Mast Cell Activation In Vivo Impairs the Macrophage Reverse Cholesterol Transport Pathway in the Mouse

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Objective—Chymase released by activated mast cells degrades high-density lipoproteins. We evaluated whether local activation of mast cells would attenuate cholesterol efflux from neighboring macrophage foam cells, thereby disrupting the entire in vivo pathway of macrophage-specific reverse cholesterol transport (RCT).

Methods and Results—C57Bl/6J mice received intraperitoneal injections of the mast cell-degranulating compound 48/80 to induce peritoneal mast cell activation, human apolipoprotein A-I (apoA-I) to stimulate RCT, and [3H]cholesterol-labeled J774 macrophages for measurement of the rate of RCT. After 3 hours, [3H]radioactivity was measured in the intestinal lumen contents. Activation of mast cells in the peritoneal cavity depleted human apoA-I pre-β-migrating species, impairing the ability of the peritoneal fluid to efficiently promote cholesterol efflux from cultured macrophages. Moreover, intact but not chymase-treated (proteolyzed) apoA-I accelerated the transfer of macrophage-derived [3H]radioactivity to the intestinal contents. Importantly, stimulation of RCT by human apoA-I was fully blocked by 48/80 in mast cell–competent wild-type C57Bl/6J mice but not in mast cell–deficient W-sash c-kit mutant mice. The ability of intraperitoneally administered phospholipid vesicles to promote RCT in wild-type mice was not blocked by 48/80, supporting the notion that mast cell–dependent proteolysis of the intraperitoneally administered apoA-I was responsible for RCT inhibition.

Conclusion—Overall, our results suggest that tissue-specific activation of mast cells with ensuing release of chymase is able to proteolytically inactivate apoA-I in the microenvironment of the activated mast cells, thus locally impairing the initiation of macrophage RCT in vivo. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: apolipoprotein A-I ■ cholesterol efflux ■ macrophages ■ mast cell chymase ■ reverse cholesterol transport

By promoting efflux of cholesterol from macrophages, high-density lipoproteins (HDL) attenuate the formation of foam cells, a hallmark of atherogenesis.1 Efflux of cellular cholesterol initiates reverse cholesterol transport (RCT), a multistep process that facilitates the transfer of cholesterol from peripheral locations to the liver and finally to feces, thus providing a major pathway for elimination of excess cholesterol from the body.2 Indeed, initiation of RCT from cholesterol-loaded macrophages in the arterial intima counteracts the formation of atherosclerotic lesions.3 Multiple studies have highlighted the key role of apolipoprotein A-I (apoA-I) structure-function relationships on the efficiency of HDL particles at promoting cholesterol efflux and in subsequent steps of RCT.4 Moreover, modulation of the macrophage-dependent RCT pathway has been recently achieved by structural and conformational modifications of apoA-I.5 Importantly, the finding of circulating HDL with attenuated antiatherogenic function in subjects with atherosclerosis reinforced the concept that dysfunctional HDL particles are generated in vivo and may predict cardiovascular risk.6 Mast cells are a potent source of neutral serine proteases.7 In the arterial intima, mast cells have been found in close proximity to macrophage foam cells.8 We have demonstrated that mast cell–derived chymase is able to modify HDL particles in vitro, thereby attenuating their ability to function as cholesterol acceptors.9 Activation of peritoneal mast cells, with ensuing exocytosis of chymase-containing granules (degranulation), also blocked cholesterol release from cholesterol-loaded macrophages generated in the peritoneal cavity of the rat.8 This suggested a role for mast cell proteases in the in vivo inhibition of RCT. Moreover, we found recently that systemic activation of mast cells by induction of anaphylactic shock in the mouse caused a mast cell–dependent proteolytic modification of HDL particles.10 This in turn compromised the ability of mouse serum and peritoneal fluid to act as cholesterol acceptors in macrophage foam cells.
cultures. Mast cell-derived chymotryptic activity was the factor generating dysfunctional HDL particles in the anaphylactic mouse. Among the mouse mast cell proteases, only mouse mast cell protease-4, the functional homologue of human chymase, remains heparin proteoglycan bound after secretion from activated mast cells, which protects it from inactivation by physiological inhibitors present in the extracellular fluids. Consequently, this enzyme was most likely responsible for the proteolytic modification of HDL particles and the degradation of apolipoprotein E observed during systemic anaphylaxis in the mouse.

In support of the antiatherogenic role of apoA-I in humans, overexpression of human apoA-I in mice has been shown to increase RCT along the pathway from macrophages injected in the peritoneal cavity to feces. Here, to evaluate the effect of mast cell activation on the apolipoprotein-mediated stimulation of macrophage RCT, we stimulated RCT by injecting human apoA-I and cholesterol-labeled macrophage foam cells into the mouse peritoneal cavity, a body compartment rich in resident mast cells. We then evaluated whether local activation of peritoneal mast cells would affect this stimulatory effect of apoA-I on RCT. This strategy allowed us to demonstrate that tissue-specific activation of mast cells is able to inhibit the macrophage RCT pathway via proteolytic inactivation of the physiological acceptors of macrophage-derived cholesterol, notably apoA-I.

Materials and Methods A detailed description of the animals, materials, and methods used in the present study is provided in the Supplemental Material, available online at http://atvb.ahajournals.org. A brief description of the methods is given below.

