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Novel mechanism of regulation of the 5-lipoxygenase/leukotriene B4 pathway by high-density lipoprotein in macrophages

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High-density lipoprotein (HDL) interacts with various cells, particularly macrophages, in functional cell-HDL interactions. Here, we found that HDL protein quality and lipid quality play critical roles in HDL functions. HDL fractions from healthy volunteers (HDLHealthy) and patients with recurrent coronary atherosclerotic disease (HDL CAD) were prepared. To analyse functional HDL-macrophage interactions, macrophages were co-incubated with each HDL, and lipid mediator production was assessed by liquid chromatography/mass spectrometry-based metabololipidomics. HDLHealthy treatment attenuated the pro-inflammatory lipid mediator production, particularly that of leukotriene (LT) B4, and this treatment enhanced lipoxin (LX) B4 and resolvin (Rv) E2 production. HDLHealthy treatment enhanced the proteasome-mediated degradation of the LT B4-producing enzyme 5-lipoxygenase (LO) in activated macrophages; however, HDL CAD did not show these anti-inflammatory effects. HDLHealthy was engulfed by macrophages via clathrin-mediated endocytosis, which was a critical step in 5-LO/LT B4 regulation. We also found that HDL CAD showed higher levels of the LT B4-producing enzymes and thus promoted LT B4 production from HDL CAD. In addition, LT B4 attenuated HDL endocytosis, HDL-mediated 5-LO degradation in macrophages, and HDL-derived augmentation of macrophage phagocytosis. These results indicated that local LT B4 produced de novo from HDL CAD regulates HDL-macrophage functional interactions and plays critical roles in dysfunctional, inflammatory HDL characteristics.

High-density lipoprotein (HDL) has diverse anti-atherosclerotic functions, such as reversing cholesterol transport1 and inhibiting inflammation1,2. Many population studies have shown that the concentration of HDL cholesterol (HDL-C) is inversely related to the risk of coronary atherosclerotic disease (CAD)3-5. However, recent unexpected results with inhibitors of cholesteryl ester transfer protein have indicated that pharmacological increases in HDL-C are not necessarily beneficial6,7 and that more attention should be focused on HDL function.

The protein quality and lipid quality of HDL play critical roles in HDL function. Recent studies have demonstrated that myeloperoxidase (MPO), a leukocyte-derived haem protein, binds to HDL. MPO generates hypochlorous acid, which oxidizes specific tyrosine and methionine residues on apoA-I and impairs apoA-I-mediated cholesterol efflux8,9. In contrast, paraoxonase 1 (PON1), an HDL-associated lipo-lactonase10, is linked to the antioxidative, anti-inflammatory, and lipid cargo-carrying functions of HDL11-13. We have recently shown that the serum MPO/PON1 ratio may indicate dysfunctional HDL and is useful for risk stratification of CAD patients14. Lipid quality, particularly the imbalance between omega-3 and omega-6 fatty acids, may be a risk factor for atherosclerosis15. We have reported that eicosapentaenoic acid (EPA)-rich HDL increases cholesterol efflux capacity and PON1 activity, thus indicating that the lipid quality of HDL may regulate its functions16.

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Chronic inflammation contributes to the development of advanced atherosclerosis. The resolution of inflammation is mediated by a phagocytic process by macrophages, known as programmed cell removal or efferocytosis. M2-differentiated macrophages contribute to the resolution of inflammation by producing pro-resolving lipid mediators (LMs) and by producing lower levels of inflammatory LMs, such as LTB₄ and prostaglandins (PGs), than those produced by M1 macrophages. Although HDL has functional interactions with macrophages during reverse cholesterol transport, little is known about HDL-macrophage interactions with regard to macrophage-dependent LM production. LMs are produced predominantly from polyunsaturated fatty acids, such as arachidonic acid (AA), EPA, and docosahexaenoic acid (DHA), and play crucial roles in the initiation and resolution of inflammatory responses. The balance between pro-inflammatory and pro-resolving mediators regulates the duration of the inflammatory response by promoting neutrophil apoptosis and macrophage efferocytosis.

Here, we prepared HDL fractions from healthy volunteers (HDL Healthy) and recurrent coronary atherosclerotic disease patients (HDL CAD). We found that HDL Healthy, through endocytic engulfment into activated macrophages, showed anti-inflammatory effects, thereby limiting pro-inflammatory LTB₄ production and enhancing anti-inflammatory, pro-resolving LXB₄ and RvE2 production, as well as enhancing macrophage phagocytosis.

Moreover, HDL CAD released de novo local LTB₄, which blocked endocytic engulfment of HDL by macrophages and did not show anti-inflammatory effects. These results provide a novel mechanism for understanding how HDL Healthy tempers pro-inflammatory responses in HDL-macrophage functional interactions and how HDL CAD becomes dysfunctional or displays pro-inflammatory characteristics.

**Results**

HDL Healthy, but not HDL CAD, decreases LTB₄ production from macrophages via proteasome-mediated degradation of 5-LO.

