo (Fig. 7D).

Cholesterol loading of the macrophage plasma membrane in vitro enhances cellular responses to TLR2 and TLR4 ligands through expansion of lipid raft microdomains (39, 40). However, it is unknown whether a similar phenomenon occurs to macrophages in vivo during hypercholesterolemia. To investigate this, we synthesized an analog (Supplemental Fig. 3) of a compound that has previously been used as a raft probe, iPEG-chol (41–43). iPEG-chol is a cholesterol derivative that intercalates into the exofacial leaflet of the plasma membrane in raft microdomains in parallel to membrane cholesterol content (41, 43). F4/80+ peritoneal exudate cells harvested from HCD-fed animals bearing increased peritoneal fluid cholesterol (Supplemental Fig. 1) indeed had greater iPEG-chol signal by flow cytometry than their counterparts from ND-fed animals (Fig. 7E, Supplemental Fig. 4), consistent with increased lipid rafts. In contrast, alveolar macrophages from HCD- and ND-fed animals, harvested from equivalent BALF cholesterol environments (Supplemental Fig. 1B), had equivalent rafts as assessed by iPEG-chol staining (Fig. 7F). F4/80+ peritoneal...
FIGURE 7. Dyslipidemia primes for more robust extrapulmonary TLR responses. A, Serum cytokines were quantified in ND- and HCD-fed mice 2 h following i.p. injection of 2 mg/kg E. coli LPS (n = 6/diet). *p < 0.05; **p < 0.001. B, TNF-α release into media by PEMs harvested from ND- and HCD-fed mice was quantified by ELISA 2 h after ex vivo treatment with LPS (10 ng/ml) or PAM3CSK4 (PAM, 10 ng/ml). Data are representative of four independent experiments. *p < 0.01. C, Cultured PEMs harvested from ND- or HCD-fed mice were exposed as shown, following which whole-cell lysates were immunoblotted for the indicated targets. Findings are representative of three independent experiments. Hatched line demarcates juxtaposition of different sections of the same gel. D, Alveolar macrophages (~3 × 10^5, >98% purity) collected from unchallenged ND- and HCD-fed mice were plated in DMEM/10% FBS and exposed to 10 ng/ml E. coli 0111:B4 LPS. TNF-α protein release into the media 16 h postexposure was quantified by ELISA (n = 8/diet). E, Lipid rafts were quantified by flow cytometry in F4/80^+ peritoneal exudate cells from ND- and HCD-fed mice after in vitro treatment with fPEG-chol, a fluorescent-labeled raft probe (representative of two experiments involving n = 7–9 animals/diet). *p < 0.001. F, Lipid rafts were quantified by flow cytometry of fPEG-chol in CD11c^+ alveolar macrophages (n = 5/diet). G, Cell surface CD14 was quantified by flow cytometry in F4/80^+ peritoneal exudate cells (n = 4–5/diet). H, Cell surface TLR4 was quantified by flow cytometry in F4/80^+ peritoneal exudate cells (left panel), and percentage of F4/80^+ cells expressing TLR4 was determined (right panel) (n = 4–5/diet). *p < 0.01. MFI, mean fluorescence intensity.

FIGURE 8. PMN recruitment to and host defense responses within the peritoneal cavity are enhanced during dyslipidemia. A and B, PMNs were quantified in peritoneal lavage fluid 4 h after i.p. injection of 2 mg/kg LPS (n = 5/diet). A and 1 h after i.p. injection of 1 × 10^8 CFU K. pneumoniae (n = 9–10/diet, B). *p < 0.05. C–F, TNF-α protein was quantified by ELISA in peritoneal lavage fluid 2 or 2.5 h after i.p. injection of LPS (C) or K. pneumoniae (E), respectively; parallel measurements were made of peritoneal fluid MIP-2 (D, F) (n = 4–10/diet/inj). *p < 0.05; **p = 0.008. G and H, Bacterial CFUs were quantified in peritoneal lavage fluid (G) and in splenic homogenate (H) 2.5 h after i.p. injection of 1 × 10^8 CFU K. pneumoniae (n = 12–19/diet over three independent experiments). *p < 0.05.