CS7Bl/6J mast cell–competent and mast cell–deficient W-shit c-kit mutant Ktsh-W-shit mice created on the C57Bl/6J background, which lack mature mast cells because of an inversion mutation of the c-Kit promoter region, were used. Lipid-free human apoA-I was degraded by chymase-containing mouse peritoneal mast cell lysates in vitro or exposed to activated peritoneal mast cells in vivo. The ability of the proteolyzed apoA-I to act as a cholesterol acceptor was evaluated in mouse macrophage foam cell cultures, and its ability to stimulate macrophage RCT was determined in vivo. A validated in vitro macrophage-specific RCT assay was applied to assess the rate of RCT in mast cell–competent and mast cell–deficient mice within 3 hours. For the RCT protocol, mice received consecutive intraperitoneal injections of (1) the mast cell–specific degranulating compound 48/80 (1 mg/kg) to achieve immediate local activation of peritoneal mast cells (stimulated group) or saline (nonstimulated group) and (2) human apoA-I (3 mg/kg) together with [3H]cholesterol-labeled macrophages. Plasma lipoproteins were fractionated by fast protein liquid chromatography using a Superose 6R column. Pre-β- and α-HDL subpopulations were detected by 2-dimensional immunoelectrophoresis. GraphPad Prism 4.0 software was used for all statistical analyses. Probability values <0.05 were considered statistically significant.

Results

In Vitro Proteolytic Inactivation of Human ApoA-I by Mouse Chymase

We first evaluated whether mouse mast cell chymase, like human chymase, would proteolyze lipid-free human apoA-I in vitro. For this purpose, we exposed apoA-I to increasing chymase activities by incubating it with increasing amounts of either chymase-containing lysate derived from mouse peritoneal mast cells or recombinant human chymase (Teijin Ltd, Japan, 80 BTEE U/μg) in TNE buffer (pH 7.4) providing increasing chymotryptic activities. Aliquots of the chymase-treated preparations were applied to SDS-PAGE, and human apoA-I was detected by Western blotting. Proteolytic fragments are highlighted in representative gels. B, Human apoA-I was incubated with increasing concentrations of the mouse mast cell lysate as described above, and the generated fragments were visualized by Western blotting (see arrows in gel shown at top). Aliquots of these chymase-treated apoA-I preparations were added to cultured [3H]cholesterol-labeled mouse peritoneal macrophage foam cells (final concentration of apoA-I in medium, 10 μg/mL) (bottom). After 4 hours of incubation, efflux of cholesterol was determined and expressed as dpmmedium/(dpmcells + dpmmedium)×100. Values are means±SD of triplicate wells.

Figure 1. In vitro mast cell–dependent proteolytic inactivation of human apoA-I. A, Human apoA-I (50 μg) was incubated for 3 hours at 37°C with a mouse mast cell lysate (15 BTEE U/10,000 mast cells) or recombinant human chymase (Teijin Ltd, Japan, 80 BTEE U/μg) in TNE buffer (pH 7.4) providing increasing chymotryptic activities. Aliquots of the chymase-treated preparations were applied to SDS-PAGE, and human apoA-I was detected by Western blotting. Proteolytic fragments are highlighted in representative gels. B, Human apoA-I was incubated with increasing concentrations of the mouse mast cell lysate as described above, and the generated fragments were visualized by Western blotting (see arrows in gel shown at top). Aliquots of these chymase-treated apoA-I preparations were added to cultured [3H]cholesterol-labeled mouse peritoneal macrophage foam cells (final concentration of apoA-I in medium, 10 μg/mL) (bottom). After 4 hours of incubation, efflux of cholesterol was determined and expressed as dpmmedium/(dpmcells + dpmmedium)×100. Values are means±SD of triplicate wells.
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Next, we tested whether the function of human apoA-I as an acceptor of macrophage cholesterol would be impaired in vivo when peritoneal mast cells were stimulated to degranulate and, thus, to secrete chymase. Importantly, mouse chymase is partially resistant to protease inhibitors found in the peritoneal fluid.10 Mice received a single intraperitoneal dose of 48/80 to activate peritoneal mast cells (stimulated mice) or saline (nonstimulated mice), immediately followed by a single intraperitoneal dose of human apoA-I. A third group of mice received only saline in the 2 injections (control group). After 3 hours, peritoneal fluid was collected, and its ability to promote cholesterol efflux from cultured macrophage foam cells was then evaluated ex vivo in macrophage foam cell cultures. Initially, we injected a high dose of apoA-I (15 mg/kg) into the peritoneal fluid of mice that had been treated with either 0.5 or 1 mg/kg of 48/80, and we observed a dose-dependent, significant (P = 0.038 for trend) inhibitory effect on cholesterol efflux (Figure 2A). Next we reduced the apoA-I dose to 3 mg/kg, which was found to also increase the cholesterol acceptor competence of peritoneal fluid, and found that pretreatment with 48/80 fully suppressed the apoA-I stimulatory effect (Figure 2B). These dose-response studies strongly suggested that the neutral protease chymase released by degranulated mast cells into the peritoneal fluid had proteolytically inactivated human apoA-I in the peritoneal cavity, thereby nullifying its ability to act as a cholesterol acceptor ex vivo. Because the 3 mg/kg dose of human apoA-I maintained an increased efflux-inducing ability of peritoneal fluid for at least 3 hours, we predicted that under these conditions the in vivo transfer of peritoneal macrophage cholesterol to the intestinal lumen might also proceed at an accelerated rate and could be measured within this period of time. Moreover, the ex vivo efflux results strongly suggested that pretreatment of the mice with the 1 mg/kg dose of 48/80 would block the overall apoA-I effect. These doses were therefore selected for the following RCT experiments.