To investigate the functional interactions between HDL and macrophages, we prepared HDL from healthy volunteers (N = 4, defined as HDL Healthy) and recurrent CAD subjects (N = 4, defined as HDL CAD) by ultracentrifugation (Supplemental Data Table 1). Macrophages (RAW 264.7 cell line, 1 × 10⁶ cells) were incubated with HDL Healthy or HDL CAD for 4 hours. The lipid mediator profiles of macrophages incubated with HDL Healthy or HDL CAD were measured using mass spectrometry. The results are shown in Table 1.

<table>
<thead>
<tr>
<th>AA Bioactive Metabolome</th>
<th>Macrophages with HDL Healthy (pg/1 × 10⁶ cells)</th>
<th>Macrophages with HDL CAD (pg/1 × 10⁶ cells)</th>
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<tr>
<td>Lipoxin A₄</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Lipoxin B₄</td>
<td>5.4 ± 0.4*</td>
<td>—</td>
</tr>
<tr>
<td>Prostaglandin D₂</td>
<td>1843.4 ± 2512.2</td>
<td>3525.7 ± 9233.8*</td>
</tr>
<tr>
<td>Prostaglandin E₂</td>
<td>2180.5 ± 652.6</td>
<td>3205.3 ± 887.5</td>
</tr>
<tr>
<td>Prostaglandin F₂₅</td>
<td>3004.3 ± 995.0</td>
<td>7887.6 ± 1096.6*</td>
</tr>
<tr>
<td>Thromboxane B₂</td>
<td>199.0 ± 44.8</td>
<td>1480.6 ± 150.0*</td>
</tr>
<tr>
<td>Leukotriene B₄</td>
<td>1.6 ± 0.3*</td>
<td>4.5 ± 2.7*</td>
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| AA pathway markers               |                                               |                                            |
|----------------------------------|                                               |                                            |
| 5 HETE                           | 10.2 ± 3.2                                    | 21.6 ± 3.3*                               |
| 12 HETE                          | 18.9 ± 6.0                                    | 11.1 ± 5.9                                 |
| 15 HETE                          | 57.6 ± 16.5                                   | 38.1 ± 9.5                                 |

| EPA Bioactive Metabolome         |                                               |                                            |
|----------------------------------|                                               |                                            |
| Resolvin E₁                      | —                                              | —                                          |
| Resolvin E₂                      | 7.0 ± 3.5*                                    | 1.9 ± 0.4                                  |
| Resolvin E₃                      | —                                              | —                                          |

| EPA pathway markers              |                                               |                                            |
|----------------------------------|                                               |                                            |
| 5 HEPE                           | 5.8 ± 1.8                                     | 13.4 ± 5.6                                 |
| 12 HEPE                          | 3.1 ± 1.1                                     | 2.5 ± 2.0                                  |
| 15 HEPE                          | 4.0 ± 1.6                                     | 4.5 ± 2.9                                  |
| 18 HEPE                          | 5.5 ± 2.0                                     | 10.0 ± 4.1                                 |

| DHA Bioactive Metabolome         |                                               |                                            |
|----------------------------------|                                               |                                            |
| Resolvin D₁                      | 1.0 ± 0.3                                     | 6.0 ± 1.8*                                 |
| Resolvin D₂                      | 0.6 ± 0.4*                                    | —                                          |
| Resolvin D₃                      | 0.5 ± 0.2*                                    | —                                          |
| Resolvin D₅                      | 2.3 ± 1.1                                     | 0.5 ± 0.4                                  |
| Maresin 1                        | —                                              | —                                          |
| Protectin D₁                     | 3.4 ± 1.5                                     | 3.0 ± 2.8                                  |

| DHA pathway markers              |                                               |                                            |
|----------------------------------|                                               |                                            |
| 4 HDHA                           | 6.0 ± 2.8                                     | 2.9 ± 2.3                                  |
| 7 HDHA                           | 4.0 ± 1.8                                     | 1.6 ± 1.2                                  |
| 14 HDHA                          | 2.9 ± 1.2                                     | 2.0 ± 1.5                                  |
| 17 HDHA                          | 2.8 ± 0.7                                     | 2.1 ± 1.3                                  |

