Cardiovascular disease including atherosclerosis continues to be one of the leading causes of mortality throughout the world (1). The accumulation of cholesterol-loaded macrophages is a critical step in the progression of atherosclerotic lesion development. This accumulation involves the uptake of free and esterified cholesterol by the cells from modified LDL (2). The excess free cholesterol is converted to cholesteryl ester by the enzymatic activity of ACAT and stored in lipid droplets. As cholesterol loading plays a critical role in atherosclerosis progression, there is great interest in understanding factors that prevent cholesterol accumulation either through decreased cellular cholesterol uptake or increased cholesterol efflux. Cholesterol loading engenders a complex network of changes in gene and protein expression, mostly furthering inflammation. Increased cholesterol efflux is the first step in reverse cholesterol transport (RCT), in which excess cholesteryl ester is hydrolyzed and transported through the plasma membrane by means of ABC transporters to extracellular acceptors, which carry the effluxed cholesterol through the plasma to the liver for conversion to bile acids for subsequent excretion in the feces (3). It is by means of this RCT that HDL and its associated apolipoproteins (e.g., apoA-I and apoE) are thought to exhibit some of their antiatherogenic properties and to furnish the basis of the epidemiologic inverse correlation between HDL levels and coronary artery disease (4–6). There is an extensive body of literature demonstrating the in vitro impact of RCT on the macrophages themselves, in other words, on the cholesterol balance within the macrophage foam cells. Recent work by Weibel and colleagues (9) has demonstrated the importance of considering both cholesterol influx and efflux when evaluating the impact of serum components on cholesterol homeostasis and atherosclerosis.

Abbreviations: A1\(^{-/-}\), apoA-I-deficient; AcLDL, acetylated LDL; BMDM, bone marrow-derived macrophages; C3\(^7\), C3\(^7\)BL/6; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; E\(^{-/-}\), apoE-deficient; LDLR\(^{-/-}\), LDL receptor-deficient; LXR, liver X receptor; PSG, penicillin/streptomycin/glutamine; RCT, reverse cholesterol transport; RPMI, Roswell Park Memorial Institute.

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The most widely used method for measuring in vivo macrophage cholesterol efflux has been the radiolabel method developed by the Rader group (10, 11). In this method, macrophages are loaded ex vivo with radiolabeled forms of nonesterified cholesterol and subsequently injected into the peritoneal cavity of the recipient animals. Variations of the protocol are also used in which the cells are injected subcutaneously rather than into the peritoneal cavity (12, 13). The levels of radiolabel in the plasma, liver, bile, and feces are then measured over time as a determinant of cholesterol transport out of the macrophages for elimination. This protocol has been valuable in determining the effects of apoA-I deletion and overexpression and other participants in HDL metabolism and RCT on cholesterol trafficking from peripheral cells to the feces. This protocol was originally developed using J774 cultured mouse macrophages; however, it is also suitable for use with primary macrophages overexpressing or lacking lipid transporters. The protocol has been used to show that ABC cholesterol transporters ABCA1 and ABCG1 promote in vivo macrophage cholesterol efflux, confirming not only the function of these transport proteins in culture but also the additive impact of their activity (14, 15). The scavenger receptor class B type I (SR-BI), however, has been the gold standard in confirming in vitro cholesterol efflux results (16–19). The scavenger receptor class B type I (SR-BI), however, was shown to have no in vivo macrophage cholesterol efflux activity using this method, despite in vitro results indicating it does promote cholesterol efflux (15). The role of other plasma apoproteins and enzymes (e.g., serum amyloid A, cholesteryl ester transfer protein, LCAT), as well as mutant forms of apoA-I (e.g., apoA-I Milano), in RCT have been confirmed in vivo using this method, and as such, it has been the gold standard in confirming in vitro cholesterol efflux results (16–19).

Other methods for determining in vivo macrophage cholesterol efflux have been examined including the centripetal cholesterol flux method in which dual radioisotopes of cholesterol precursors and labeled LDL cholesterol are administered and used to estimate the cholesterol efflux from the peripheral cells, with the assumption that the synthesis of cholesterol and uptake by peripheral cells is equal to the efflux (20). However, this method failed to show an effect of apoA-I and HDL on in vivo cholesterol transport and may not be specific enough to reveal cholesterol transport effects unique to macrophages (21–23). Recently, Turner et al. (24) have used constant infusion of [13C]cholesterol in humans to monitor the tissue free cholesterol efflux, esterification of free cholesterol in the plasma, and excretion of plasma-derived free cholesterol as fecal sterols. The measurement of fecal steroid levels has also been used to compare RCT on different genetic backgrounds or following interventions aimed at increasing RCT. The administration of apoA-I increased fecal steroid content in human studies, while the deletion of ABCA1 in mice had no effect on fecal steroid output (22, 23).

Despite the usefulness of the radiolabeled macrophage injection method, there are drawbacks. Recent studies suggest that the radiolabeled cholesterol does not accurately reflect the total cholesterol pool found in the macrophages (26). As only the injected macrophages are radiolabeled, a combination of radiolabel efflux and changes in cellular cholesterol mass are necessary to measure the net ingress/egress of cholesterol from the cells over the course of the experiment (9). Finally, as the injected cells move out of the peritoneal cavity, they are not recoverable for measurement of cellular cholesterol homeostasis. Weibel and colleagues (27) have addressed these issues with a novel method for assessing macrophage cholesterol trafficking in which nonlabeled macrophages are inserted into hollow fibers (0.2 µm pore size), which are then surgically implanted into the mouse peritoneal cavity for recovery after 24-48 h. They demonstrated that this method allows for modification of both macrophage and recipient genetic background, recovery of macrophages, and measurement of free and esterified cholesterol mass from the recovered macrophages without the need of radiolabel. Given the pore size of the fibers used, LDL particles were able to pass through the fiber allowing for evaluation of net uptake/efflux of cholesterol. We attempted to use this method and found the injection of the recommended 100 µl (3.5 × 10^6 cells) to be very difficult in 2 cm of 1 mm inner diameter Microkros hollow fiber, as was the recovery of viable cells. Given that the fiber is cylindrical, the total inner volume of the fiber \( \pi \times 20 \text{mm} \times (0.5 \text{mm})^2 \) would be 15.7 mm^3 or 15.7 µl. Additionally, this method requires surgical insertion of the hollow fibers rather than the simple injection of macrophages used with the Rader method.