Validation of Short-Time RCT Assay From Intraperitoneally Injected Macrophages to Intestinal Lumen Contents

To study the in vivo effect of peritoneal mast cell degranulation on the rate of RCT, we adapted an established macrophage-specific RCT assay.15 This RCT assay evaluates the transfer of [3H]cholesterol from macrophages injected into the peritoneal cavity to feces within periods of 24 or 48 hours, and it has been widely applied in genetically modified mice.19,20 Given the relatively short period of our in vivo incubation model, we first evaluated the reliability of the RCT assay when the time between the intraperitoneal injection of labeled macrophages and the collection of samples was shortened to 3 or 6 hours (Supplemental Figure I). Within these time limits, the assay proved to be an accurate measure of the time-dependent transfer of macrophage radioactivity to plasma and intestinal lumen contents, even within the 3-hour time frame. We then evaluated whether the modified assay would be sufficiently sensitive to measure changes in the
RCT efficiency induced within this relatively short time. Because activation of liver X receptor significantly increases macrophage-derived \[^3H\]cholesterol tracer contents in feces,\textsuperscript{21,22} we injected labeled macrophages into the peritoneal cavity of mice treated with the liver X receptor agonist T0901317 and measured \[^3H\]tracer levels in plasma, liver, and intestinal contents after 3 hours. In line with previous results,\textsuperscript{21} even though liver X receptor activation did not affect plasma or liver \[^3H\]cholesterol levels, the \[^3H\]tracer transfer to the intestinal lumen contents increased \(\approx 2\)-fold compared with control mice (Supplemental Figure II). Overall, application of the 3-hour macrophage RCT assay appeared to be valid for quantification of cholesterol efflux from peritoneal macrophages to the intestinal contents, and so it could be applied to our experimental model in which mast cell-derived proteolysis of a single dose of human apoA-I is acutely triggered in the peritoneal cavity.

**Effect of Mast Cell Degranulation on In Vivo RCT Stimulated by Human ApoA-I**

To test our hypothesis on the in vivo mast cell proteolytic inactivation of RCT, we evaluated RCT in both stimulated (48/80-treated) and nonstimulated mice that received intraperitoneal injection of human apoA-I (3 mg/kg) and in control mice that received saline only. The experimental protocol was identical to that described in Figure 2, but in addition, \[^3H\]cholesterol-labeled J774 macrophages were administered together with apoA-I during the second injection. Three hours after injection of the macrophages, we collected blood, liver, and total intestinal lumen contents and measured \[^3H\]tracer radioactivity of the samples (Figure 3). We found that compared with the control group, the levels of \[^3H\]cholesterol in plasma were reduced both in the nonstimulated and in the stimulated group, whereas the \[^3H\]tracer levels in liver were similar in all 3 groups of mice (Figure 3A and 3B). More importantly, the \[^3H\](cholesterol+bile acids) radioactivity in intestinal lumen contents was significantly higher in the nonstimulated mice (Figure 3C). This indicated that the intraperitoneally injected human apoA-I increased the transfer of macrophage-derived cholesterol to the intestinal lumen, most likely by promoting ABCA1-dependent cholesterol efflux from the J774 cholesterol-laden macrophages. In sharp contrast, the apolipoprotein stimulus on RCT was completely blocked in 48/80-treated mice, ie, in mice in which peritoneal mast cells had been activated (Figure 3C).

To clarify the mechanism underlying RCT inhibition by 48/80, we evaluated the 48/80 effect on the mRNA expression of \(\text{J}774\) macrophage cholesterol transporters in vitro and found that a 3-hour incubation with 48/80 did not change the gene expression levels of ABCG1 or SR-BI but did increase that of ABCA1 (Supplemental Figure III). This finding suggested that in the 48/80-treated mice, the observed inhibitory effect on RCT was not attributed to inhibition of ABCA1 transporter but rather to inhibition of the ligand of this transporter (ie, apoA-I). The overall results concurred...
with the notion that exocytosed chymase released from 48/80-activated peritoneal mast cells had degraded human apoA-I in the peritoneal cavity, thus impairing the ABCA1-dependent cholesterol efflux from the cojected macrophages and ultimately leading to diminished transfer of cholesterol from the peritoneal cavity to the intestinal lumen.

To ascertain that activation of peritoneal mast cells was responsible for the attenuated transfer of macrophage-derived cholesterol to the intestinal lumen, we repeated the above RCT experiment using genetically mast cell–deficient sash mice. Trends similar to those found in the wild-type mice were observed for the \(^{3}H\)cholesterol levels in plasma and liver (Figure 3D and 3E). In sharp contrast, \(^{3}H\)tracer levels were significantly increased in the intestinal lumen in both groups relative to the control group (Figure 3F), indicating that the compromised RCT rate in the 48/80-treated wild-type mice (Figure C) was mast cell dependent.

