Leukocyte low density lipoprotein receptor (LDL-R) does not contribute to LDL clearance in vivo: bone marrow transplantation studies in the mouse

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Abstract The targeted disruption of the low density lipoprotein (LDL) receptor gene in mice results in accumulation of plasma LDL cholesterol and in predisposition to diet-induced aortic atherosclerosis. Although the liver is the central organ for receptor mediated clearance of LDL, the in vivo role of other organs and tissues in LDL catabolism has not been directly studied. Since bone marrow-derived cells such as blood leukocytes and tissue macrophages express LDL receptors and contribute a large cell mass to the body, we designed bone marrow transplantation (BMT) experiments to reconstitute LDL receptor null mice [LDL-R(−/−)] with marrow obtained from LDL-R wild-type mice [LDL-R(+/+)] and evaluate the effects on parameters of plasma lipid metabolism. Although reconstitution of the transplanted mice with donor bone marrow cells was complete, no differences in plasma lipid levels and lipoprotein distribution were found between groups, irrespective of the diet used, and turnover studies using ^{125}I-labeled LDL showed that LDL receptor expression by leukocytes and macrophages does not significantly contribute to plasma LDL clearance. The complementary experiment of transplanting LDL-R(−/−) marrow into C57BL/6 recipients [LDL-R(−/−)→LDL(+/+)], performed to evaluate the role of leukocyte LDL-R in normocholesterolemic conditions, also produced no effects on plasma lipid parameters. LDL binding studies using macrophages isolated from transplanted mice showed a lack of LDL-R expression. Thus, despite their large number and wide distribution, bone marrow-derived cells do not significantly influence receptor-mediated clearance of plasma LDL.—**Fazio, S., A. H. Hasty, K. J. Carter, A. B. Murray, J. O. Price, and M. F. Linton.** Leukocyte low density lipoprotein receptor (LDL-R) does not contribute to LDL clearance in vivo: bone marrow transplantation studies in the mouse. *J. Lipid Res.* 1997. **38:** 391–400.

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The clearance of plasma LDL in the body is mostly controlled by the LDL receptor (LDL-R) pathway (1, 2). The LDL-R is expressed by all cell types so far studied in humans, mice, rabbits, dogs, cows, hamsters, and swine (3). Due to its large mass, the liver is responsible for about 70% of the total body LDL-R mediated uptake of LDL, with the remaining one third being cleared by extrahepatic tissues (4). The hematopoietic system contributes a large mass of cells to the body: circulating leukocytes, lymphocytes in lymph nodes, thymus, and spleen, and resident macrophages in all tissues and organs. Hematopoietic cells and their circulating products (such as lymphocytes and monocytes) express high affinity LDL-R activity in vivo. Lymphocytes were the first cells in which LDL-R activity was proven, both in unstimulated (after isolation from blood) and stimulated (after 3 days in LDL deprived medium) conditions (5, 6). In addition, proliferation of normal lymphocytes in conditions of blocked cholesterol synthesis is LDL-R dependent (7), and blood monocytes exhibit saturable degradation of LDL during the period of greatest growth (8). LDL-R activity is also expressed by human monocyte-derived macrophages (9) and arterial macrophage-derived foam cells (10). It is thus plausible that cells of hematopoietic derivation might contribute to the extrahepatic LDL clearance and to the regulation of plasma cholesterol levels. On the other hand, it has long been known that freshly isolated lymphocytes express minimal LDL-R activity, which can be up-regulated by incubation in lipoprotein-deficient serum (5, 11), and that in the presence of high levels of LDL, lym-

Abbreviations: LDL, low density lipoproteins; LDL-R, low density lipoprotein receptor; PCR, polymerase chain reaction; ROSA 26, reverse orientation splice acceptor (line 26); BMT, bone marrow transplantation; GVDH, graft-versus-host disease; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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phocytes down-regulate the expression of the LDL receptor and derive most of their cholesterol from neosynthesis (11). In addition, human and mouse macrophages have a very low affinity for LDL in vitro (12, 13). However, evidence indicating that suppression of LDL-R activity does in fact occur in vivo in cells of hematopoietic origin is lacking, and the potential of the hematopoietic system to impact plasma cholesterol levels is underscored by the observation that administration of granulocyte-macrophage colony-stimulating factor to patients with severe aplastic anemia results in an average reduction of 37% in non-HDL cholesterol, which is concomitant with the increased blood cell count and disappears after discontinuation of the drug (14). If expression of the LDL-R by cells of the hematopoietic system is a significant contributor to LDL clearance from plasma, then bone marrow transplantation could be developed as a therapeutic option for familial hypercholesterolemia (15).