We here present a novel method for assessing macrophage participation in cholesterol homeostasis that satisfies the above concerns. The method involves the in vivo entrapment of the macrophages in alginate. Alginites are naturally occurring polysaccharides derived from seaweed made up of copolymers of nonbranched \( \beta \)-D-mannuronic acid and \( \alpha \)-L-guluronic acid (28). The addition of a divalent cation such as calcium to alginic acid solution results in the formation of a gel of increasing rigidity depending on alginate concentration. The gel can additionally be disrupted with the addition of a citrate buffer to chelate the calcium ions. Because of its high biocompatibility, alginate is the most common material used in entrapment of islet cells for transplantation, with alginate implants being at least 50% recoverable after as long as 12 weeks in vivo (29). This combination of biocompatibility, stability, and ease of cell entrapment and harvest suggested that entrapment of macrophages in alginate might be a suitable method for the in vivo measurement of macrophage cholesterol homeostasis with the subsequent recovery and analysis of macrophages. This alginate-based method that we describe requires no surgery, and the macrophages are readily recovered at >95% cell viability.

**MATERIALS AND METHODS**

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. The J774 mouse macrophage cell line (J774A.1) was purchased from American Type Culture Collection (Manassas, VA). Acetylated human LDL (AcLDL) was

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**In vivo macrophage cholesterol homeostasis** 471
Prepared as described previously (30). Mouse plasma was run on fast-protein liquid chromatography as described previously (31).

Mice

WT C57BL/6j (C57), apoA-I-deficient (A1−/−), apoE-deficient (E−/−), and LDL receptor-deficient (LDLR−/−) mice were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were bred in a specific pathogen-free facility. Mice at 7–10 weeks of age were used for all experiments. All animals used in these experiments for in vivo analysis or as sources of plasma for in vitro experiments were maintained on standard chow diet. All mouse studies and euthanasia were performed in accordance with The Guide for the Care and Use of Laboratory Animals and National Institutes of Health guidelines and approved by the University of Chicago Institutional Animal Care and Use Committee.

Bone marrow-derived macrophage preparation

Bone marrow-derived macrophages (BMDMs) were prepared as described previously (32). Briefly, bone marrow cells were isolated by flushing the hind leg femur and tibia of WT C57 mice with DMEM + 10% FCS + 1% penicillin/streptomycin/glutamine (PSG). Cells were strained through a 100 µm strainer and pelleted by centrifugation at 40 g for 7 min. The pellet was resuspended in DMEM + 10% FCS + 1% PSG + 50% L929 conditioned media (33) and plated in 8 100 mm petri dishes per mouse (15 ml media/plate). After 4 days, 15 ml additional media was added. On day 7, the cells were washed 3× with room temperature PBS to remove nonadherent cells, and the adherent macrophages were detached by 30 min incubation on ice in cold PBS followed by scraping and counting. BMDM yield was ~50 million/mouse.

[^]H]cholesterol macrophage cholesterol efflux

Measurement of macrophage cholesterol efflux using [^3]H] cholesterol-loaded macrophages was done based off of the method of the Rader laboratory (10). J774 cells grown in DMEM + 10% FCS + 1% PSG were scraped from culture dishes and counted. The cells were resuspended in Roswell Park Memorial Institute (RPMI) media + 10% FCS + 1% PSG + 30% L929 conditioned media (33) and plated in 8 100 mm petri dishes per mouse (15 ml media/plate). After 4 days, 15 ml additional media was added. On day 7, the cells were washed 3× with room temperature PBS to remove nonadherent cells and the adherent macrophages were detached by 30 min incubation on ice in cold PBS followed by scraping and counting. BMDM yield was ~50 million/mouse.

Determination of ideal alginate concentration

To measure the ability of HDL or VLDL to move through the alginate bulbs, human HDL or VLDL was isolated from human plasma by density gradient centrifugation (HDL density 1.063–1.21, VLDL density <1.006) (34); 1 × 10^7 J774 cells were suspended in 0.5 ml alginate of varying concentrations (0.6–1.2% w/v in 0.9% NaCl), and the suspensions were polymerized by the addition of 1 ml 50 mM CaCl₂. For the HDL experiments the cells were loaded with 0.1 mg/ml AcLDL and 0.3 mM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (cGMP) for 24 h prior to entrapment in order to stimulate ABCA1 and ABCG1 cholesterol efflux. The alginate bulbs were transferred to individual wells of 24-well plates and incubated in 2 ml DMEM ± either 0.1 mg/ml human HDL or 0.2 mg/ml VLDL. After 24 h, the alginate bulbs were transferred to 2 ml polypropylene tubes and dissolved in 1.5 ml citrate buffer (20 mM Na citrate/150 mM NaCl pH 7.8) by 30 min rotation at room temperature to release the cells. The cells were washed 2× with PBS, and cell pellets frozen for later quantitation of the cell protein and total and free cholesterol in each sample as described below.