Dyslipidemia enhances PMN influx into and host defense within the peritoneum

To address whether dyslipidemia globally impairs PMN transvascular migration into all peripheral loci or, alternatively, whether it exerts effects more specific to PMN accumulation in the airspace, two models of neutrophilic peritonitis were next investigated. Contrary to responses in the lung, and despite the HCD-associated PMN chemotactic defect, i.p. LPS (Fig. 8A) and i.p. K. pneumoniae (Fig. 8B) both induced greater i.p. PMN accumulation in HCD- than ND-fed mice. HCD-fed mice also had increased peritoneal lavage fluid levels of TNF-α and MIP-2 following both i.p. LPS and K. pneumoniae (Fig. 8C–F). Also contrary to the lung, HCD-fed mice cleared i.p.-injected K. pneumoniae more effectively from their peritoneum than their ND-fed counterparts (Fig. 8G); in parallel, HCD mice had lesser dissemination of peritoneal K. pneumoniae to the spleen (Fig. 8H). To determine whether dyslipidemia modulates intrinsic PMN bactericidal capacity in vivo, we pre-elicited PMNs into the peritoneum with thioglycollate, exposed the elicited PMNs in vivo to K. pneumoniae by i.p. injection, and then harvested the PMNs for further ex vivo incubation, as described previously (25). Quantification of intracellular K. pneumoniae CFUs revealed similar time-dependent killing curves between PMNs from ND- and HCD-fed mice (Supplemental Fig. 6), indicating no diet-induced modification of intracellular bactericidal function.

Discussion

Serum lipoproteins influence innate immunity, but whether and how innate immune responses in the airspace are sensitive to dyslipidemia...
has, to a large extent, not been described. In this paper, we report that dyslipidemia induces widely contrasting effects upon innate immunity and host defense within versus outside of the lung. Airspace fluid, as evaluated in the form of BALF, maintains cholesterol levels under conditions inducing a doubling of serum cholesterol. This suggests that the airspace may be a privileged compartment during dyslipidemia and confirms that intra-alveolar and circulating immune cells are exposed to divergent environments during dyslipidemia. Dyslipidemia primes systemic innate immune responses by 1) inducing basal serum cytokines through TLR4/MyD88; 2) enhancing LPS induction of serum cytokines in vivo and macrophage cytokines ex vivo; and 3) expanding circulating PMNs and monocytes; together, 4) enhancing bloodstream killing of bacteria. In contrast, dyslipidemia attenuates intrapulmonary innate immunity in the form of reduced: 1) LPS-stimulated airspace cytokines; 2) migration of PMNs into the airspace in response to intra-alveolar LPS, KC, and bacteria; and 3) bacterial killing within the lung. Enhanced bloodstream killing and deficient pulmonary killing of *K. pneumoniae* are unified by the dysregulation of blood/airspace PMN compartmentalization that occurs during dyslipidemia. This, in turn, appears to be due, at least in part, to a PMN migration defect. Of interest, dyslipidemic animals with pulmonary bacterial overgrowth nevertheless clear bacteria that have egressed from lung to blood and peripheral sites more effectively than their ND-fed counterparts. Opposite to the airspace, PMN migration into the peritoneum in response to both LPS and *K. pneumoniae*, and i.p. clearance of the latter, is enhanced in the dyslipidemic state. Taken together, these findings indicate that, despite impairing chemotaxis, dyslipidemia does not impose a global defect upon transvascular migration of the PMN into all inflammatory loci. Rather, dyslipidemia selectively impairs host defense in the airspace.