In the course of the above RCT experiments, we found that the levels of \(^{3}H\)cholesterol in plasma and liver did not follow a consistent trend when related to the net transfer of \(^{3}H\)cholesterol from macrophages to the intestine (see Figure 3 and Supplemental Figure II). This lack of association between \(^{3}H\)cholesterol levels in plasma, liver, and feces has also consistently been observed by others when using the original RCT assay of 24/48 hours’ duration.\(^{21–24}\) Because plasma and liver are transit compartments for cholesterol from the peritoneal cavity to feces, it appeared that they only incompletely reflected the rate of the whole RCT process at any single given time point, thus giving a poor readout also in the present 3-hour in vivo RCT assay. It is likely that in our model, the intermediate RCT steps were also modulated by restoration of homeostasis, which followed the temporary burst of RCT, whereas a cumulative effect was clearly observed in the cholesterol transfer to the intestinal lumen.

Validation of the Mast Cell–Derived Proteolytic Inhibitory Effect on In Vivo RCT

To validate that proteolysis of the injected human apoA-I was responsible for the observed RCT inhibition, we next applied the 3-hour RCT assay in mice that received intraperitoneally a human apoA-I preparation that had been preincubated for 3 hours in the absence (control) or presence (proteolyzed) of mouse chymase. Such proteolyzed apoA-I is not competent in promoting cholesterol efflux in vitro (Figure 1B). Similarly, the preproteolyzed apoA-I was not able to stimulate the transfer of the tracer to intestine (Figure 4). This finding further strengthened the notion that declined RCT in mice in which peritoneal mast cells had been stimulated by 48/80 was caused by chymase-dependent proteolysis of the intact human apoA-I.

We further evaluated RCT in 48/80-stimulated mice that received a type of cholesterol acceptor that is not susceptible to proteolysis. This was assessed by intraperitoneal injection of cholesterol-free phospholipid vesicles, which are efficient inducers of aqueous diffusion of cellular cholesterol.\(^{25}\) For this purpose, we injected intraperitoneal phosphatidyl choline as small unilamellar vesicles to nonstimulated and 48/80-stimulated mice. Phosphatidyl choline as small unilamellar vesicles (30 mg/kg) significantly increased the transfer of \(^{3}H\)cholesterol to intestine, and importantly, activation of peritoneal mast cells totally failed to alter the rate of RCT (Figure 4). Together, these results provided strong evidence that decline of RCT in vivo in 48/80-treated wild-type mice (Figure 3C) was mediated by mast cell–dependent proteolytic degradation of human apoA-I rather than by other consequences of mast cell activation.

We also examined the effect of 48/80 (1 mg/kg) on the basal RCT component covered by the endogenous cholesterol acceptor system, ie, in the absence of exogenously administered human apoA-I, and found similar magnitudes of RCT in the 2 groups of mice (Figure 4). Considering the present model of local mast cell activation, this finding might reflect a lower in vivo efficiency of chymase in degrading mature \(\alpha\)-HDL species, as it has been found in vitro.\(^{17}\) Indeed, mouse apoA-I in the peritoneal fluid exists predominantly (≈80%, our measurements) as \(\alpha\)-migrating species and is constantly supplied from plasma.

ApA-I in Peritoneal Fluid

To better assess the metabolic fate of intraperitoneally injected human apoA-I, we analyzed human apoA-I concentrations in the peritoneal fluid at different time points after mast cell stimulation. The results revealed a significantly faster decay of human total apoA-I in stimulated compared with nonstimulated mice, and, moreover, it closely reflected the
Discussion

Although mast cell degranulation leads to massive release of active proteases to the extracellular environment, its potential role to modify RCT by degrading apolipoproteins in vivo has not been studied before. In the present study, we demonstrate that targeted activation of mast cells in the peritoneal cavity of mice from which RCT was accelerated by a single dose of human apoA-I was sufficient to fully block the in vivo apolipoprotein-mediated stimulation of macrophage RCT. This inhibitory effect was mast cell–dependent because administration of the mast cell–specific degranulating compound 48/80 to human apoA-I-treated mast cell–deficient sash mice did not compromise the RCT. Moreover, the inability of activated peritoneal mast cells to affect RCT rate in mice that received an intraperitoneal injection of phospholipid vesicles further suggested that proteolysis was the mechanism involved, by which mast cells reduced human apoA-I-induced macrophage RCT in vivo.

As in the peritoneal cavity, mast cells and macrophages coexist in the arterial wall. Because both peritoneal and intimal fluids are filtrates from blood plasma, the levels of HDL particles in these 2 compartments are likely to be similar. Therefore, the peritoneal cavity can be used as a reasonable surrogate when one studies potential effects of activated mast cells on macrophage RCT in vivo. Like other extracellular fluid compartments, the peritoneal cavity is an open system from which ultrafiltration of soluble low–molecular weight solutes through the peritoneal membrane rapidly occurs. Therefore, a rapid flux of the small 48/80 molecule (molecular mass, 520 Da) into the circulation made it unlikely that sustained activation of peritoneal mast cells had occurred. Similarly, the intraperitoneally injected apoA-I (molecular mass, 28 000 Da) can also diffuse from the peritoneal cavity, albeit at a slower filtration rate. Because of the highly dynamic nature of the cholesterol efflux assay used here most likely led to only a transitory sequence of events that targeted activation of mast cells in the peritoneal cavity, albeit at a slower filtration rate. Because of the dynamic nature of the cholesterol efflux assay (Supplemental Figure II). This novel methodological application opens up further possibilities to study other short-term effects on macrophage RCT in vivo.

