Supplement Methods

Materials

[1,2 $^3$H]-cholesterol (Perkin-Elmer Analytical Sciences, Boston, MA), LPS (Sigma Aldrich, St. Louis, MO), Macrophage Colony Stimulating Factor (M-CSF) (Peprotech, Rocky Hill, NJ), RPMI 1640 (GIBCO, Invitrogen, Eugene, OR), RNAlater (Ambion, Austin, TX) and fetal bovine serum (FBS) (HyClone, Logan, UT) were obtained commercially. J774 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, Ca). ACAT inhibitor was donated from Pfizer Pharmaceuticals, Groton, CT. Wild-type female C57BL/6 mice (8-12 weeks old) were purchased from Jackson Laboratories (Bar Harbour, Maine). Human monocytes were obtained from the Human Immunology Core (HIC) of the Penn Center for AIDS Research (CFAR). All other reagents purchased from Sigma Aldrich (St Louis, MO). Human endotoxin studies were performed at Clinical and Translational Research Center (CTRC) at the University of Pennsylvania.

Methods

J774 macrophage preparation for RCT studies

For murine RCT studies and ex vivo efflux studies, J774 macrophages were labeled and loaded identically - 48h in labeling media containing acetylated LDL (25µg/mL) and $^3$H-cholesterol (5µCi/mL). Under these experimental conditions (25µg/ml acLDL loaded for 48h time), we have found that loaded J774 macrophages have foam-cell like
characteristics (total cholesterol of $87.1 \pm 4.5 \mu g/mg$ protein; $47.9 \pm 5.1$ % cholesterol ester, analysis of cells from n=4 studies) and that J774 macrophages express abundant ABCA1 protein (Supplement figure 2a) and support robust efflux to the major ABCA1 acceptor apoA-I (Supplement figure 2b), consistent with work by Favari et al\textsuperscript{1}.

**J774 macrophage preparation for human HDL *ex vivo* efflux studies**

For human *ex vivo* efflux studies, the established J774 macrophage model (non acLDL loaded, ± cAMP (0.3mM), + ACAT inhibitor (2µg/ml)\textsuperscript{2,3}) maximizes ABCA1 expression and provides a ready pool of free cholesterol (ACAT inhibition) for short term efflux studies which are not confounded by variation in macrophage cholesterol esterification and hydrolysis. In this model, efflux values from non-cAMP treated cells are subtracted from efflux to cAMP treated cells to measure ABCA1-dependent efflux.

**In vivo RCT Studies**

*Rationale for choice of LPS dose and route of administration*

Overall, we chose doses and routes of LPS administration based on published literature (mouse and human)\textsuperscript{4-10}, pilot studies (mouse) and experimental needs (mouse) in order to elicit a reproducible, non-lethal, non-toxic acute inflammatory response. In rodents, we wished to employ a sub-acute model that induced a slower rate of endotoxin release into the blood-stream and therefore eliminated the intravenous (IV) route of administration (ROA). Because we reserve the intraperitoneal (IP) cavity for labeled macrophage administration, we chose the subcutaneous (SQ) route. Given the paucity of published data\textsuperscript{11 4-7}, we performed pilot studies comparing inflammatory and lipid responses across
different LPS administrations, IV LPS (0.1 and 0.3 mg/kg) vs. SQ LPS (3 and 10 mg/kg). All doses produced robust increases in plasma TNFα levels (Supplement figure 3A). Notably, peak inflammatory responses with 3mg/kg SQ were comparable to a much lower IV dose (0.1 mg/kg). FPLC profiles of plasma at 48h revealed a marked induction in LDL-C peak with all LPS doses and ROA with little change in HDL-C mass peak (Supplement figure 3B). We chose 3 mg/kg SQ LPS for our initial studies because this produced a robust, non-toxic (no increase in liver enzymes), acute systemic inflammatory response accompanied by changes in plasma lipoproteins (Supplement figure 1A-F). We chose a lower dose LPS (0.3 mg/kg SQ) in rodents in order to assess the effect of modest hepatic inflammation (~2 fold elevation in plasma levels of hepatic derived SAA) on RCT (especially on the final liver to bile steps) in the setting of low grade systemic inflammation (<3 fold increase in plasma cytokines).

Parenthetically, LPS at 3mg/kg SQ induced ~20 fold peak-increase in plasma cytokines and ~112 fold increase in plasma SAA (Figure 2B) levels in mice, which is of the same order of magnitude as the increases in plasma cytokines (~50 fold) and SAA (~39 fold) (Figure 5) observed with LPS 3ng/kg IV in humans\textsuperscript{12}. In contrast, low dose LPS (0.3 mg/kg SQ) in rodents induced ~2-fold increase in plasma cytokines (Figure 4) and ~2 fold increase in plasma SAA.