Preparation of macrophages for alginate entrapment

A 0.8% (w/v) solution of sodium alginate (Sigma #71283) was prepared in 0.9% (w/v) NaCl and sterilized by autoclave. J774 cells grown in DMEM + 10% FCS + 1% PSG were scraped from culture dishes and counted. To prepare cells for entrapment into alginate, J774 or BMDM cells were resuspended at 1–3 million cells/ml DMEM + 10% FCS + 1% PSG. For experiments comparing the effects of the liver X receptor (LXR) agonist TO901317, no FCS was included in the media. Resuspended cells were incubated in 250 ml Teflon-coated growth flasks with gentle swirling three to four times over the course of 24 h to prevent readherence. CPT-cAMP (0.3 mM) was added to the J774 media during the 24 h incubation to induce ABCA1 cholesterol transporter expression. To compare the effects of cholesterol transporter expression in BMDM, TO901317 (10 µM) was added to the BMDM media during the 24 h incubation. For cholesterol enrichment, 0.05–0.1 mg/ml AcLDL was added to the 24 h media. Total cellular cholesterol levels following enrichment were dependent on AcLDL batch. For radiolabeled [^3]H]cholesterol experiments, 1 µg/ml[^3]H]cholesterol was included in the 24 h media. After 24 h, the cells were washed 3× with PBS and counted. Cells were resuspended at 2–5 million cells/ml in 0.8% alginate solution and immediately used in either in vitro or in vivo cholesterol-trafficking experiments.

In vitro cholesterol efflux from alginate-entrapped macrophages

Macrophages in 0.5 ml alginate were entrapped in 2 ml polypropylene tubes by the addition of 1 ml sterile 50 mM CaCl₂, which immediately converted the liquid alginate solution into a gel. No cells were lost in the remaining liquid solution. The bulbs were transferred using sterile forceps to individual wells of 12-well tissue culture dishes containing 2 ml DMEM + 2.5% freshly isolated serum from each mouse genetic background as a cholesterol donor/acceptor. After a 24 h incubation, the bulbs were harvested from the media and dissolved in Na citrate buffer and washed, and the cell pellets were frozen as described above. Cell viability after 24 h was >95% as determined by trypan blue exclusion.

In vivo macrophage cholesterol trafficking by the alginate method

Mice of varying genetic backgrounds were anesthetized by isoflurane inhalation and injected subcutaneously along the dorsal region midway between the head and tail with 1 ml sterile 50 mM CaCl₂ with the needle inserted parallel to the spinal column. This created a liquid bubble into which macrophages in 0.5 ml alginate were injected, again keeping the needle parallel to the spinal column. A solid gel was detectable by touch immediately following the alginate injection. Twenty-four hours postinjection, the mice were euthanized by CO₂ inhalation, and the alginate bulbs removed by making a dorsal incision in the skin proximal to the tail. The skin was then peeled back to reveal the membrane-coated alginate bulb. An incision was made in the membrane coating allowing the alginate bulb to be removed with forceps. Cells were harvested by dissolving the alginate bulb in 7 ml of citrate buffer rotating 30 min at room temperature. The cell suspension was then filtered through a 100 µm cell strainer,
cholesterol loss from the alginate-entrapped macrophages revealed that HDL was able to pass through the pore sizes of all of the alginate concentrations (Fig. 1A). The ability of VLDL to load alginate-entrapped macrophage cells with cholesterol was determined by incubating 0.6–1.2% alginate-entrapped J774 mouse macrophage cells in media containing isolated human VLDL. There was significant cholesterol loading into the cells at all of the tested alginate concentrations with 0.8% and 1.0% alginate allowing the most significant increase in cellular cholesterol.

Cholesterol extraction and quantitation

Cell pellets were extracted 2× with 2 ml 3:2 hexane-isopropanol with a 1 min vortex followed by rotating 1 h at room temperature. Cell pellets were spun down 5 min at 16,000 g, and the solvent was transferred to a 2 ml borosilicate screw cap vial and dried under air, combining sequential extractions. Dried extracts were resuspended in 0.2 ml 9:1 isopropanol (molecular biology grade)-Tergitol and frozen for later analysis (35). Extracted cell pellets were resuspended in 2 ml 0.1 N NaOH and incubated 1 h at 37°C to dissolve protein and frozen for later analysis.

For [3H]cholesterol-label measurements, the resolubilized extracts were run on TLC plates (Baker-flex silica gel IB2-F; Thermo Scientific, Waltham, MA) using 70:30:1 hexane-diethyl ether-acetic acid as a running solvent. Plates were stained with iodine vapor, and the free and esterified cholesterol dots cut out and counted in UltimaGold scintillation fluid (Perkin Elmer, Waltham, MA) as described previously (31).

For cholesterol mass measurements, total cholesterol was measured using 20 µl extracted sample and 20 µl cholesterol standard (0–1,000 mg/ml) in 9:1 isopropanol-Tergitol and 200 µl Chol reagent (Roche Diagnostics, Indianapolis, IN). Free cholesterol was measured using 200 µl Free Chol E reagent (Wako Diagnostics, Richmond, VA) in place of Chol reagent. Total protein was measured using Pierce BCA protein assay (Pierce, Rockford, IL).

Statistical analysis

Statistical analysis was done by ANOVA using StatView software. Both Fisher’s protected least significant difference and post hoc Tukey/Mann were used in analysis. A P value of <0.05 was considered significant.

RESULTS

Alginate matrices (2% w/v) have been shown to have a pore size of 8–10 nm, which is within the size range of both mouse and human HDL (36). Lowering the alginate concentration increases the pore size (37). Leal-Egaña and colleagues (38) have determined the pore size distribution of different concentrations of alginate gels using both N₂ adsorption and image analysis. At both 0.8% and 1.4% alginate (w/v), the pore-size distribution ranged from <10 nm up to 70 nm, with the average pore size being greater at 0.8% alginate. The size of the largest plasma lipoprotein, VLDL, ranges from 51 to 62 nm depending on fasting status (39). To confirm that alginate would be suitable for monitoring lipoprotein transport, J774 cells were entrapped in alginate bulbs using alginate concentrations ranging from 0.6% to 1.2%. Alginate concentrations at 0.6% and below did not form a gel that was easily grasped with forceps. The bulbs were incubated for 24 h in media containing either human HDL or VLDL. For the HDL experiments, the cells were loaded with 0.1 mg/ml AcLDL and CPT-cAMP for 24 h prior to entrapment in order to stimulate ABCA1 and ABCG1 cholesterol efflux. Human HDL is expected to be the major lipoprotein involved in cholesterol export from macrophages, while VLDL is the largest expected cholesterol donor. Measurement of cholesterol loss from the alginate-entrapped macrophages revealed that HDL was able to pass through the pore sizes of all of the alginate concentrations (Fig. 1A). The ability of VLDL to load alginate-entrapped macrophage cells with cholesterol was determined by incubating 0.6–1.2% alginate-entrapped J774 mouse macrophage cells in media containing isolated human VLDL. There was significant cholesterol loading into the cells at all of the tested alginate concentrations with 0.8% and 1.0% alginate allowing the most significant increase in cellular cholesterol.