Consistent with the well-known apoA-I stimulatory role on the ABCA1-mediated cholesterol efflux pathway, we found that a single massive intraperitoneal dose of human apoA-I (providing \( \approx 100 \mu g \) per mouse) significantly accelerated macrophage RCT. As noted above, the single dose of human apoA-I used here most likely led to only a transitory sequence of events in the peritoneal cavity. Mechanistic data on ABCA1-mediated lipidation of apoA-I indicate that pre-\( \beta \)-migrating subfractions of multiple sizes are formed in vitro (Supplemental Figure V). Of note, small apoA-I/PL complexes

reduction in pre-\( \beta \)-HDL particles, as evaluated by 2-dimensional IE (Figure 5A and 5B). Importantly, the decline of human apoA-I levels in the pre-\( \beta \)-migrating fraction of stimulated mice was most marked within the first hour after mast cell stimulation. Accordingly, we searched for human apoA-I degradation products in peritoneal fluid derived from 48/80-stimulated mice at early stages after apoA-I administration (up to only 20 minutes). However, despite the significant reduction in total and pre-\( \beta \)-migrating human apoA-I, Western blotting failed to detect any human apoA-I fragments in stimulated mice even at the shortest feasible time of collecting peritoneal fluid (3 to 5 minutes). Among the experimental groups, analysis of mouse plasma did not indicate differences in human apoA-I levels or in any other parameter studied (Supplemental Table I and Supplemental Figure V).
stimulated diffusional cholesterol efflux that also significantly promoted RCT was not inhibited by mast cell activation. Additional studies in suitable animal models are required to assess whether activation of the protease-containing mast cells in the atherosclerotic arterial wall would reduce the efflux of macrophage cholesterol promoted by the actual blend of acceptors present in the intimal fluid and so attenuating the initiation of the RCT pathway from vulnerable lesions to ultimate excretion of cholesterol from the body.

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Disclosures
None.

References


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Supplemental Material

Materials and Methods

Animals

C57Bl/6J mast cell-competent mice were obtained from the Viikki Laboratory Animal Center, University of Helsinki, and mast cell-deficient mice W-sash c-kit mutant KitW-sh/W-sh created on the C57Bl/6J background were purchased from the Jackson Laboratory (Bar Harbor, Maine). KitW-sh/W-sh mice lack mature mast cells due to an inversion mutation of the c-Kit promoter region\(^1\). The mice (females, 10 to 12 weeks old) were housed 5 per cage in conditions controlled for light/dark cycle, temperature, and humidity, and were provided ad libitum with a standard chow diet and water. The experiments were conducted in accordance with published regulations, and the protocols were approved by The National Animal Care and Use Committee of Finland and by the Institutional Animal Care Committee of the Hospital de la Santa Creu i Sant Pau, Spain.

Chymotryptic activity in mouse peritoneal mast cell lysates

Peritoneal mast cells were collected from non-stimulated mice as described\(^2\). Aliquots of the mast cell suspension were stained in a 1:1 volume of Moore & James staining solution and counted in a Burker chamber. Mast cell suspensions were sonicated on ice for 10 sec at a constant duty cycle (Branson sonicator, model 250) to obtain a mast cell lysate. Most of the chymotryptic activity in mouse peritoneal mast cells is due to the chymase protease referred to as mouse mast cell protease 4 (mMCP-4)\(^3\). Chymase
activity in the mast cell lysates was determined with the chromogenic substrate for chymotrypsin-like proteases S-2586, as described\textsuperscript{2}.

**In vitro proteolysis of apoA-I by mouse chymase**

Human apoA-I (50 µg) was incubated at 37°C with various concentrations of the mast cell lysate (chymase activity 20 U/µL) in a final volume of 250 µL in 5 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 1 mM EDTA (TNE buffer). At 3 h post-incubation, the vials were centrifuged at 10,000 x g for 10 min at 4°C to stop the reaction. ApoA-I in the supernatant was analyzed by 15% SDS-PAGE and western blotting. The ability of the chymase-treated mixtures to induce cellular cholesterol efflux \textit{ex vivo} (10 µg/mL final concentration in medium) and to induce RCT \textit{in vivo} (3 mg/kg) was evaluated as described below. In a separate experiment human apoA-I was proteolyzed by recombinant human chymase (20 U/µL) kindly provided by Dr. Hidenori Kasai from the Teijin Ltd Co, Japan. The human chymase reaction was fully stopped by addition of 100 µg/mL of soybean trypsin inhibitor (Sigma).

**Peritoneal mast cell activation in vivo and i.p. administration of human apoA-I**

Mice (5 animals/group) received intraperitoneal (i.p.) injections of the non-cytotoxic mast cell degranulator compound 48/80 (Sigma-Aldrich) at a dose of 1 mg/kg to induce local mast cell degranulation (stimulated group) or NaCl 0.9% (non-stimulated group), followed by apoA-I (3 mg/kg). Another group of mice received equal volumes of NaCl 0.9% alone for both injections (control group). After 3 h, mice were euthanized and peritoneal fluid was collected by gentle aspiration using a plastic pipette and centrifuged
200 x g for 10 min at 4°C to sediment detached peritoneal cells. The supernatants were immediately used or stored at -80°C for structural and functional analysis.