Table 1. Lipid mediator profiles of macrophages incubated with HDL Healthy or HDL CAD. — = below detection limits. Values were represented as mean ± SEM. *P < 0.05 between two groups.
Figure 1. HDL<sub>healthy</sub> but not HDL<sub>CAD</sub>-decreased LTB<sub>4</sub> production from macrophages through proteasome-mediated degradation of 5-LO. Macrophages (RAW 264.7 cell line, 1 × 10<sup>6</sup>) were activated by ZyA (30 min at 37 °C), then treated with HDL<sub>healthy</sub> or HDL<sub>CAD</sub> (10 μg protein). (a) LTB<sub>4</sub> production from macrophages was quantified by LC/MS/MS. The results are expressed as pg/1 × 10<sup>6</sup> cells, mean ± SEM, N = 4-5 in each group. (b) Representative MRM-chromatograph and MS-MS spectrum are presented for the identification of LTB<sub>4</sub>. (c) Macrophage lysates were processed for western blot analysis of 5-LO. The results are shown as fold changes from N = 4 experiments. (d) 5-LO degradation by HDL<sub>healthy</sub> is proteasome-dependent. ZyA-activated macrophages were pretreated for 30 min with the proteasome inhibitor MG 132 (1 μM) or vehicle, then incubated with HDL<sub>healthy</sub> for 30 min at 37 °C. Lysates were collected for western blot analysis of 5-LO. The results are shown as the mean ± SEM from N = 4 experiments. *P < 0.05, **P < 0.01, ***P < 0.005. (e) HDL<sub>healthy</sub> enhanced ubiquitination of 5-LO in macrophages. ZyA-activated macrophages were pretreated for 30 min with the proteasome inhibitor MG 132 (1 μM) or vehicle, then incubated with HDL<sub>healthy</sub> or HDL<sub>CAD</sub> for 30 min at 37 °C.
were activated by zymosan (ZyA) (0.1 mg, 30 min at 37 °C), then incubated with HDL Healthy or HDL CAD (10 μg protein, 30 min at 37 °C). After incubation, total RNA was extracted, and then, 12/15-LO expression was analysed by real-time PCR. The results are shown as fold change compared with the vehicle group; mean ± SEM, N = 4. *P < 0.05 vs the other groups.

HDL Healthy particle engulfment and localization in macrophages through clathrin-mediated endocytosis. We found that HDL Healthy and HDL CAD have a distinct effect on LM production by macrophages. To investigate the details of HDL-macrophage interactions, we tracked HDL within macrophages, because specific cells other than macrophages have recently been reported to endocytose HDL holoparticles24–26. Macrophages were incubated with Dil-labelled HDL for 30 min at 37 °C, and this was followed by nuclear staining with DAPI. Here, we detected the engulfment of HDL Healthy into macrophages; however, low uptake of HDL CAD was observed (Fig. 2a). To confirm the uptake of human-derived HDL into macrophages, we investigated human apoA-I protein expression in the murine macrophage cell line RAW 264.7. After co-incubation with human-derived HDL, macrophages were extensively washed with phosphate-buffered saline, and then the cell lysates were analysed by western blot using an antibody specific to human apoA-I. Negligible human apoA-I protein expression was observed in murine macrophages (Fig. 2b, 1st lane). Co-incubation with human apoA-I protein (10 μg) showed human apoA-I expression in murine macrophages (Fig. 2b, 2nd lane). Macrophages co-incubated with HDL Healthy (10 μg) showed substantial human apoA-I expression (Fig. 2b, 3rd lane); however, co-incubation with HDL CAD (10 μg) did not show detectable human apoA-I protein expression in murine macrophages (Fig. 2b, 4th lane). We also confirmed that the apoA-I protein expression levels were similar between HDL Healthy and HDL CAD (Supplemental Fig. 4).

To address the mechanism underlying macrophage engulfment of HDL Healthy, we next used the clathrin-mediated endocytosis inhibitor Pitstop 227. Macrophages were incubated with Dil-labelled HDL with or without pretreatment with 20 μM Pitstop 2 for 30 min. HDL Healthy engulfment into macrophages was confirmed by confocal microscopy (Fig. 2c, left panel), and Pitstop 2 treatment was found to completely abolish HDL uptake (Fig. 2c, right panel). In the same experiment, macrophage cell lysates were obtained and subjected to western blot analysis of apoA-I and 5-LO (Fig. 2d). Pitstop 2 treatment attenuated human apoA-I protein expression in murine macrophages incubated with HDL Healthy (Fig. 2d, upper panel). Additionally, Pitstop 2 treatment augmented 5-LO protein expression within macrophages (Fig. 2d, lower panel). Furthermore, HDL Healthy particle engulfment was competitively blocked by excess unlabelled HDL (Fig. 2e) or apoA-I (Fig. 2f).
Figure 2. HDL\textsubscript{Healthy} but not HDL\textsubscript{CAD} was engulfed by macrophages through clathrin-mediated endocytosis. (a) Macrophages were incubated with DiI-labelled HDL (shown as red) for 30 min at 37 °C, then subjected to nuclear staining with DAPI (shown as blue). The arrows indicate HDL engulfment into macrophages (Scale bar = 10 μm). (b) Activated macrophages (1 x 10\(^6\)) were co-incubated with human apoA-I (10 μg) or each HDL (10 μg) for 30 min at 37 °C. After extensive washing with PBS\(^{-/-}\), macrophages were harvested. Cell lysates were processed for western blot analysis of human apoA-I. The image is representative of results from N = 3 experiments. (c) Macrophages were incubated with DiI-labelled HDL (10 μg) with or without 30 min pretreatment with the clathrin-mediated endocytosis inhibitor Pitstop 2 (20 μM). The arrowheads indicate HDL engulfment into macrophages (Scale bar = 10 μm). (d) Activated macrophages (1 x 10\(^6\)) were treated with HDL\textsubscript{Healthy} (10 μg) for 30 min at 37 °C with or without pretreatment with 20 μM Pitstop 2. After extensive washing, cell lysates were processed for western blot analysis of apoA-I and 5-LO. The image is representative of results from N = 3 experiments. (e) HDL\textsubscript{Healthy} engulfment was competitively blocked by unlabelled HDL.
Macrophages were incubated with Dil-labelled HDL (shown as red) alone or in the presence of 40-fold excess of unlabelled HDL for 30 min at 37 °C, and this was followed by DAPI staining (shown as blue). The Dil-positive area in the macrophages was analysed by ImageJ as μm²/cell. N = 6. (f) Macrophages were incubated with Dil-labelled HDLHealthy (shown as red) in the presence or absence of a 40-fold excess of unlabelled apoA-I for 30 min at 37 °C, (Scale bar = 10 μm), and the Dil-positive area in macrophages was analysed by ImageJ as μm²/cell. The data are shown as the mean ± SEM of 3 independent experiments. **P < 0.01.