Our findings that HCD modulates LPS-induced BALF cytokines and lung parenchymal NF-κB suggest that lung-resident cells are indeed sensitive to dyslipidemia and divergently so compared with extrapulmonary LPS-responsive cells (Figs. 3, 7, 8). Cholesterol-loaded macrophages have been detected in lung tissue of hypercholesterolemic rabbits, although the interstitial versus alveolar localization of these cells is uncertain (44). Airspace fluid appears to be relatively sheltered from changes in circulating cholesterol, as the lipid environments of the serum and of the alveolus, to which alveolar macrophages and alveolar epithelium are exposed, diverge during dyslipidemia (Supplemental Fig. 1). Complicating the matter, dyslipidemic serum contains an array of mediators with conflicting activities upon innate immunity. Serum-lung compartmentalization of these mediators has not been determined; in addition, it is not known whether alveolocapillary disruption compromises mediators’ compartmentalization during lung injury. We recently reported that pharmacologic activation of liver X receptor (LXR), a cell sensor of oxysterols expressed in both alveolar macrophages and alveolar epithelium, reduces LPS induction of TNF-α and MIP-2 in BALF and airspace recruitment of PMNs in mice (23). As dyslipidemia and oxLDL stimulate LXR, it is possible that the inhibitory effects of dyslipidemia on airspace cytokine expression in the current study may reflect an LXR-dependent counterregulatory response in alveolar cells.

Given the complex milieu during dyslipidemia, it is unlikely that a single mediator is sufficient for the HCD phenotype. Elevated LDL is unlikely by itself to explain our findings. Although *ldlr* null mice and nLDL-infused WT mice have a similar pulmonary LDL phenotype to that of HCD-fed mice (Fig. 2), neutralization of LPS by excess serum LDL in *ldlr* null mice is thought to account for their reported dampened induction of serum cytokines following i.p. LPS (10). Also unlike the HCD phenotype, *ldlr* null mice are reported to have normal, not enhanced, clearance of i.v. *K. pneumoniae* from blood, liver, and spleen (10). Interestingly, contrary to the in vivo *ldlr* null i.p. LPS phenotype, *ldlr* null murine macrophages (10) and mutant *ldlr* monocytes from familial hypercholesterolemia patients (45), like HCD peritoneal macrophages in the current study, are hyperresponsive to LPS in vitro. This suggests their intrinsic modification in vivo by a mediator not requiring interaction with the LDL receptor. OxLDL is induced in serum by HCD (3) and taken up by scavenger receptors, but several papers and our own findings (data not shown) indicate that oxLDL inhibits LPS induction of cytokines in macrophages (6). Thus, although circulating oxLDL also recapitulates some features of our model, reducing PMN influx into the LPS-exposed airspace (Fig. 2D), it does not appear sufficient to mediate the full phenotype.

Cellular cholesterol loading per se also exerts complex effects upon innate immune function. Increasing plasmalemmal-free cholesterol through either genetic manipulation or in vitro cholesterol loading reportedly enhances TLR2 and TLR4 activation through expanding lipid raft mass (39, 40). We provide the first paper, to our knowledge, that macrophage lipid rafts are increased in vivo during hypercholesterolemia and that there is an associated increase in cell surface TLR4. These findings raise the interesting possibility that cellular raft cholesterol mass, and with it, TLR4 expression, may also vary with lipid status in human subjects and act as a determinant of cellular inflammatory phenotype. Although we found significant differences between alveolar and peritoneal elicited macrophages from the HCD condition, caution is warranted in drawing direct comparisons between these two substantially different cell types. Thus, future studies are required to more carefully characterize the phenotype of different macrophage populations during the dyslipidemic state.