**Biochemical analysis of apoA-I in mouse serum and peritoneal fluid**

The ability of peritoneal fluid to induce cellular cholesterol efflux *ex vivo* (2.5%, v/v, final concentration in medium) was evaluated as described below. In other experiments, samples of blood and peritoneal fluid were collected for periods of times up to 3 h (3 mice/group) and subjected to quantitative and qualitative apoA-I analysis. Aliquots of peritoneal fluid collected in 50-100 µL PBS were applied to SDS-PAGE 15% and protein bands were immunoblotted with both human (Rabbit R314 antibodies, bleeding 4, dilution 1:2000, home-made antibodies against purified human apoA-I) and mouse (Rabbit anti-mouse A-I, cat.no K23001R, 1:2500 dilution, Meridian Life Science Inc., ME, USA) apoA-I polyclonal and specific monoclonal antisera. Secondary antibody was goat anti-rabbit IgG-HRP conjugate, Cat.no. 170-6515, Bio-Rad, CA, USA. Peritoneal fluid samples were further analyzed by two-dimensional immunoelectrophoresis (IE) to determine apoA-I-containing preβ- and α-HDL subpopulations. Rabbit 314 as antiserum (7.5 %, v/v) and rabbit 226 (home-made antiserum against purified mouse apoA-I, 2.5 % (v/v) were used in two-dimensional IE.

**Measurement of [3H]cholesterol efflux from cultured mouse macrophage foam cells**

Peritoneal macrophages isolated from non-stimulated mice were loaded with cholesteryl esters by incubation for 18 h in the presence of 20 µg/mL of [3H]CE-acetyl-LDL (80 dpm/ng protein) in DMEM supplemented with 20% FCS, as described previously.
Aliquots of chymase-treated apoA-I or mouse peritoneal fluid were added to the
[3H]cholesterol-labeled macrophage foam cell cultures. After 4 h of incubation, the cell
medium was analyzed for [3H]radioactivity by liquid scintillation counting (LSC).
Fractional cholesterol efflux (%) was calculated as \( \frac{\text{dpm}_{\text{medium}}}{(\text{dpm}_{\text{cells}} + \text{dpm}_{\text{medium}})} \times 100 \). Cholesterol efflux into the incubation medium in the absence of cholesterol
acceptors was considered as basal efflux, and was subtracted from the efflux values
obtained in the presence of the acceptors.

**Evaluation of the rate of RCT from macrophages to intestine within 3 h**

We applied the *in vivo* RCT procedure previously described\(^6\text{,}\text{7}\) with some modifications,
as indicated. The J774 mouse macrophage cell line (ATTC; Manassas, VA) was cultured
in 75-cm\(^2\) cell culture flasks at 5 x 10\(^6\) cells/flask and grown to 90% confluence in RPMI
1640 supplemented with 10% FBS. Cells were then incubated for 48 h in the presence of
5 \( \mu \text{Ci/mL} \) of \([1\alpha,2\alpha(n)-3H]\)cholesterol (specific activity of 44 x 10\(^3\) cpm/pmol; GE
Healthcare, Germany), 100 \( \mu \text{g/mL} \) of acetylated LDL, and 10% lipoprotein-depleted
serum. Macrophage foam cells were washed, equilibrated, detached by gently pipetting,
resuspended in NaCl 0.9\%, and pooled. Prior to i.p. injection, cell count and viability
were measured by acridine orange and ethidium bromide staining. Mice were
exsanguinated by cardiac puncture at 3 or 6 h and livers removed after extensive
perfusion with saline. Plasma radioactivity was determined by LSC. HDL-associated and
non-HDL-associated \([^3\text{H}]\)cholesterol radioactivities were measured after precipitation of
apoB-lipoproteins with phosphotungstic acid and magnesium chloride (Roche
Diagnostics GmbH, Germany)\(^8\). Small intestines, reaching from duodenum to ileum,
were isolated and flushed with 5 mL of sterile NaCl 0.9% to collect the luminal contents. Fecal material was collected as well from the large intestine lumen. Liver and intestinal lipids were extracted with isopropyl alcohol-hexane. The lipid layer was collected, solvent was evaporated, and $[^3\text{H}]$tracer radioactivity measured by LSC. The $[^3\text{H}]$tracer detected in fecal bile acids was determined in the remaining aqueous portion of fecal material extracts. A known amount of $[1\alpha,2\alpha(n)-[^3\text{H}]]$cholesterol (GE Healthcare) and $[^3\text{H}(G)]$taurocholic acid (PerkinElmer LAS, Boston) was used as control. The amount of $[^3\text{H}]$tracer was expressed as a fraction of the injected dose (results shown in Supplemental Figure I). In the various experiments performed, cell viability of the macrophage preparations ranged from 87 to 91% and each mouse received an i.p. dose of $[^3\text{H}]$cholesterol-labeled mouse macrophages (3.25-5.14 x 10$^6$ cells/mouse, 1.95-2.92 x 10$^6$ cpm/mouse). The small intestine accounted for 77-85% of the total $[^3\text{H}]$tracer in the whole intestinal lumen. HDL-associated $[^3\text{H}]$cholesterol accounted for 70-79% of the total plasma $[^3\text{H}]$cholesterol in all groups. A pilot experiment to test the sensitivity of the shortened 3 h-RCT assay was performed using female C57BL/6 mice treated with a liver X receptor (LXR) agonist (results shown in Supplemental Figure II). As described previously$^7$, mice (8 animals/group) were given one daily oral gavage of vehicle (1.0% v/v ethanol and 1.0% w/v carboxymethylcellulose medium viscosity) or the LXR agonist T0901317 (Cayman Chemicals, Ann Arbor, MI) at a dose of 20 mg/kg body weight dissolved in the vehicle solution, at 16:00 h, for 5 consecutive days, after which the animals were used for the experiment.