These findings indicated that engulfment of HDLHealthy into macrophages is regulated by clathrin-mediated endocytosis, presumably through a specific receptor that binds to apoA-I and that HDL endocytosis regulates HDL-mediated degradation of 5-LO in macrophages. HDL CAD was not endocytosed into macrophages and thus did not regulate 5-LO expression in macrophages. To explore the localization of macrophage-engulfed HDL, we performed immunohistochemical analysis of macrophages with anti-human apoA-I and anti-EEA1 (early endosomal-antigen-1) antibodies (Supplemental Fig. 5a) or anti-human apoA-I and anti-LAMP1 (lysosomal marker) antibodies (Supplemental Fig. 5b). ApoA-I and EEA1 did not co-localize; however, after 120 min of incubation with HDL, we observed partial co-localization of apoA-I and LAMP1 (Supplemental Fig. 5b), thus indicating that the engulfed HDL was localized in lysosomes at this time point.

**De novo** LTB₄ production from HDL CAD. Local bioactive LMs play critical roles in controlling various macrophage functions⁵⁸. After observing that HDLHealthy and HDL CAD had distinct effects on HDL-macrophage interactions, we examined the differences in the bioactive LMs released from each HDL. Each HDL type (10 μg protein) was incubated with a mixture of 1 μM each of deuterium-labelled AA, EPA, and DHA as substrates for 2 h at 37 °C, and then, deuterium-labelled LM production was analysed by LC/MS/MS. The deuterium-labelling enabled us to quantitatively analyse LM production from extra-HDL substrates. The profiles of LM production by HDLHealthy and HDL CAD during the 2-h incubation are shown in Table S2. We found that HDL CAD produced significantly higher levels of PGD₂ and LTB₄, as well as 5-LO-related pathway markers, including 5-HETE, 5-HEPE, 4-HDHA, and 7-HDHA, than those produced by HDLHealthy.

We focused on de novo LTB₄ synthesis from each HDL (Fig. 3a) and found distinct differences in LTB₄ production between HDLHealthy and HDL CAD. As shown in Fig. 3b, LTB₄ synthesis from AA requires sequential enzymatic conversion driven by 5-LO and LTA₄ hydrolase. Next, we investigated whether HDL CAD contains these critical enzymes for LTB₄ production. Western blot analysis of HDLHealthy and HDL CAD revealed that HDL CAD showed an approximately 55-fold increase in the 5-LO protein levels and an approximately 90-fold increase in LTA₄ hydrolase protein levels (Fig. 3c-e). Additionally, HDL CAD, but not HDLHealthy contained FLAP (Supplemental Fig. 6). These results indicated that HDL CAD carries the neutrophil-like, functional enzymatic machinery that produces pro-inflammatory LTB₄ from extra-HDL AA.

We hypothesized that exosome components might be transferred to HDL in circulating plasma. To provide evidence for this concept, PlasmaHealthy and Plasma CAD were immunoprecipitated with anti-CD9 antibody to collect exosomes using an ExoTrap Exosome Isolation Spin Column Kit, and samples were then immunoblotted with anti-CD9 and anti-ApoA-I antibodies. As shown in Supplemental Fig. 7, ExoTrap successfully isolated plasma-derived exosomes from each type of plasma (upper panel), and we found that these exosomes also possessed ApoA-I, thus indicating the presence of HDL particles. In this experiment, we did not use separation by ultracentrifugation; therefore, this result suggests that HDL might acquire exosome-derived components during systemic circulation.