**Sample Processing for rodent in vivo RCT studies:** Blood, collected via the retro-orbital plexus from anesthetized mice, was centrifuged for plasma supernatant. At 48h mice were sacrificed by cervical dislocation, liver and gall bladder isolated; liver portions (50mg) snap-frozen for lipid and RNA extraction. Tissue lipids were extracted according
to Bligh and Dyer procedure and $^3$H-cholesterol levels in plasma, bile, and extracted lipids measured by liquid scintillation counting (LSC). Feces (0-48h) were collected and soaked overnight in ddH$_2$O (100mg feces/1mL), ethanol added, samples homogenized and free cholesterol isolated by hexane extraction. The aqueous phase was adjusted to pH $\leq$ 1 for bile acid extraction with ethyl acetate. $^3$H-label counts in plasma, liver and feces are expressed as a percentage of total $^3$H-cholesterol injected.

**General Laboratory Methods**

**Lipoprotein Analysis and Enzyme Linked Immunosorbent Assays (ELISA):** Pooled plasma from mice (150µL, N=6) was separated by Fast Protein Liquid Chromatography (FPLC) (Amersham Pharmacia Biotech). Plasma and FPLC fraction total cholesterol levels were measured enzymatically (Wako Pure Chemical Industries). Counts in FPLC fractions (cpm/100µl) were measured by LSC. In humans, following ultracentrifugation, plasma lipoproteins, apolipoproteins, phospholipids and hsCRP were measured enzymatically (Wako Diagnostics, Richmond, VA) on a Hitachi 912 analyzer (Roche Diagnostics, Basel, Switzerland). Levels of murine TNF$\alpha$ and IL6 (R&D systems, Minneapolis, MN) and SAA (Life Diagnostics, West Chester, PA) in plasma and FPLC fractions and human plasma SAA (BioSource, Invitrogen, Eugene, OR) were measured by ELISA.

**RNA isolation and quantitative Real-time PCR analysis:** Liver tissue was homogenized in Trizol using a tissue lyser (Qiagen), RNA (500ng) isolated, and reverse transcribed using an Applied Biosystems High Capacity cDNA archive kit. Levels of mRNAs, using
Applied Biosystems primers, probes and 7300 sequence detector, were assessed by quantitative Real-Time PCR and normalized to β-actin levels.

**Immunoblot analysis:** FPLC fractions were reduced, separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with apoA-I, apoE and apoB primary antibodies (BIODESIGN International, Meridian Life Science, Maine). Blots were visualized by chemiluminescence (ECL plus kit, GE Healthcare). Liver tissue (50mg) was homogenized in PBS (+protease inhibitors) and centrifuged at low speed (2000xg for 10min). Liver membrane proteins were isolated from supernatant by high-spin ultracentrifugation (56,806xg for 1h at 4°C). The remaining pellet was resuspended in lysis buffer, quantitated by bicinchoninic acid (BCA) assay and equal quantities (10µg/lane) separated by SDS-PAGE electrophoresis prior to transferring to nitrocellulose membranes. Membranes were blocked and probed with ABCA1, ABCG1, SR-BI, ABCG8 and β-actin antibodies (Novus Biologicals, Littleton, CO).
Supplement Tables

Supplement Table 1. Fold change in hepatic mRNA expression of genes at 48h post LPS (3mg/kg, SQ) as assessed by Real-Time PCR (n=6 mice, data presented as mean ± SEM).

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Saline</th>
<th>LPS (3mg/kg)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>1.06 ± 0.17</td>
<td>0.41 ± 0.06</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>ApoB</td>
<td>1.04 ± 0.15</td>
<td>0.65 ± 0.04</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>ApoE</td>
<td>1.08 ± 0.22</td>
<td>0.41 ± 0.06</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>LDLR</td>
<td>1.01 ± 0.07</td>
<td>0.47 ± 0.04</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>LXR</td>
<td>1.01 ± 0.07</td>
<td>0.78 ± 0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>LCAT</td>
<td>1.01 ± 0.09</td>
<td>0.69 ± 0.02</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>HL</td>
<td>1.05 ± 0.17</td>
<td>0.45 ± 0.08</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>PLTP</td>
<td>1.07 ± 0.18</td>
<td>0.38 ± 0.03</td>
<td>P&lt;0.01</td>
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<td>PON1</td>
<td>1.06 ± 0.15</td>
<td>0.62 ± 0.04</td>
<td>P&lt;0.05</td>
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</table>

* For comparison of Real-Time data between LPS and saline groups unpaired t-tests were performed.
Supplement Table 2. Endotoxin Reduces HDL Cholesterol Acceptor Function in vivo

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>4 Hours</th>
<th>24 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n=3)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>LPS (3mg/kg) (n=3)</td>
<td>0.61 ± 0.14</td>
<td>0.78 ± 0.13</td>
<td>0.85 ± 0.16</td>
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<tr>
<td><strong>P value</strong></td>
<td>P&lt;0.05</td>
<td>P=0.17</td>
<td>P=0.41</td>
</tr>
</tbody>
</table>

*Data representative of 3 independent studies (n=6 mice per treatment group per study).