Fig. 1. Lipoprotein diffusion through alginate bulbs. A: HDL transfer through 0.6–1.2% alginate bulbs. AcLDL-loaded J774 cells stimulated with 0.3 mM CPT-CAMP were entrapped in 0.6% to 1.2% alginate and incubated in DMEM ± 0.1 mg/ml HDL as a cholesterol acceptor. After 24 h, the bulbs were dissolved, and the cells washed 3× and extracted with 3:2 hexane-isopropanol. Cell cholesterol and protein was measured as described in Materials and Methods. B: VLDL transfer through alginate bulbs. J774 cells entrapped in 0.6% to 1.2% alginate were incubated in DMEM ± 0.2 mg/ml VLDL as a cholesterol donor. After 24 h, the bulbs were dissolved and the cells washed 3× and extracted with 3:2 hexane-isopropanol. Cell cholesterol and protein was measured as described in Materials and Methods. P values for total cholesterol are above the column bar. P values for free and esterified cholesterol are within the column bars. * P < 0.05 versus control; † P < 0.005 versus control; ‡ P < 0.0005 versus control. Data are expressed as mean ± standard deviation of three to four samples.
(Fig. 1B). Given these results and the difficulty in grasping alginate gels at or below 0.6%, 0.8% alginate was chosen as the alginate concentration for all further experiments. Cell viability by trypan blue exclusion was found to be >95% after 24 h of cell entrapment (data not shown).

We next sought to determine whether we would be able to detect a difference in free and esterified cholesterol mass remaining in alginate-entrapped macrophages following incubation with serum containing cholesterol acceptors from WT mice or serum deficient in the cholesterol acceptor apoA-I from *Apoa1* knockout (A1*/*−−*) mice. The plasma cholesterol and lipoproteins of the WT and A1*/*−−* mice have been widely characterized, and our data are in agreement with the previously reported results (40, 41).

The serum cholesterol levels of the two mice were significantly different (WT = 67.5 ± 6.9 mg/dl, A1*/*−−* = 24.7 ± 2.8 mg/dl), with the majority of the cholesterol found in the HDL fraction of both mice (87% HDL in WT, 73% HDL in A1*/*−−*), although A1*/*−−* HDL is larger in size (data not shown). The majority of the cholesterol in the serum is esterified in both mice (WT = 54.5 ± 6.3 mg/dl, A1*/*−−* = 17.8 ± 2.2 mg/dl). [3H]cholesterol-labeled J774 macrophages with or without cholesterol preloading with AcLDL were either plated without alginate or entrapped in 0.8% alginate and incubated for 24 h in media ± 2.5% serum from the WT or knockout mice. Comparisons between plated cells and alginate-entrapped cells incubated 24 h in serum free media demonstrated that cell entrapment alone does not alter cellular cholesterol levels (data not shown). In the presence of WT or A1*/*−−* serum, the overall % efflux was higher in the nonencapsulated cells. However, in both plated cells and cells entrapped in alginate, there was significantly less [3H]cholesterol efflux to the serum that lacked apoA-I (Fig. 2A). This was true in both the cholesterol normal and cholesterol-enriched macrophages indicating that although the alginate does restrict some cholesterol movement out of the cells, it still allows for meaningful comparison between the diffusing mouse sera. The differences in [3H]cholesterol efflux between WT and A1*/*−−* plasma did not have a dramatic impact on cellular [3H]cholesterol levels, especially in the +AcLDL cholesterol-loaded cells (Fig. 2B). In the absence of AcLDL, cholesterol loading, the postefflux cellular [3H]cholesterol was measurably different between WT and A1*/*−−* serum treatment, but the difference was only significant in the nonentrapped cells. Recent studies by Weibel and colleagues (9, 26) have indicated that the measurement of [3H]cholesterol efflux from cells is not necessarily a good indicator of changes in cellular cholesterol mass because the [3H]cholesterol efflux does not account for cholesterol influx. We additionally measured the changes in cellular cholesterol mass in both free and alginate-entrapped J774 cells after incubation with WT and A1*/*−−* serum. In agreement with the results of Weibel and colleagues, the cholesterol mass changes do not reflect the changes in [3H]cholesterol label. In the absence of AcLDL cholesterol loading, incubation of the cells with WT serum produced no measureable change in cellular cholesterol levels, while A1*/*−−* serum resulted in a loss of cholesterol (Fig. 2C). Although this loss did not reach significance in the alginate-entrapped cells, the trend was similar to the nonentrapped cells. In cells preloaded with cholesterol, cellular cholesterol loss was similar for both WT and A1*/*−−* in both free and alginate-entrapped cells (Fig. 2D). The nonentrapped cells had a greater loss of cholesterol relative to controls due mainly to a greater loss of esterified cholesterol. Taken together, the data do indicate that the impact of apoA-I deficiency on [3H]cholesterol efflux and cellular cholesterol mass can be assessed in cells entrapped in alginate, but the magnitude of the cholesterol mass effect may be impacted by the cell entrapment at the levels of serum used here. It is clear that the alginate-entrapped cells were able to undergo measurable lipoprotein-mediated changes in cellular cholesterol, and so we proceeded to test our methodology in vivo.