**LTB₄ attenuates HDL Healthy particle engulfment into macrophages and HDL-mediated 5-LO degradation.** Given that HDL CAD locally releases de novo LTB₄, we investigated the effect of HDL-derived LTB₄ on the functional interactions between HDL and macrophages. Macrophages were incubated with 1-100 nM LTB₄ for 30 min, then incubated with HDLHealthy for 30 min at 37 °C. Macrophages in specific experiments were preincubated with the LTB₄ receptor antagonist U75302 (300 nM) for 30 min at 37 °C. We also confirmed BLT1 mRNA expression in RAW macrophages (Supplemental Fig. 8.) Here, we found that 1-100 nM LTB₄ significantly suppressed HDL engulfment into macrophages (Fig. 4a,b), whereas U75302 treatment rescued HDL engulfment (Fig. 4a,b, 5th lane). LTB₄-mediated suppression of macrophage engulfment appeared to be specific for clathrin-mediated endocytosis. LTB₄ selectively attenuated transferrin engulfment into macrophages (Supplemental Fig. 9, upper panels), which was endocytosed via clathrin-mediated machinery⁶⁰,⁶³; however, LTB₄ had no effect on engulfment of dextran (Supplemental Fig. 9, lower panels), which is internalized via macropinocytosis⁶²,⁶³. Notably, LTB₄ treatment did not affect the expression of ApoA-I receptors in macrophages, including ABCA1, ABCG1, SR-B1, and SR-A (Supplemental Fig. 10). Next, we investigated whether pretreatment of LTB₄ might reverse HDL-initiated 5-LO degradation in macrophages. We pretreated macrophages with LTB₄ (1-100 nM) or vehicle for 30 min, then incubated them with HDLHealthy for 30 min at 37 °C. Cell lysates were processed for western blot analysis of 5-LO (Fig. 4c). We confirmed HDL-mediated reduction of 5-LO (Fig. 4c, 2nd lane). Additionally, 1-100 nM LTB₄ reversed this HDL-mediated decrease in 5-LO in a dose-dependent manner, resulting in augmented expression of 5-LO in macrophages (Fig. 4c, 3rd-5th lanes). Pretreatment with U75302 (300 nM) inhibited the effects of 100 nM LTB₄, thus resulting in lower 5-LO expression in macrophages by HDL-mediated degradation (Fig. 4c, 6th lane).

**LTB₄ receptor antagonist promoted HDL CAD engulfment into macrophages.** After showing that HDL CAD locally produced LTB₄ de novo and that local LTB₄ suppressed HDL engulfment into macrophages in a dose-dependent manner, we investigated the effect of the LTB₄ receptor antagonist on HDL CAD-macrophage interactions. Macrophages were pretreated with the BLT1 antagonist U75302 (300 nM), or left untreated, before...
incubation with Dil-labelled HDL Healthy and HDL CAD. Pretreatment with U75302 had no effect on HDL Healthy engulfment into macrophages (Fig. 5a, upper panels and Fig. 6b, 1st-2nd lanes); however, HDL CAD engulfment was significantly increased by U75302 pretreatment, and the particle engulfment was similar to that of HDL Healthy (Fig. 5a, lower panels and Fig. 6b, 3rd-4th lanes).

HDL Healthy enhances macrophage phagocytosis. Because macrophage phagocytosis plays critical roles in the anti-atherogenic machinery, we evaluated whether each HDL type might have distinct effects on macrophage phagocytosis. Pretreatment with HDL Healthy significantly enhanced macrophage phagocytosis, as compared with treatment with the vehicle (Fig. 6). Notably, pretreatment with HDL CAD and pretreatment with HDL Healthy + LTB4 (100 nM) resulted in significant decreases in phagocytosis, and treatment with either BLT1 antagonist LY293111 or U75302 rescued macrophage phagocytosis. These results indicated that functional interactions between HDL Healthy and macrophages contribute to enhanced macrophage efferocytosis, and local low-dose LTB4 may suppress these anti-atherogenic functions.

Proposed HDL functions: regulation of LTB4 from activated macrophages via HDL holoparticle endocytosis. To address the functional interactions between HDL and macrophages, we propose novel HDL-initiated regulation of macrophages via HDL holoparticle endocytosis. HDL Healthy was engulfed by macrophages via clathrin-mediated endocytosis and 5-LO expression attenuated by ubiquitin proteasome degradation, thus resulting in lower LTB4 production from activated macrophages (Fig. 7a). HDL CAD also enhanced LXB4 and RvE2 production, as well as phagocytosis in macrophages. In contrast, HDL CAD carries neutrophil-like enzymatic machinery, which produced higher levels of local de novo LTB4. This enzymatic machinery may be transferred to HDL particle from neutrophil-derived exosomes. Locally produced de novo LTB4 interfered with HDL engulfment into macrophages (Fig. 7b). Here, 5-LO escaped from HDL-mediated degradation, thus resulting in continuous LTB4 production from activated macrophages.
Figure 4. LTB4 attenuated HDL engulfment and HDL-mediated 5-LO degradation. (a) Macrophages were pretreated for 30 min at 37 °C with the BLT1 inhibitor U75302 (300 nM) or vehicle, and then incubated with LTB4 (100 nM) for 30 min at 37 °C and subsequently with Dil-labelled HDLHealthy (shown as red) for 30 min at 37 °C. (b) The Dil-positive area in macrophages (μm²/cell) was quantified by ImageJ (Scale bar = 10 μm). The data are shown as the mean ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005. (c) Macrophages were pretreated for 30 min at 37 °C with U75302 (300 nM) or vehicle and were then incubated with LTB4 (1–100 nM) for 30 min at 37 °C and subsequently with 10 μg of HDLHealthy for 30 min at 37 °C. Lysates were collected for western blot analysis of 5-LO. The data are shown as the mean ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01.
Discussion
In the present study, we demonstrated that HDL\textsubscript{Healthy} contributes to anti-inflammatory and pro-resolving functions during HDL-macrophage interactions through HDL holoparticle endocytosis. We also found that HDL\textsubscript{CAD} releases de novo pro-inflammatory LM LTB\textsubscript{4}, which locally interferes with the anti-inflammatory function of HDL by suppressing HDL particle engulfment into macrophages.