In our study, unchallenged HCD-fed mice displayed evidence of basal systemic inflammation in the form of circulating neutrophilia and TLR4-/MyD88-dependent induction of serum MIP-2 and IL-17, although it should be noted that the serum cytokine induction was modest and of uncertain biological impact. Of interest, in humans, dyslipidemia, sometimes in the context of obesity and/or “metabolic syndrome” is also associated with peripheral leukocytosis and increased serum cytokines (46). Obesity was not a factor in the phenotype in our study, because HCD-fed animals had modestly lower body weight than ND-fed animals did (data not shown). We are unaware of any investigations of alveolar inflammation in dyslipidemic humans, although we recently reported an inverse relationship between serum cholesterol and asthma, an inflammatory airways disease, in a national survey (47). Thus, the findings of the current study may well have relevance to human disease.

Other mouse models may provide insight into the pathogenesis in our dyslipidemia model. Interestingly, circulating neutrophilia, increased plasma IL-17, and impaired PMN transvascular migration are hallmarks of murine models of adhesion molecule deficiency (i.e., CD18, E/P-selectin knockouts) (31, 32). Indeed, granulopoiesis and trafficking of PMNs to tissues are homeostatically linked insofar as successful PMN trafficking to tissues including the lung curbs their induction of IL-17/G-CSF (32). Contrary to the deficit in PMN migration to the peritoneum reported in adhesion molecule-deficient models, we found that HCD feeding was associated with enhanced inducible migration of PMNs into the peritoneum. We also found PMN surface expression of CD18 to be unchanged by HCD (data not shown).

We speculate that the basal systemic TLR4-/MyD88-dependent inflammation induced by dyslipidemia may contribute causally to the deficit in inducible PMN transvascular migration into the airspace. Reminiscent of our findings, PMN recruitment into the airspace in response to i.t. LPS is attenuated during endotoxemia because of impaired PMN chemotaxis to MIP-2 (48). Systemic administration of bacterial lipoprotein, a MyD88-dependent
stimulus, also impairs PMN migration to peripheral inflammatory loci through downregulation of chemokine receptors including CXCRR2 (49). Long-term exposure to circulating CXC chemokines, a feature of our HCD model (Fig. 1), was also previously reported in the context of genetic overexpression to impair PMN transvascular emigration through CXCRR2 desensitization (50). Taken together, these studies suggest that dyslipidemia-induced systemic inflammation has the potential to produce several features of the PMN compartmentalization phenotype in the current study through effects upon PMN migration. Despite many features of PMN transvascular emigration that are unique to the lung (e.g., transcapillary instead of transvenular transit, absence of rolling) (20), we are unaware of other reported models in which PMN migration into airspace and peritoneum are oppositely regulated. One important feature of dyslipidemia that may contribute to this lung-selective phenotype is the parallel, discordant effect of dyslipidemia upon local intra-alveolar versus i.p. induction of cytokines that act to attract the circulating PMN.

The lung has not been widely conceived of as an organ sensitive to systemic dyslipidemia. We report that dyslipidemia regulates innate immunity and host defense discordantly between lung and blood/peritoneum and connect this to dysregulated compartmental levels of PMNs and cytokines, as well as to a tissue-divergent LPS response phenotype that occurs during dyslipidemia. The “unique” relationship between the airspace and serum lipids likely arises from unique features of lung biology, including the requirement for maintenance of alveolar surfactant lipid, and the unique and incompletely understood cellular/molecular features of the transcapillary passage of PMNs from blood to interstitium to alveolus.

For maintenance of alveolar surfactant lipid, and the unique and incompletely understood cellular/molecular features of PMN transvascular emigration that are unique to the lung (e.g., transcapillary instead of transvenular transit, absence of rolling) (20), we are unaware of other reported models in which PMN migration into airspace and peritoneum are oppositely regulated. One important feature of dyslipidemia that may contribute to this lung-selective phenotype is the parallel, discordant effect of dyslipidemia upon local intra-alveolar versus i.p. induction of cytokines that act to attract the circulating PMN.