The methodology used for in vivo macrophage entrapment is described in Materials and Methods and depicted in Fig. 3. Using this method, the alginate bulb formed almost instantaneously as measured by tactile manipulation of the bulb that is formed subcutaneously. The injected mice fully recovered in the same amount of time as noninjected mice that were subjected to isoflurane anesthesia (data not shown). The total time from the start of injection until full recovery was less than 2 min per mouse. The harvest of the alginate bulb is also rapid, with the bulb at the time of harvest being a firm gel that is easily removable by forceps after cutting into the subcutaneous space (Fig. 3C). All of the experiments reported here were harvested after a 24 h time postinjection. Cells were counted before injection and in the alginate bulbs harvested 24 h postinjection, and no significant difference in cell number was observed, indicating minimal migration of cells into and out of the gel (data not shown). Forty-eight-hour time points were also tested, and no significant difference in cell survival was found (data not shown).

When intraperitoneally injected J774 cells loaded with AcLDL and [3H]cholesterol are used for the standard macrophage-to-feces cholesterol transport assay of the Rader group using WT and *Apoa1*/*−−* mice, less [3H]cholesterol label from the injected macrophages is found in the apoA-I deficient mouse plasma than in the WT (Fig. 4A). Similar results have been reported by other laboratories (19, 42). This suggests less efflux from the macrophages injected into mice deficient in apoA-I, although the macrophages cannot be recovered for cholesterol measurement using this method. We expected then that using the alginate method, there would be more cholesterol in the reisolated macrophages that were injected into mice deficient in apoA-I. Similar to the standard in vivo macrophage efflux assay of the Rader group, which uses nonrecoverable macrophages, using the alginate method with J774 cells loaded with [3H]cholesterol, over 24 h the WT mice had significantly more [3H]cholesterol in the plasma than the knockout mice (Fig. 4B). Similar results were observed regardless of whether the cells were preloaded with cholesterol using AcLDL. Reisolation of the macrophages from the alginate bulbs after 24 h demonstrated the loss of [3H]cholesterol from the entrapped cells (Fig. 4C). There was a significant
In vivo macrophage cholesterol homeostasis.

Indeed, in the in vivo alginate-entrapped cells, the cholesterol mass changes do not reflect the observed cellular \(^{3}H\)cholesterol loss, with the total \(^{3}H\)cholesterol radiolabel demonstrating much greater differences between WT and A1\(^{-/-}\) recipients than the total cholesterol (Fig. 4D). In the absence of cholesterol preloading with AcLDL, there is significantly greater loss of macrophage total cholesterol mass in A1\(^{-/-}\) mice than in WT, mainly due to less cholesteryl ester mass in the A1\(^{-/-}\) mice. In fact, despite no significant change in total cholesterol in the cells injected into WT mice (compared with 0 h controls), there is an unexpected gain in the amount of esterified cholesterol mass. When the macrophages are preloaded with AcLDL, there is a significant loss of total cell cholesterol mass in both WT and A1\(^{-/-}\) recipients compared with 0 h controls, but no difference in total cholesterol between WT and A1\(^{-/-}\) recipients. The cellular cholesterol balance, however, is different, with macrophages
Fig. 3. In vivo alginate cell cholesterol-trafficking assay. Mice of varying genetic background are injected subcutaneously with 50 mM CaCl₂ (A) followed immediately by injection of macrophages in 0.8% alginate (B), which entrap the macrophages in a subcutaneous solid alginate gel. After 24-48 h, the mice are euthanized by CO₂ inhalation, and the dorsal skin is peeled back to reveal the solid alginate bulb gel in which the macrophages are entrapped (C1). Bulbs harvested from triplicate mice shown (C2). C3: Light microscopy 20× magnification of 1.5 mm cross-section of 0.8% alginate bulb containing 3 million cells. The bulb is dissolved in citrate buffer to release the macrophages (D), which are washed and measured for protein and free and esterified cholesterol after lipid extraction (E).

Injected into WT mice yielding no net change in cholesteryl ester but a significant net loss of free cholesterol mass. The macrophages injected into A1⁻/⁻ mice had a net loss in both free and esterified cholesterol.

The differences in cell [³H]cholesterol loss and changes in cellular cholesterol mass were assessed by measuring the change in specific activity consequent upon the presence of both cholesterol donor and acceptor lipoproteins in the in vivo environment. While we have shown that cholesterol is removed from the sequestered cells, they are also capable of both taking up plasma lipoprotein cholesterol or synthesizing cholesterol, both of which would result in a dilution of specific activity of the cellular cholesterol (Table 1). Indeed the cellular cholesterol specific activity is substantially diluted in vivo in cells both with and without cholesterol preloading. The specific activity reduction is more profound in the WT animals than in those lacking apoA-I. These changes in specific activity are important considerations in examining the impact of genetic background on macrophage cholesterol homeostasis, and the results highlight the versatility of this methodology. Overall these results may be a reflection of the overall effects of genetic background on cholesterol influx/eflux and rate of cholesterol esterification/hydrolysis or cholesterol synthesis. These results also support the need for the combined measurements of both cholesterol radiolabel and cholesterol mass in gaining an accurate understanding of in vivo macrophage cholesterol homeostasis.

The impact of a hypercholesterolemic environment is an important consideration in methodologies that investigate macrophage cholesterol homeostasis. Murine apoE deficiency results in elevated plasma VLDL and LDL levels and increased atherosclerotic lesion development (43). The impact of apoE deficiency was investigated in alginate-entrapped macrophages in culture using serum from E⁻/⁻ mice as well as in vivo using the alginate methodology (Figs. 5 and 6). In both plated [³H]macrophages and [³H]alginate macrophages entrapped in 0.8% alginate, [³H]cholesterol % efflux and cellular [³H]cholesterol loss were similar between serum from WT and E⁻/⁻ mice (Fig. 5A, B). As also observed in Fig. 2, cells entrapped in alginate had less overall loss of [³H]cholesterol, but this did not greatly impact the comparison between WT and E⁻/⁻. In both plated and alginate-entrapped cells, incubation with apoE-deficient serum resulted in only a small increase in cellular total cholesterol mass (Fig. 5C).