The availability of mice carrying a targeted disruption of the LDL-R gene makes it possible to address the question of whether leukocyte LDL-R expression has a role in vivo in maintaining plasma LDL levels. Homozygous LDL-R deficient (−/−) mice show a significant increase in plasma LDL levels and enhanced susceptibility to diet-induced aortic atherosclerosis (16). Long-term reconstitution of the entire hematopoietic system in LDL-R (−/−) mice, achieved by transplantation of murine bone marrow expressing two functional LDL-R alleles, would re-establish LDL-R expression in blood leukocytes and tissue macrophages, and thus create a viable setting to study the in vivo effects of extrahepatic LDL-R activity on turnover and levels of LDL in plasma. Similarly, LDL-R (−/−) marrow can be transplanted into C57BL/6 normal mice to determine whether the elimination of the LDL-R from the cells of the hematopoietic system would have an effect on plasma lipid parameters. To this end, we used LDL-R(−/−) mice as recipients of either LDL-R(+/+) marrow (LDL-R(+/+)→LDL-R(−/−) mice; experimental group) or LDL-R(−/−) marrow (LDL-R(−/−)→LDL-R(−/−) mice; control group). Donor mice were derived from ROSA β-geo 26 mice, an engineered strain which show ubiquitous expression of *E. coli* β-galactosidase, providing a marker to assess the extent of reconstitution of recipient mice with donor-derived hematopoietic cells (17). After transplantation, plasma lipids and lipoprotein levels and LDL turnover rates were compared in the two groups. In parallel studies, we used LDL-R(+/+) C57BL/6 mice as recipients of either LDL-R(−/−) marrow (LDL-R(−/−)→LDL-R(+/+) mice; treated group) or C57BL/6 marrow (LDL-R(+/+)→LDL-R(+/+) mice; control group). The results support the lack of an effect of leukocyte LDL-R expression on LDL clearance from plasma.

MATERIAL AND METHODS

Animals

A colony of C57BL/6 mice is established in our animal facility. The LDL-R(−/−) mice were obtained from Jackson Laboratories (Bar Harbor, ME) as hybrids between the strains C57BL/6 and 129, which share the same major histocompatibility antigen, H-2b (18). Before use in bone marrow transplantation studies, these mice were backcrossed into a C57BL/6 background for five generations. ROSA β-geo 26 mice (C57BL/6 × 129 hybrids) (17) at the fifth backcross in C57BL/6 were mated with LDL-R(−/−) mice and normal C57BL/6 mice to obtain LDL-R(−/−) and LDL-R(+/+) donor mice bearing the ROSA 26 marker gene. All mice were maintained in microisolator cages, and were fed one of two diets (all percentages are w/w): PMI Autoclavable Rodent Diet (#5010), 4.5% fat or PMI Mouse Diet (#5015), 11.0% fat. Animal care and experimental procedures involving animals were conducted in accordance with institutional guidelines.

Bone marrow transplantation

Recipient mice were housed in autoclaved microisolator cages and were maintained on acidified water (pH 2.0) containing 100 mg/l neomycin and 10 mg/l polymyxin B for 3 days before and 14 days after transplantation. Four hours prior to transplantation, recipient mice were lethally irradiated with 900 rads from a cesium gamma source. Bone marrow was harvested from femurs and tibias of donor mice as described (19). Bone marrow cells were washed and resuspended in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 2% fetal bovine serum and heparin 5 U/ml. Lethally irradiated recipients received 5–10 × 10^6 bone marrow cells in 0.3 ml by tail vein injection.