**RCT studies in mast cell-stimulated mice**
To evaluate the effect of mast cell activation on RCT, we conducted separate experiments using mast cell-competent C57Bl/6J and mast cell-deficient Kit\textsuperscript{W-sh/W-sh} mice. In each experiment, mice received consecutive i.p. injections of 48/80 (1 mg/kg) to stimulate peritoneal mast cells (stimulated group) or the same volume of saline (non-stimulated group), immediately followed by the apoA-I dose (3 mg/kg) administered to both groups. Another group of mice received only saline for both injections (control group). To measure the rate of RCT, [\textsuperscript{3}H]cholesterol-labeled J774 macrophages were administered in the second i.p. injection to all mice and the transfer of the radioactivity to plasma, liver and intestine luminal contents was measured 3 h after macrophage injection. Using the setup described above, RCT was also examined in 48/80-stimulated and non-stimulated C57Bl/6J wild-type mice upon receiving i.p. doses of (a) proteolyzed human apoA-I (3 mg/kg) i.e., after being incubated for 3 h with chymase derived from mouse peritoneal mast cell lysates, (b) phosphatidyl choline (30 mg/kg) administered in small unilamellar vesicles (PC-SUV), or (c) only saline. PC-SUV vesicles (26 µmol PC/mL) with average diameter of 25 nm were prepared as previously described\textsuperscript{9}.

**Quantitative real-time RT-PCR analyses**

J774 mouse macrophage foam cells as described for RCT experiments were incubated for an additional period of 3 h in RPMI 1640 in the absence or presence of 48/80 (30 µg/10\textsuperscript{6} macrophages in 0.5 mL of culture medium). RNA was isolated using the trizol RNA isolation method (Gibco/BRL, Grand Island, NY, USA). Total RNA samples were repurified, checked for integrity by agarose gel electrophoresis and reverse-transcribed with Oligo(dT)\textsubscript{15} using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant to
generate cDNA. Predesigned validated primers (Assays-on-Demand, Applied Biosystems, Foster City, CA) were used with Taqman probes. PCR assays were performed on an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described. All analyses were performed in duplicate and relative RNA levels were determined using GAPDH as internal control (results shown in Supplemental Figure III).

**Other analyses**

Total cholesterol and HDL-cholesterol in serum were determined enzymatically using commercial kits (Boehringer Mannheim, Germany). Serum lipoprotein fractions were separated by FPLC system using a Superose 6R column, as previously described. Mouse and human apoA-I were determined by ELISA.

**Statistical Methods**

Unpaired Student’s t-test or non-parametric Mann–Whitney U test were used to compare data obtained from two groups. One-way ANOVA with Tukey's multiple comparison test or a non-parametric Kruskal-Wallis test with Dunn’s posttest was used to compare data from three groups. GraphPad Prism 4.0 software (GraphPad, San Diego, CA) was used for all statistical analyses. A p value <0.05 was considered statistically significant. All parameters were expressed as the mean ± SD.
Supplemental Results

Analysis of mouse plasma showed that the experimental treatments did not modify the levels of total or HDL-cholesterol, nor mouse apoA-I (Supplemental Table I). FPLC fractionation of plasma (Supplemental Figure IV) showed similar cholesterol profile in the 3 groups, and a slight increase in phospholipids in the groups of mice that received human apoA-I. Whereas mouse apoA-I eluted with large-sized HDL in control plasma, administration of human apoA-I particularly in 48/80-stimulated mice, modified mouse apoA-I distribution by generation of a smaller-size HDL subpopulation, causing HDL to elute in two peaks (panel C). FPLC analysis of plasma from mice treated with 48/80 in the absence of injected human apoA-I revealed that 48/80 alone induced mouse apoA-I to elute in two peaks (not shown) despite the fact that HDL cholesterol and phospholipid profiles remained unchanged. This qualitative change in mouse apoA-I profile was not, however, associated to RCT modification in mice treated only with 48/80 (Figure 4). Minor amounts of human apoA-I (~5% of total apoA-I) were found associated with α-HDL in unfractionated plasma of the apoA-I-treated groups after 3 h of treatment (Supplemental Figure VA). No fragments of apoA-I of either mouse (Supplemental Figure IV, bottom panel) or human (Supplemental Figure VB) origin were detected by SDS-PAGE and WB analyses of plasma from the various experimental groups. Time-course of human apoA-I concentrations in plasma showed no difference between the groups at any time point (Supplemental Figure VC).
References


Online Supplemental Figure Legends

**Supplemental Figure I. Time-course (3 and 6 h) of RCT from macrophages to intestinal lumen contents in C57Bl/6J mice**

C57Bl/6J mice were i.p. injected with \[^{3}\text{H}]\text{cholesterol-labeled} J774 mouse macrophages, and macrophage-derived \[^{3}\text{H}]\text{cholesterol radioactivity} was measured after 3 and 6 h of macrophage injection in plasma, and in the luminal contents of the whole intestine (\[^{3}\text{H}]\text{cholesterol+}\[^{3}\text{H}]\text{bile acids}). The amount of \[^{3}\text{H}]\text{tracer} was expressed as a fraction of the injected dose. Three female C57BL/6 mice were used in each experimental point. Measurement of macrophage-derived cholesterol radioactivity was time-dependent in plasma and intestine, and accounted for 2.4% and 1.0% of the injected doses, respectively, already within 3 h.