Given the higher plasma cholesterol levels of E⁻/⁻ mice compared with WT (WT = 67.5 ± 6.9 mg/dl, E⁻/⁻ = 409 ± 48 mg/dl), we expected the cellular cholesterol mass increase to be much greater in cells incubated with serum from E⁻/⁻ mice. However, the circulating cholesterol in E⁻/⁻ mice is mainly found in VLDL/remnants (43, 44). Whitman and colleagues (45) have previously demonstrated that incubation of E⁻/⁻ VLDL with macrophages results in little change in cellular cholesterol, whereas incubation of macrophages with LDL results in a significant increase of cellular cholesterol, mainly as esterified cholesterol. Additionally, Perrey and colleagues (46) have demonstrated that while VLDL from E⁻/⁻ mice is poorly taken up by macrophages, VLDL from LDLR⁻/⁻ mice (which possess apoE) is actively taken up into macrophages. To be certain that our alginate system was functional in a cholesterol-enriched in vivo environment, we used the alginate method to compare WT and E⁻/⁻ mice, as well as LDLR⁻/⁻ mice (total plasma cholesterol: 209.5 ± 8.5 mg/dl) in which the plasma cholesterol is mainly found in LDL (47) (Fig. 6). All mice were maintained on a standard chow diet (Harlan Laboratories diet #2918, Madison, WI). The [³H]cholesterol in the plasma after 24 h was modestly higher in the LDLR⁻/⁻ mice and lower in the E⁻/⁻ mice when compared with WT mice (Fig. 6A), which may be a reflection of the increased HDL levels in LDLR⁻/⁻ mice and decreased HDL levels in E⁻/⁻ mice (48, 49). These changes were not significantly reflected in cellular [³H] cholesterol levels (Fig. 6B). There was no significant difference in cholesterol mass between the WT and E⁻/⁻ recipient mice, although the total cholesterol mass tended to be higher in E⁻/⁻ mice (P = 0.0586) (Fig. 6C). On the other hand, entrapment of the macrophages in LDLR⁻/⁻ mice resulted in nearly a 2-fold increase in cellular cholesterol mass, mainly as cholesteryl ester. One of the goals of this methodology would be to use it in measuring cholesterol trafficking from primary macrophages with the idea that macrophages with genetic deletions or overexpression of proteins involved in cholesterol trafficking could be examined.
cholesterol mass changes between WT and A1/H11002-injected cells are similar. The impact of alterations in macrophage protein expression on cholesterol homeostasis was also examined using the alginate method. Expression of the LXR inducible cellular cholesterol transporters ABCA1 and ABCG1 has been shown to increase the in vivo efflux of [3H]cholesterol out of macrophages (15). We compared the cholesterol homeostasis of BMDM injected into WT mice using the alginate method with and without pretreatment of the BMDM with the LXR agonist TO901317 (Fig. 8). This agonist increases both ABCA1 and ABCG1 cellular expression and increases cellular cholesterol efflux (15, 50). Preincubation of BMDM with TO901317 prior to alginate entrapment resulted in significantly increased plasma [3H]cholesterol after 24 h in WT mouse recipients but no increase in A1/H11002 recipients (Fig. 8A). This was reflected in.

As such, the methodology was further tested with C57 WT BMDM (Fig. 7). In nonloaded cells (−AcLDL) in the WT recipient background, although there was no impact on total cellular cholesterol, there was a significant effect on overall cholesterol balance, with a significant increase in the cellular cholesterol that was esterified and a concomitant decrease in cellular free cholesterol. This change also resulted in significantly more esterified cholesterol in the cells injected into WT mice than into A1−/− mice. The A1−/− mice had less total cholesterol, although the difference did not reach significance. When the BMDM cells were preloaded with AcLDL cholesterol, they lost similar levels of total cholesterol mass when injected into WT or A1−/− mice; however, the resulting esterified cholesterol levels were significantly less when injected into A1−/− than into WT mice. Additionally, when comparing the results of J774 macrophages (Fig. 4D) with BMDMs, the pattern of cholesterol mass changes between WT and A1−/−-injected cells are similar.

The impact of alterations in macrophage protein expression on cholesterol homeostasis was also examined using the alginate method. Expression of the LXR inducible cellular cholesterol transporters ABCA1 and ABCG1 has been shown to increase the in vivo efflux of [3H]cholesterol out of macrophages (15). We compared the cholesterol homeostasis of BMDM injected into WT mice using the alginate method with and without pretreatment of the BMDM with the LXR agonist TO901317 (Fig. 8). This agonist increases both ABCA1 and ABCG1 cellular expression and increases cellular cholesterol efflux (15, 50). Preincubation of BMDM with TO901317 prior to alginate entrapment resulted in significantly increased plasma [3H]cholesterol after 24 h in WT mouse recipients but no increase in A1−/− recipients (Fig. 8A). This was reflected in
cellular \[^3\text{H}\]cholesterol levels, with +TO901317 cells having decreased \[^3\text{H}\]cholesterol levels in WT mice but no change in A1\(^{-/-}\) mice (Fig. 8B). Total, free, and esterified cellular cholesterol mass were significantly decreased in +TO901317 cells entrapped in WT mice, but no change was seen between cells entrapped in A1\(^{-/-}\) (Fig. 8C). Additionally, preincubation with TO901317 altered the free:esterified cholesterol ratio in BMDM entrapped in WT versus A1 mice.

### DISCUSSION

We believe that the method described here has the potential to provide a comprehensive approach to in vivo cholesterol trafficking at the level of the macrophage that is the result of the net flux of cholesterol in and out of these cells. Numerous methods have been developed in an attempt to quantify RCT in vivo. These methods include the most commonly used peritoneal injection of macrophages labeled with \[^3\text{H}\]cholesterol, the centripetal cholesterol efflux method, the fecal sterol output method, and the hollow fiber method (10, 20, 27). Although each of these methods have their merits, there are several concerns. Both the peritoneal injection method and the centripetal cholesterol efflux method involve the use of radiolabeled cholesterol, which alone may not serve as an accurate reflection of the bidirectional flow of cholesterol into and out of peripheral cells, and more specifically macrophages. Additionally, the use of radiolabeled cholesterol alone does not give dynamic indication of changes in the free and esterified cholesterol mass of the macrophages. Of all of these methods, only the hollow fiber method allows for recovery of macrophages for analysis of cholesterol flux at the cellular level (27).