Only hepatocytes and endothelial cells have previously been reported to engulf HDL holoparticles\textsuperscript{24-26}. Here, we reported human HDL engulfment into macrophages by tracking Dil-stained HDL (Fig. 2a) and human apoA-I protein expression within murine macrophages (Fig. 2b). After the addition of the clathrin inhibitor Pitstop 2, HDL engulfment was nearly completely abrogated (Fig. 2c), thus indicating that HDL engulfment is driven by clathrin mediated endocytosis. We observed that excess non-labelled HDL or apoA-I competitively decreased Dil-labelled HDL engulfment (Fig. 2f), thus suggesting the presence of an HDL-specific, apoA-I-dependent receptor for HDL engulfment. We next investigated the intracellular localization of HDL

**Figure 5.** LTB\textsubscript{4} receptor antagonist promoted HDL\textsubscript{CAD} engulfment into macrophages. (a) Macrophages were pretreated for 30 min at 37 °C with or without LTB\textsubscript{4} receptor (BLT1) antagonist, U75302 (300 nM) and then were incubated with Dil-labelled HDL\textsubscript{Healthy} (upper panels) or Dil-labelled HDL\textsubscript{CAD} (lower panels) for 30 min at 37 °C. Scale bars = 10 μm. (b) The Dil-positive area in macrophages (μm\textsuperscript{2}/cell) was quantified by ImageJ. The data are shown as the mean ± SEM of 3 independent experiments. *P < 0.05.

**Figure 6.** HDL\textsubscript{Healthy} enhanced macrophage phagocytosis. Macrophages (0.5 × 10\textsuperscript{5}) were pretreated with each HDL (10 μg) for 1 h or 2 h at 37 °C and then incubated with fluorescent-labelled zymosan for 30 min at 37 °C. In specific experiments, LTB4 (100 nM) or BLT1 antagonist LY293111 (300 nM) or U75302 (300 nM) were used as pretreatment. The results are percentage increases of phagocytosis compared with that after vehicle treatment and are shown as the mean ± SEM, N = 4. †P < 0.05 compared to vehicle. *P < 0.05, **P < 0.01, versus HDL\textsubscript{Healthy}. 

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particles after engulfment into macrophages; however, we did not observe co-localization of HDL with early endosomes at 10-120 min after co-incubation (Supplemental Fig. 5a). HDL showed partial co-localization with lysosomes after 120 min co-incubation (Supplemental Fig. 5b). Further studies are required to identify the receptor for HDL engulfment and HDL trafficking within macrophages.

HDL engulfment into macrophages attenuated 5-LO expression by proteasome degradation (Fig. 1c,d), thus decreasing LTB₄ production from activated macrophages (Fig. 1a,b). It remains unclear how HDL engulfment regulates proteasome-mediated degradation of 5-LO. Notably, engulfment of HDLHealthy promoted anti-inflammatory, pro-resolving LXB₄ and RvE2 release from macrophages (Table 1), and this release orchestrates resolution programs20,21, including enhanced phagocytosis of macrophages, as shown in Fig. 6. HDLCAD did not enhance macrophage phagocytosis but instead resulted in decreased phagocytic functions. Recent studies have indicated that advanced atherosclerotic lesions are characterized by the pathological accumulation of diseased vascular cells and apoptotic cellular debris and that the removal of these cells and cellular debris appears to be significantly impaired in diseased blood vessels34,35. HDL CAD may contribute to impaired macrophage phagocytosis and pathogenesis of atherosclerosis.

HDL carries several enzymes involved in lipid metabolism, such as lecithin-cholesterol acyltransferase and lipoprotein-associated phospholipase A₂ (also known as platelet-activating factor acetylhydrolase)36. Using our LC/MS/MS-based metabololipidomics approach, we confirmed de novo LM production from HDL particles. Notably, we identified pro-inflammatory LTB₄ production from HDLCAD (Fig. 3a), as well as critical enzymes for LTB₄ biosynthesis, particularly 5-LO, LTA₄ hydrolase, and FLAP (Fig. 3c and Supplemental Fig. 6), which are enriched in activated neutrophils37. Our results indicated that the HDL CAD carries LTB₄-related enzymes and that this enzymatic machinery may be transferred to HDL CAD via activated leukocyte-derived exosomes and microparticles, which may contain 5-LO, LTA₄ hydrolase, and FLAP.