Plasma from control or LPS treated mice was pooled in each individual study and separated by FPLC. Data presented as mean ± SEM.
Supplement Figures

Supplement Figure 1: Subcutaneous endotoxin (3mg/kg SQ) induces a robust systemic inflammatory response in vivo

Increased plasma protein levels of (A) TNFα and (B) IL6 during endotoxemia (3mg/kg SQ). (C) Induction of hepatic IL6 and TNFα mRNA following LPS. (D) Plasma levels of the liver enzyme, alanine aminotransferase (ALT) were measured enzymatically (Wako Diagnostics, Richmond, VA) on a Hitachi 912 analyzer (Roche Diagnostics, Basel, Switzerland). There was no evidence for increase in ALT at any time point post-LPS (n=3 mice per group). (E) Total cholesterol levels in mice following endotoxin (n=6). (F) FPLC cholesterol profiles at 48h. A marked increase in the LDL-cholesterol peak was observed with no change in HDL-cholesterol mass peak. An increase in cholesterol in the “shoulder” region between LDL and HDL peaks (Fractions 22-28) was also observed. Statistical significance is presented as *p<0.05, **p<0.01 and ***p<0.001 in all figures.

Supplement Figure 2: AcLDL-loaded J774 macrophages express abundant ABCA1 and support robust efflux to ApoA-I

(A) J774 macrophages were treated ± acLDL (20µg/ml) ± cAMP (0.3mM) for 24h prior to harvesting in RIPA buffer. Immunoblot analysis revealed an increase in ABCA1 protein levels with both acLDL (20µg/ml) and cAMP (0.3mM) treatment. (B) J774 macrophages were loaded and labeled for 24h (RPMI, 50µg/ml gentamicin, 1µCi/ml ³H-cholesterol and 50µg/ml acLDL) prior to equilibration for a further 24h (RPMI, 50µg/ml gentamicin and 0.2 % BSA). Cells were washed and efflux to apoA-I (20µg/ml), HDL3...
(25µg/ml) or media alone (MEM) was monitored over a 4h efflux period. Loaded J774 macrophages support robust efflux to ApoA-I and HDL3 compared with efflux to MEM (n=4, *p<0.05, **p<0.01 and ***p<0.001).

**Supplement Figure 3: Pilot studies of route and dose of LPS administration**

(A) The effect of different doses of LPS administered SQ and IV on inflammatory markers and lipoprotein changes were compared. All doses increased plasma TNFα levels; however peak effects of 3mg/kg LPS SQ were similar to a much lower IV dose (0.1 mg/kg) (n=4, *p<0.05, **p<0.01 and ***p<0.001). (B) FPLC profiles of plasma at 48h revealed a marked induction in LDL-C peak with all LPS doses and little change in HDL-C mass peak.
References


Supplement Figure 1

A

**TNFα (pg/ml)**

Saline | LPS
---|---
1h | 8h

Time post LPS

B

**IL6 (pg/ml)**

Saline | LPS
---|---
1h | 8h

Time post LPS

C

**Hepatic cytokine (Fold change)**

Saline | 6h | 48h
---|---|---
IL6 | TNFα

Time post LPS

D

**ALT (U/L)**

Saline | 6h | 24h | 48h
---|---|---|---

Time post LPS (3mg/kg, SQ)

E

**Total cholesterol (mg/dL)**

Saline | LPS
---|---
Pre-LPS | 6h | 24h | 48h

Time post LPS

F

**Cholesterol (mg/dL)**

Saline | LPS
---|---

Fraction number
Supplement Figure 2

A

<table>
<thead>
<tr>
<th>acLDL (20µg/ml)</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
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<tbody>
<tr>
<td>cAMP (0.3mM)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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B

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>% Efflux/4h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>0.5</td>
</tr>
<tr>
<td>ApoAI</td>
<td>1.5</td>
</tr>
<tr>
<td>HDL3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

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**
Supplement Figure 3

A

![Bar chart showing TNFα levels in different treatment groups.](chart_TNFalpha)

B

![Line chart showing cholesterol levels across fraction numbers.](chart_cholesterol)