Prior to the development of the alginate method reported here, we attempted to recover macrophages injected into the peritoneal cavity by several methods. We first used a protocol in which macrophages were labeled by phagocytosis of fluorescent beads prior to peritoneal injection. At early time points (less than 1 h following injection), the cells were largely recoverable. However, at longer time points necessary to observe cholesterol-trafficking activity, the cells were not recoverable from the peritoneal cavity, presumably due to migration of the injected cells out of the peritoneum.

We also attempted to use the hollow fiber method to measure macrophage cholesterol trafficking with the recovery of the injected macrophages. We found the injection of the recommended volume of cells into the Microkros hollow fiber to be very difficult, as was the recovery of viable cells. Additionally, the hollow fiber method requires training and institutional animal care and usage approval in surgical procedure for each technician who would be utilizing the protocol, as well as monitoring of the animals throughout the surgical recovery time. Using the alginate method, we have routinely used anywhere from \(1 \times 10^6\) to \(5 \times 10^6\) cells in 500 \(\mu\)l of alginate for injection without problem. Alginate volumes as low as 250 \(\mu\)l have also been found to produce an easily excisable bulb in vivo. Alginate volumes larger than 500 \(\mu\)l are likely acceptable because the bulbs easily form in vitro, although we have not tested this in vivo. Additionally, the alginate method requires no survival surgery with the whole injection procedure done on an isoflurane-anaesthetized mouse in less than 1 h. Mouse recovery time is no different than a noninjected isoflurane-anaesthetized mouse (1–2 min). The time of training for our technicians was minimal, as the procedure requires only two simple subcutaneous injections. There is also no manual handling of the samples as occurs with the hollow fiber method, which aids in maintaining the sterility of the samples. In addition, the presence of the alginate bulb in the back of the animal had no effect on the observed mobility of the animal.

The use of alginate in biological roles has been increasing with its use in controlled release of small-molecule drugs and peptides as well as in cell entrapment for continual delivery of therapeutics (51–53). The results of the alginate method when using \[^3\text{H}\]cholesterol as the tracer were in agreement with both our results and the previously reported results using the peritoneal injection of nonentrapped radiolabeled J774 cells, with apoA-I deficiency resulting in decreased plasma \[^3\text{H}\]cholesterol (19, 42). Each of these reports makes use of AcLDL-loaded macrophages as the injected cells. Our results indicate that the preloading of the macrophages with AcLDL does not greatly affect the relative levels of free cholesterol transport of the macrophage \[^3\text{H}\]cholesterol to the plasma in WT or A1\(^{-/-}\) mice over 24 h but significantly alters the changes in the injected macrophage cellular cholesterol mass in these mice (Fig. 4).

### TABLE 1. Specific activity of 24 h in vivo alginate-entrapped cells as a percent of 0 h control

|                  | Specific Activity (% of 0 h) | P versus 0 h | P versus WT | P versus AcLDL |
|------------------|-----------------------------|--------------|-------------|-----------------|
| −AcLDL 0 h       | 100 ± 14.26                 |              |             |                 |
| WT 0 h           | 26.96 ± 5.46                | \(P < 0.0001\) | \(P < 0.0001\) | \(P < 0.0001\) |
| ApoA1\(^{-/-}\)  | 60.52 ± 8.76                | \(P < 0.0001\) | \(P < 0.0001\) |                 |
| +AcLDL 0 h       | 100 ± 8.07                  | \(P < 0.0001\) | \(P < 0.0001\) | \(P < 0.0001\) |
| WT 0 h           | 36.41 ± 9.00                | \(P < 0.0001\) | \(P < 0.0001\) | \(P = 0.1430\) |
| ApoA1\(^{-/-}\)  | 62.19 ± 11.63               | \(P < 0.0001\) | \(P = 0.0007\) | \(P = 0.8023\) |

Specific activity is measured as total cell \[^3\text{H}\]cholesterol (cpm) per total cell cholesterol (mg). Data are expressed as mean ± standard deviation of four to five samples.
This results in the total cholesterol specific activity being more profoundly affected in cells that were not pre-loaded with AcLDL. That there was no difference in total cholesterol between AcLDL-loaded cells injected into WT and A1/−/− was surprising. Also surprising was the greater loss of total cholesterol in nonloaded cells injected into A1/−/− mice than into WT mice. It is possible that under conditions in which the macrophages are highly loaded with cholesterol, plasma apoproteins other than apoA-I are able to compensate for their deficiency. Although the reasons for this observed effect are outside the scope of this study, it does merit future investigation, and the alginate entrapment methodology described here provides a useful tool for exploring these questions.

The large differences in total cholesterol mass compared with [3H]cholesterol in the macrophages as manifested by the changes in cell cholesterol specific activity following the alginate entrapment method (Fig. 4C, D) highlights the limitations of using only radiolabeled cholesterol as a surrogate for cholesterol homeostasis. The bidirectional flow of cholesterol into and out of the cell is not fully appreciated using the radiolabel alone, and the total cholesterol mass at the end of the in vivo culture may be unchanged while the free:ester cholesterol ratio has changed. The combination of mass measurements with the use of labeled cholesterol and the determination of ending cholesterol specific activity allows for an assessment of the dynamics of cholesterol movement in and out of macrophages under a variety of physiological circumstances, which may be critical in determining the impact of plasma components on cholesterol metabolism and atherosclerosis (9). The WT and A1/−/− animals have very different plasma lipid levels and lipoprotein composition. Although HDL is the major lipoprotein in both WT and A1/−/− mice, the protein and lipid components of the HDL are altered with A1/−/− mice having lower total HDL cholesterol, larger HDL particle size, and an increase in apoE, apoA-IV, apoCs, and apoA-II compared with WT HDL (40, 41, 54, 55). Additionally, in WT HDL, apoA-I acts to increase SR-BI selective uptake of cholesteryl ester compared with HDL from A1/−/− mice (41). It is striking that the specific activity of the residual cholesterol is much higher in the cells transferred to the A1/−/− mouse. This is likely due to less influx into cells in this context, and this in turn may in part reflect the lower plasma cholesterol of the A1/−/− mice that could contribute to the influx into the macrophages. Other factors such as increase in non-apoA-I apoproteins, which can act as cellular cholesterol efflux agents, combined with the decrease in selective uptake of plasma cholesteryl ester could also contribute to changes in cholesterol homeostasis in these cells. These factors merit further investigation.