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Disclosures

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References


Supplementary Figure Legends

Figure S1. Effect of high-cholesterol diet on serum and airspace cholesterol levels.
(A) C57BL/6 mice were fed ND or HCD (5 wks), following which serum lipoprotein cholesterol (A), BALF total cholesterol (B), and peritoneal lavage fluid cholesterol (C) were quantified, as described in the Methods. TC, total cholesterol; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein (n=8/diet for A and B; *, P<0.0001; n=20/diet for C; †, P=0.01).

Figure S2. Effect of dyslipidemia upon airspace protein levels. (A) BALF protein was quantified by Bradford assay 24 hrs following i.t. delivery of 2000 CFUs of K. pneumoniae (A), or before and 8 and 24 hours following exposure to aerosolized LPS (B) in ND- and HCD-fed C57BL/6 mice (n=10-13/diet)(*, P<0.05; **, P<0.001).

Figure S3. Chemical structure of fPEG10000-chol.

Figure S4. Flow cytometry of peritoneal and alveolar macrophages. (A) Peritoneal exudate cells from ND- and HCD-fed mice treated with fPEG-chol ex vivo were quantified for fluorescein signal and for F4/80 (PE); alveolar lavage cells were similarly treated and quantified for CD11c (PE-Cy5). (B) Peritoneal exudate cells were quantified for CD14 (PE) and F4/80 (PE). (C) Peritoneal exudate cells were exposed to isotype control antibody (shaded peak) or to anti-TLR4 (PE), and F4/80 (APC)+ cells gated for TLR4-positivity as shown.
Figure S5. *In vitro* cholesterol loading alters macrophage membrane and LPS response. A) RAW 264.7 macrophages were treated with buffer or 5 mM methyl-β-cyclodextrin-cholesterol complex (CD-cholesterol)(30 min), stained with fPEG-chol, and then assayed by flow cytometry. Data are representative of 3 independent experiments. B) RAW 264.7 macrophages were treated with buffer or 5 mM CD-cholesterol (30 min), washed, and exposed to buffer or LPS (5 ng/ml, 2 hrs). Media was then assayed for TNFα by ELISA. Data are representative of 3 independent experiments performed in triplicate (*, P<.001 compared to - / buffer; †, P<.001 compared to - / LPS).

Figure S6. Effect of dyslipidemia upon PMN intracellular killing of *K. pneumoniae*. Mice received i.p. thioglycollate, followed by 1x10⁸ CFUs of *K. pneumoniae* i.p. 4 hrs later, and then underwent peritoneal cell harvest with gentamicin-containing buffer 30 min afterward. Peritoneal cells were then washed, incubated (37°C) ex vivo for the durations shown, and then lysed for quantification of intracellular CFUs (n=9-11/diet/timepoint).
Figure S1

A

Serum Cholesterol

mg/dl

TC LDL VLDL HDL

ND HCD

B

BALF Cholesterol

mg/dl

ND HCD

C

Peritoneal lavage fluid cholesterol

µg/ml

ND HCD
Figure S2

A

**BALF Protein**

μg/mL

ND  HCD

B

**BALF Protein**

μg/mL

**ND**  **HCD**

0  8  24  Hrs

** Significant difference
Figure S3
Figure S4

A

B

C

CD11c

F4/80

CD14

F4/80

TLR4

F4/80

Isotype anti-TLR4

anti-TLR4

anti-TLR4

Peritoneal Exudate Cells

Alveolar Cells

ND HCD

ND HCD

ND HCD

ND HCD
Figure S5

A figure showing a histogram with the x-axis labeled as 'PEG-chol' and the y-axis labeled as 'Count'. The histogram includes two lines labeled 'Buffer' and 'CD-cholesterol'.

B figure showing a bar graph with the x-axis labeled as 'CD-cholesterol' and the y-axis labeled as 'TNFα (ng/ml)'. The graph compares 'Buffer' and 'LPS' conditions with data points at '+'.
Figure S6

CFU (X 10^3/ml)

0 30 60
Incubation ex vivo (min)

ND
HCD