**Figure 7.** Proposed HDL functions: regulation of LTB₄ production from activated macrophages via HDL engulfment. (a) HDLHealthy is engulfed by macrophages via clathrin-mediated endocytosis and attenuates 5-LO expression by the ubiquitin proteasome system, thus resulting in decreased LTB₄ from activated macrophages. HDLHealthy enhances LXB₄ and RvE2 production, as well as phagocytosis in macrophages. (b) HDL CAD produces de novo LTB₄, which interferes with HDL engulfment into macrophages. Here, 5-LO escapes from HDL-mediated degradation, thereby resulting in continuous LTB₄ production from activated macrophages.
LTB₄ promotes monocyte chemotaxis and conversion of monocytes to foam cells, thus resulting in accelerated atherosclerosis. The LTB₄ receptor (BLT1) is expressed in macrophages. The contribution of the LTB₄/BLT1 signalling pathway to atherosclerosis has been demonstrated by using a BLT1 antagonist and BLT1-deficient mice. Additionally, 5-LO gene expression in peripheral blood mononuclear cells and LTB₄ concentration in the plasma were augmented in patients with carotid atherosclerosis. In our study, local de novo LTB₄ release from HDL_CAD suppressed HDL engulfment into macrophages, thereby inhibiting HDL-mediated 5-LO degradation. The LTB₄ antagonist U75302 rescued HDL engulfment (Figs 4a,b and 5a,b) and HDL-mediated 5-LO degradation (Fig. 4c) in macrophages, thus indicating a specific contribution of LTB₄/BLT1 signalling pathways to HDL-macrophage functional interactions.

In summary, our results suggested that HDL holoparticle endocytosis plays critical roles in functional HDL-cell interactions. Additionally, HDL carries specific LM-producing enzymes that may be transferred from specific exosomes, and it releases de novo local LM, which controls HDL holoparticle engulfment and HDL-mediated regulation of cellular functions. Notably, de novo local LTB₄ release from HDL_CAD interferes with HDL-macrophage interactions. The new pathway elucidated here suggests that therapeutic administration of an LTB₄/BLT1 pathway antagonist may be beneficial for improving HDL-mediated anti-inflammatory and pro-resolving functions in cardiovascular disease patients.

Methods
Clinical participants and HDL preparation. HDL Healthy (n = 4) and HDL CAD (n = 4) were prepared from our previous study, on the basis of serum MPO/PON1 ratios. Plasma was stored at −80 °C until use, HDL was isolated by ultracentrifugation as previously described, and the purity of HDL isolation was confirmed by SDS-PAGE and subsequent Coomassie staining. This study was conducted in accordance with the Declaration of Helsinki. The study protocols complied with the Guidelines of the Ethical Committee of the Kobe University Graduate School of Medicine and was approved by the Institutional Review Board of Kobe University Graduate School of Medicine. Written informed consent for participation was obtained from all subjects before the study.

LC/MS/MS-based LM metabololipidomics. Deuterated internal standards d₄-LTB₄, d₅-5-HETE, d₇-PGE₂, and d₇-RvD2, representing each chromatographic region of identified LMs, were added to the samples (500 ng each) to facilitate quantification. The samples were extracted by SPE on C18 columns as previously described and were subjected to LC-MS/MS. The system consisted of a Q-Trap 6500 (Sciex) equipped with a Shimadzu LC-30AD HPLC system. A ZORBAX Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 μm, Agilent Technologies) was used with a methanol/water/acetic acid gradient of 55:45:0.01 to 98:2:0.01 (v/v/v) at a 0.4 ml/min flow rate. For monitoring and quantifying the levels of targeted LMs, the multiple reaction monitoring (MRM) method was developed with signature ion pairs Q1 (parent ion)/Q3 (characteristic fragment ion) for each molecule. Identification was conducted with published criteria using the LC retention time, specific fragmentation patterns, and at least six diagnostic fragmentation ions. Quantification was carried out on the basis of the peak area of the MRM chromatograph, and the linear calibration curves were obtained with authentic standards for each compound.

Macrophage-HDL interactions. RAW 264.7 macrophages were cultured in DMEM supplemented with 10% FBS. In total, 1 × 10⁶ macrophages were activated by opsonized Zym particles (100 μg, 30 min at 37 °C) in PBS, and this was followed by co-incubation with HDL Healthy or HDL CAD (10 μg protein, 30 min at 37 °C). After incubation, a 2 × volume of ice-cold methanol was added for targeted LM metabololipidomics, and 200 μl of lysis buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 1% NP40, 1% SDS) was added for western blot analysis. The expression levels of 5-LO were monitored using an anti-5-LO antibody (3289, Cell Signaling Technology). In select experiments, macrophages were pretreated with the proteasome inhibitor MG132 (10012628, Cayman Chemical) before co-incubation with HDL.