Another surprising result was obtained with the in vivo incubation of nonloaded macrophages in mice deficient in apoE or the LDLR. Given the high levels of apoB-containing lipoproteins found in the plasma of E/−/− mice, we initially expected the total cholesterol mass to increase in
macrophages injected into the mice. However, only the cells injected into LDLR−/− mice demonstrated a significant increase in cellular cholesterol mass compared with WT mice. This agrees with the work of Whitman et al. (45) in which VLDL from E−/− mice did not greatly increase the cellular cholesterol levels of J774 cells in vitro while LDL greatly increased the cellular cholesterol, mainly esterified cholesterol. Cholesterol in E−/− mice is mainly found in VLDL, whereas in LDLR−/− mice it is mainly found in LDL (43, 44, 47). Furthermore, the presence of apoE on the VLDL is important for its efficient cellular uptake (46). This again highlights the benefit of the alginate method’s ability to examine cellular cholesterol mass changes rather than only effluxed labeled cholesterol. The results obtained using BMDMs in the presence or absence of TO901317 further emphasize the utility of this methodology, indicating that this method is sensitive not only to the effects of the recipient animal genetic background on cellular cholesterol homeostasis, but also to changes in protein expression levels within the macrophages themselves.

It is clear that this methodology can serve as a platform for a more intensive analysis of cholesterol homeostasis in macrophages in vivo. The ability to recover injected macrophages for measurement of the final cellular cholesterol radiolabel and mass is a valuable tool for investigating animal models in which the fecal sterol data and cholesterol efflux data do not agree, with the goal of determining how each step in the RCT system...
In vivo macrophage cholesterol homeostasis may relate to atheroprotection. The findings obtained with this approach may possibly represent a closer parallel to the cholesterol homeostasis in the intracellular macrophages of atherosclerotic vessels than can be deduced from the more conventional approaches to RCT.

Overall, the alginate entrapment methodology that we describe here provides a simple and effective method for the measurement of in vivo macrophage cholesterol homeostasis. This method does not require survival surgery, is simple to learn, and requires negligible recovery time of the mice. Additionally, both cultured cells as well as primary macrophages can be used with this method, which allows for the exploration of the effects of gene deletion or overexpression in the cells used for entrapment. The analysis of the free and esterified cellular cholesterol mass following harvest of the entrapped cells can be done using simple enzymatic colorimetric assays without the need for mass spectrometry or radioactivity. The ability of both HDL and LDL/VLDL to move into the alginate bulbs allows for the measurement of both cholesterol uptake as well as efflux to and from the entrapped cells. Alginate is one of the most widely used substrates for in vivo cell entrapment, and its biocompatibility and stability have been widely documented. Although the studies done here have been in mice, this method can easily be modified for use in other animals. Studies using alginate-entrapped cells have shown that the bulbs are at least partially recoverable even after as much time as 12 weeks, indicating that this methodology is amenable to long-term diet or drug treatment studies (29) and a useful tool in the assessment of novel antiatherogenic therapies.

This alginate methodology has been developed to probe macrophage cholesterol homeostasis, but it has great potential for many other investigations of the behavior of macrophages and other cells in particular genetic environments. For example, the influence of in vivo environments on gene expression in macrophages of WT or mutated genetic phenotype can be probed. Indeed, the cross-talk between the genes of the sequestered cells and of the host can be studied. The one limitation of this approach is that communication between the sequestered cells and the host must be mediated by molecules capable of passing the alginate bulb wall. However, more than one cell type may be sequestered and recovered after being subject to a particular host environment. This emphasis is significant because it allows communication between the sequestered cells and the host to be mediated by molecules capable of passing the alginate bulb wall. However, more than one cell type may be sequestered and recovered after being subject to a particular host environment.

Fig. 8. In vivo cholesterol trafficking in BMDM induced with LXR agonist TO901317 using alginate entrapment method. BMDMs from WT mice were prepared as described in Materials and Methods and grown in suspension for 24 h in the presence of 1 μCi/ml [3H]cholesterol and with or without 20 μM TO901317 to induce cholesterol transporter expression. Equal cell numbers (5 × 10⁶) in 0.8% alginate of BMDM ±TO901317 were injected into WT mice using the in vivo alginate entrapment method. After 24 h, the alginate bulbs were harvested and dissolved, and the cells washed 3× and extracted with 3:2 hexane-isopropanol. Cell cholesterol and protein was measured as described in Materials and Methods. A: Plasma [3H]cholesterol label at 24 h using the alginate entrapment method. B: BMDM cellular radiolabel after 24 h following the alginate entrapment method. C: BMDM cellular free and esterified cholesterol mass after 24 h following the alginate entrapment method. Cellular cholesterol values are expressed relative to respective 0 h ±TO901317 controls. P values for total cholesterol are above the column bar. P values for free and esterified cholesterol are within the column bars. * P < 0.05 versus WT; ** P < 0.005 versus WT; *** P < 0.0005 versus WT; * P < 0.05 versus –TO901317; ** P < 0.005 versus +TO901317. Data are expressed as mean ± standard deviation of three to four samples.
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