Flow cytometry

Hematopoietic cell populations from C57BL/6, ROSA 26, and LDL-R(−/−)→LDL-R(+/+) mice and LDL-R(+/+)→LDL-R(+/+) mice were analyzed by flow cytometry. Red blood cells in the bone marrow and spleen were lysed by ammonium chloride. Single cell suspensions were washed twice in PBS with 2% BSA. Activity of β-D-galactosidase (β-gal) was measured as intracellular accumulation of fluorescein from the hydrolysis of fluorescein di-β-galactopyranoside (FDG; Molecular

Probes), as described (20). Cells were loaded with FDG by hypotonic shock: 1×10^6 cells in 100 µl of staining media (PBS, 4% fetal calf serum, 10 mM HEPES, pH 7.2) were incubated with 100 µl of 2 mM FDG in dH_20 at 37°C for 1 min, and the reaction was stopped by adding 1.8 ml of ice-cold staining media. The cells were incubated on ice prior to flow cytometry. During staining with lineage-specific antibodies all incubations were on ice and centrifugations were at 4°C. The FDG stained cells (1×10^6) were incubated with phycoerythrin- (PE) conjugated MAb for 30 min, washed two times, and two-color flow cytometry analyses were performed on a FACScan (Becton-Dickinson, San Jose, CA). Gating for viable cells was performed using 7-amino-actinomycin D exclusion (Molecular Probes, Eugene, OR). In each sample $0.5–1.0 \times 10^4$ cells were analyzed. The antibodies to lineage specific differentiation antigens were: CD11b (Mac-1) for myeloid cells, B220 for B lymphocytes, and TER for erythrocytes (all from PharMingen, San Diego, CA).

Cell culture studies

Mice were prepared with a peritoneal injection of 1 M thioglycollate (3 ml). Two days later peritoneal macrophages were collected according to a published protocol (21), and plated at a density of 10^6 cells per 35-mm dish (in 6-well plates) in DME containing 10% FBS. Viability was about 50%. Cells were washed and the media was changed to DME with 10% murine LPDS. Binding studies were carried out on ice for 4 h, with at least 6 duplicates of each macrophage type [C57BL/6, LDL-R(−/−)→LDL-R(−/−), and LDL-R(+/+)→LDL-R(−/−)], using 10 µg of ^{125}I-labeled mouse LDL prepared as described below. The media was then aspirated and the cells were washed 3 times with DME containing 2% BSA, 3 times with PBS, and finally extracted with 0.1 N NaOH for detection on a γ-counter and for determination of protein concentration. Specific binding was calculated as the difference between total binding and the binding shown by control cells incubated with the same amount of radioactive LDL and an excess (300 µg) of cold LDL (nonspecific binding). The specific activities of the LDL preparations varied between experiments from 124 to 614 cpm/ng.

Serum cholesterol determinations

Non-fasting mice were anesthetized with methoxyflurane and blood was collected by puncturing the retro-orbital venous plexus. Serum cholesterol determinations were performed using Kit #352 (Sigma, St. Louis, MO) adapted for a microplate assay (22). In brief, 10 µl of serum was diluted 1:100 in sterile water, and a 100-µl aliquot (equivalent to 1 µl of serum) was loaded on the microplate well and mixed with 100 µl of freshly prepared cholesterol reagent. After a 20-min incubation at 37°C, the absorbance at 490 nm was read on a Molecular Devices (Menlo Park, CA) microplate reader. Cholesterol levels on chromatography fractions were determined similarly, except that 100 µl from each tube was loaded directly in the microplate well and mixed 1:1 with the Sigma cholesterol reagent.