HDL engulfment into macrophages. Each HDL was stained with Dil (1704526, Molecular Probes) as previously described. Briefly, HDL was incubated with CM-Dil for 15 min at 37 °C, and the mixture was dialyzed overnight to remove the residual staining solution. Macrophages (1 × 10⁶) were incubated with each Dil-labelled HDL (10 μg) for 30 min at 37 °C. HDL particle uptake into macrophages was investigated using confocal microscopy (LSM700, LEICA). For detection of human HDL-derived apoA-I from murine macrophages, macrophages (1 × 10⁶) were co-incubated with human apoA-I (SLBN8688V, SIGMA-ALDRICH, 10 μg) or each HDL (10 μg) for 30 min at 37 °C. After extensive washing with PBS, macrophages were harvested. Cell lysates were processed for western blot analysis of human apoA-I with an anti-human apoA-I antibody (230030485, CHEMICON INTERNATIONAL). In specific experiments, macrophages were pretreated with the clathrin-mediated endocytosis inhibitor Pitstop 2 (20 μM, ab120687, Abcam) for 30 min or the LTB₄ receptor antagonist U75302 (300 nM, 70705, Cayman CHEMICAL). For analysis of the effects of LTB₄ on HDL uptake into macrophages, the latter was pretreated with 1, 10, or 100 nM LTB₄ (20110, Cayman CHEMICAL) for 30 min at 37 °C, and this was followed by incubation with Dil-labelled HDL Healthy or HDL CAD for 30 min. HDL uptake was analysed with confocal microscopy. In the same experiment, 5-LO protein expression in raw macrophages was also monitored by western blot analysis. After 2-h co-incubation with macrophages and HDL at 37 °C, the cells were washed in PBS, fixed with 4% formaldehyde for 15 min at 4 °C, and rinsed three times in PBS. The cells were permeabilized in 0.1% Triton X-100 for 10 min, blocked in 5% BSA/PBS for 1 h, and incubated with primary antibodies to apoA-I (230030485, CHEMICON INTERNATIONAL), EEA-1 (ab2900, Abcam) and LAMP-1 (816001, BioLegend) in 5% BSA/PBS overnight. Proteins were detected with Alexa Fluor-labelled secondary antibodies.

HDL-mediated regulation of cellular functions. Notably, local LTB₄ release from HDL CAD interferes with HDL holoparticle interactions. Additionally, HDL carries specific LM-producing enzymes that may be transferred from specific exosomes, and it releases de novo local LM, which controls HDL holoparticle engulfment and HDL-mediated regulation of cellular functions. Notably, de novo local LTB₄ release from HDL CAD interferes with HDL-macrophage interactions. The new pathway elucidated here suggests that therapeutic administration of an LTB₄/BLT1 pathway antagonist may be beneficial for improving HDL-mediated anti-inflammatory and pro-resolving functions in cardiovascular disease patients.
HDL-derived LM production. For analysis of HDL-derived LM production, each HDL (10 µg) was dialyzed with PBS to remove EDTA and then was incubated with 1 µM deuterium-labelled substrates (d-AA, d-EPA and d-DHA) for 2 h at 37 °C. Deuterium-labelled LMs were analysed with our targeted LM metabololipidomics. HDL-carrying proteins were analysed by western blotting. Briefly, 10 µg of each HDL was boiled for 5 min at 95 °C in SDS buffer, then subjected to SDS-PAGE. Specific proteins were detected using primary antibodies against 5-LO (3289, Cell Signaling) and LTA4 hydrolase (ab133512, Abcam).

Detection of ubiquitinated 5-LO. ZyA-activated macrophages were pretreated for 30 min with MG 132 (1 µM) or vehicle, and this was followed by incubation with HDL at 37 °C for 30 min at 37 °C. Lysates were collected with 1% SDS containing lysis buffer, boiled for 5 min and sonicated. Immunoprecipitation (IP) was performed with anti-5-LO Ab (#3289, Cell Signaling) using Dynabeads Protein A IP Kit (Thermo Fisher Scientific). After IP, western blot analysis was carried out with poly-UB antibody (ab140601, linkage-specific K48 antibody, Abcam).

PCR analysis. Macrophage total RNA was extracted with TRizol reagent (Invitrogen). cDNA was prepared from 1 µg total RNA by using PrimeScript RT reagent (RR047, Takara). Real-time polymerase chain reaction (real-time PCR) was performed with SYBR™ Premix Ex Taq II (RR820, Takara). Primers were obtained from Takara Bio Inc. Amplification reactions were performed in duplicate using a LightCycler 96 Real-Time PCR system (Roche), and fluorescence curves were analysed with the included software. GAPDH was used as an internal control. Relative quantification was performed on the basis of the ΔΔCt method.

Macrophage phagocytosis. Macrophages (0.5 × 10^6) were cultured on 96-well plates and preincubated with or without 5 µg of HDL for 1 h or 2 h at 37 °C, and cells were treated with 5 µl of fluorescent-labelled opsonized zymosan (Molecular Probes Z2850) at a 1:1 ratio (zymosan:macrophages) for 30 min at 37 °C. In specific experiments, LTB4 (100 nM) or BLT1 antagonist LY293110 or U75302 (300 nM) were used as pretreatment. After the incubations, macrophages were gently washed, extracellular fluorescence was quenched by addition of a 5-fold diluted trypan blue solution, and phagocytosis was measured with a fluorescent plate reader (EnSpire, PerkinElmer).

Statistical Analysis. Results are expressed as the mean ± SEM. Statistical significance was determined using two-tailed Student’s t test for two-group comparisons and one-way ANOVA for multiple comparisons with post hoc analysis using Tukey’s test (GraphPad Prism). A P value < 0.05 was considered to be significant.

References

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**Author Contributions**

S.T. and M.S. designed the study, performed the experiments, analyzed data and wrote paper. T.O., M.N., N.T. and T.M. analyzed data. T.H., Y.I., R.T., T.I. and K.H. edited the manuscript. All authors discussed the results and commented on the manuscript.

**Additional Information**

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