**Supplemental Figure II. RCT from macrophages to intestinal lumen contents in mice treated with LXR agonist T0901317**

C57Bl/6J mice were given one daily oral gavage of a dose of 20 mg/kg body weight of the LXR agonist T0901317 or only vehicle (control) for 5 consecutive days. Mice were i.p. injected with \[^{3}\text{H}]\text{cholesterol-labeled} J774 mouse macrophages, and macrophage-derived \[^{3}\text{H}]\text{cholesterol radioactivity} was measured after 3 h of macrophage injection in plasma, liver, and in the intestinal lumen material collected from the whole intestine (\[^{3}\text{H}]\text{cholesterol+}\[^{3}\text{H}]\text{bile acids}). The amount of \[^{3}\text{H}]\text{tracer} was expressed as a fraction of the injected dose. Statistical significance between LXR agonist-treated and vehicle mice was determined with the two-tailed Student’s \(t\) test. Eight mice were used in each experimental group. Treatment with the LXR agonist led to a significant increase in
the % of total injected macrophage-derived labeled cholesterol dose recovered within 3 h in intestinal lumen material compared to control mice (P<0.05).

**Supplemental Figure III. Real-time RT-PCR quantification of relative mRNA expression in J774 mouse macrophages**

J774 macrophage foam cells were incubated for 3 h in the presence of 48/80 (60 µg/mL) or saline. Values in the figure correspond to the relative mRNA expression of macrophage ABCA1, ABCG1 and SR-BI genes. The signal of control J774 mouse macrophages was set at a normalized value of 100 arbitrary Units. GAPDH was used as internal control. Results are expressed as mean ± SEM of 3 independent samples per group.

**Supplemental Figure IV. Lipids and mouse apoA-I in plasma lipoproteins**

Aliquots of plasma pools derived from C57Bl/6J mice treated as described in Figure 2B (3 mice/group) were applied to a Superose 6HR size-exclusion chromatography column. The eluted fractions were analyzed for (A) cholesterol, (B) choline-containing phospholipids, and (C) mouse apoA-I. Total mouse apoA-I accounted for 1.01±0.02, 1.01±0.01, and 0.91±0.05 mg/mL in plasma derived from the control, the non-stimulated, and the stimulated group, respectively. Western blotting images of mouse apoA-I from HDL-containing fractions are shown at the bottom panel.

**Supplemental Figure V. Human apoA-I in mouse plasma**
(A) Plasma derived from C57Bl/6J wild-type mice treated as described in Figure 2B legend was analyzed by two-dimensional crossed immunoelectrophoresis to analyze human apoA-I-containing preβ- and α-HDL subpopulations. (B) FPLC fractions corresponding to the HDL elution volume range (see Supplementary Figure IV) were applied to SDS-PAGE and subjected to western blotting analysis to search for human apoA-I degradation fragments. (C) Human apoA-I was quantitated by Elisa in plasma from stimulated (+48/80) and non-stimulated (-48/80) mice, which were used for the time-course study described in Figure 5 legend.
### Supplemental Table I. Lipid and apolipoprotein A-I levels in mouse serum

**Intraperitoneal injection**

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<th>PBS</th>
<th>Human apoA-I</th>
<th>Human apoA-I+48/80</th>
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<td>Total cholesterol (mmol/L)</td>
<td>1.68 ± 0.14</td>
<td>1.61 ± 0.07</td>
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<td>HDL-cholesterol (mmol/L)</td>
<td>1.23 ± 0.24</td>
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<td>Mouse apoA-I (mg/mL)</td>
<td>1.05 ± 0.02</td>
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<td>Human apoA-I (µg/mL)</td>
<td>0</td>
<td>43 ± 9</td>
<td>44 ± 4</td>
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</table>

Data are presented as mean ± SEM, 6 mice/group.
Supplemental Figure I

![Graph showing plasma and fecal radiolabeled cholesterol and bile acids](image-url)
Supplemental Figure II

A. Plasma

B. Liver

C. Intestinal contents

$[^3]H$ radioactivity (% injected dose)

Vehicle  T0901317

p< 0.05
Supplemental Figure III

Expression of cholesterol transporters in J774 cells

Relative mRNA levels (% of control)

ABCA1   ABCG1   SR-BI

-48/80   +48/80

p= 0.05
Supplemental Figure IV

A

Cholesterol (mM)

0.00 0.05 0.10 0.15

B

Phospholipids (mM)

0 25 30 35 40

C

Peak I

Peak II

Mouse apoA-I (µg/mL)

Control
Non-stimulated
Stimulated

Mouse apoA-I
Supplemental Figure V

Human apoA-I in mouse plasma

A. HDL sub-populations

C. Total apoA-I in plasma

B. Human apoA-I WB of FPLC fractions

<table>
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<th># FPLC fraction</th>
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<th>33</th>
<th>34</th>
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<tbody>
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<tr>
<td>ApoA-I+48/80</td>
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