Separation of lipoproteins

Sequential ultracentrifugation of lipoproteins was done on 200-µl aliquots of individual mouse serum using solid KBr to increase the density of the solution. The following density cut-off points were used: VLDL, d < 1.019 g/ml; LDL, d 1.019–1.040 g/ml. Since mouse lipoprotein classes show density overlaps, the classical LDL region (d 1.019–1.063) also contains large HDL (23). Mouse lipoproteins were also prepared by FPLC analysis of serum using a Superose 6 column (Pharmacia, Piscataway, NJ) on a system model 600 from Waters (Milford, MA). A 100-µl aliquot of serum from individual mice was injected onto the column and separated with a buffer containing 0.15 M NaCl, 0.01 M Na_2HPO_4, 0.1 mM EDTA, pH 7.5, at a flow rate of 0.5 ml/min. Fifty fractions of 0.5 ml each were collected, with the lipoproteins being contained within tubes 15–33. Fractions 15–19 = VLDL and chylomicrons; fractions 20–26 = IDL, LDL, and large HDL; fractions 27–33 = HDL.

Turnover of LDL in transplanted LDL receptor-deficient mice

Ultracentrifugation on a Beckman TL-120 table-top was used to prepare LDL (d 1.019–1.040 g/ml) from pooled plasma from LDL-R(−/−) mice on a normal chow diet. After dialysis and protein quantitation, 200 µg of each lipoprotein was iodinated according to a modification of the McFarlane procedure (24, 25), diluted to 5 µg/100 µl of saline solution, and each mouse received a dose of 200 µl. The study was performed in the following groups of mice: 1. LDL-R(+/+)→LDL-R(−/−) mice (experimental, or treated, group, n = 4); 2. LDL-R(−/−)→LDL-R(−/−) mice (negative control group, n = 4); 3. C57BL/6 mice (nontransplanted positive controls, n = 4). The experiment was performed 8 weeks after transplantation to allow for complete marrow reconstitution in the recipient and for the complete expression of the bone marrow derived LDL-R to occur. Lipoproteins were injected through the right jugular bulb, which was prepared by a 2-cm incision from the midclavicular line up to the neck. After injection the wound was closed with two automatic staples. Blood was collected by retro-orbital puncture at 10, 60, and 200 min. Data are reported as percent of the injected dose,

assuming that in the mouse plasma volume is 3.5% of body weight (26).

Polymerase chain reaction (PCR) of genomic DNA for LDL-R analysis

Genomic DNA was prepared from the tail of C57BL/6 mice and from peripheral blood cells of BMT mice, according to a published procedure (27). Two different PCR amplifications were performed: the first to amplify the normal LDL-R allele, and the second for the amplification of the disrupted allele. In the first reaction, the upstream primer was 5′ AGG ATC TCG TCG TGA CCC ATG GCGA 3′, and the downstream primer was 5′ GAG CGG CGA TAC CGT AAA GCA CGAGG 3′. This reaction yields a 383-bp band which is specific for the normal allele. In the second reaction, the 5′ primer was the same as for the reaction described above, and the 3′ primer was 5′ CGC AGT GCT CCT CAT CTG ACT TGTC 3′, which hybridizes to a region in the Neo-R gene close to the 5′ end of the coding region. This reaction yields a 1127-bp band from the disrupted LDL-R allele, and no amplification from the normal allele. Reactions were done in 100 µl, using 50 pmol of the primers and 100 ng of the template, and the following cycling conditions: denaturing, 1 min at 94°C; annealing, 1 min at 62°C; extension, 1 min at 72°C (for 35 cycles). Final extension was done at 72°C for 7 min.

RESULTS

After lethal irradiation (900 rads) male LDL-R(−/−) mice were transplanted with 5×10^6 bone marrow cells either from ROSA 26 mice with the normal LDL-R gene (LDL-R(+/+)→LDL-R(−/−); experimental group; n = 12) or from LDL-R(−/−) mice (LDL-R(−/−)→LDL-R(−/−); control group; n = 15). The ROSA 26 mice and the LDL-R(−/−) mice used in these experiments originated as (C57BL/6 × 129) hybrids and were backcrossed into C57BL/6. The C57BL/6 and 129 strains share the same major histocompatibility complex (H-2^b) (18). After transplantation, there was no evidence of rejection or graft-versus-host disease (GVHD), and the mice were viable, active, and maintained their normal body weight.

Six weeks after BMT, the extent of reconstitution of the bone marrow in three LDL-R(−/−)→LDL-R(−/−) mice by donor-derived (β-gal$^+$) myeloid (Mac-1$^+$) and lymphoid (B220$^+$) cells was examined by flow cytometry **(Fig. 1).** The percentages (mean ± SD) of Mac-1$^+$ and B220$^+$ bone marrow cells staining positive for β-gal$^+$ in the LDL-R(−/−)→LDL-R(−/−) mice were 99.2 ± 0.03 and 98.7 ± 1.30, respectively, indicating

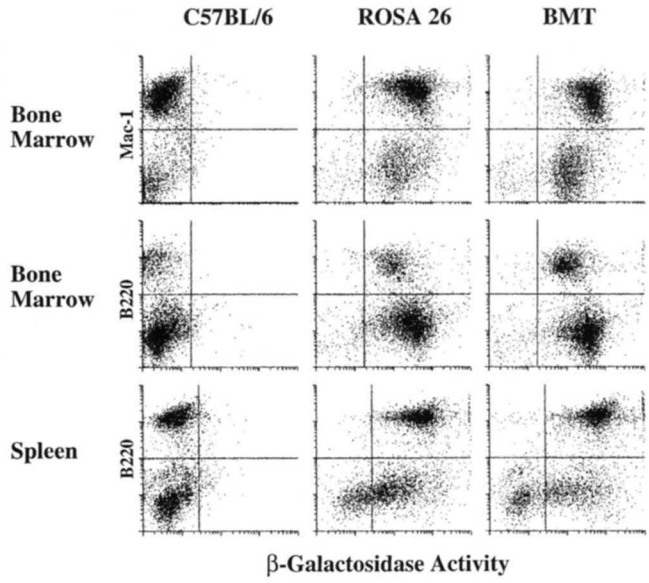

Fig. 1. FACS-Gal analysis of reconstitution of myeloid and lymphoid cell lines in the bone marrow and spleen of LDL-R(−/−)→LDL-R(−/−) mice. Bone marrow and spleen cells were collected 6 weeks after BMT for flow cytometry analysis from LDL-R(−/−)→LDL-R(−/−) BMT mice, and marrow from C57BL/6 and ROSA26 mice was added as control. Myeloid cells were identified using a rat anti-mouse monoclonal antibody (CD11b, or MAC-1) conjugated to R-phycoerythrin (PE), while β-gal activity was detected by staining with fluorescein di-β-galactopyranoside. B lymphocytes were identified in bone marrow and spleen by means of the antibody B220-PE. Nonviable cells were excluded from analysis by staining with 7-amino actinomycin D, and results represent analysis gated on viable cells (>75% viability in all samples). The double-negative staining cells (β-gal$^-$/Mac-1$^-$ or β-gal$^-$/B220$^-$) seen in the left lower quadrant in ROSA26 and BMT mice are unlysed erythrocytes as evidenced by positive staining with TER (data not shown).

that, 6 weeks post-BMT, both myeloid and lymphoid cells in the bone marrow of recipient mice were essentially completely reconstituted by the donor marrow. The percentage of B220$^+$ cells in the spleen that were β-gal$^+$ was 98.7 ± 0.36, indicating that reconstitution of the recipient spleens with lymphoid (B220$^+$) cells of donor origin was also essentially complete.

Flow cytometry was again used to compare the extent of reconstitution of the bone marrow in experimental and control mice by donor-derived (β-gal$^+$) myeloid (Mac-1$^+$) and lymphoid (B220$^+$) cells at a later stage after BMT (16 weeks). The percentages (mean ± SD) of Mac-1$^+$ bone marrow cells staining positive for β-gal$^+$ in ROSA 26 mice (n = 3), LDL-R(+/+)→LDL-R(−/−) mice (n = 5), and LDL-R(−/−)→LDL-R(−/−) mice (n = 5) were 98.0 ± 0.7, 97.6 ± 2.0, and 98.0 ± 1.3, respectively, indicating that the myeloid cells in the bone marrow of both the experimental and control mice were essentially completely donor-derived. The percentages of B220$^+$ cells in the bone marrow that were β-gal$^+$ were 92.1 ± 2.8 in ROSA 26 mice (n = 3),

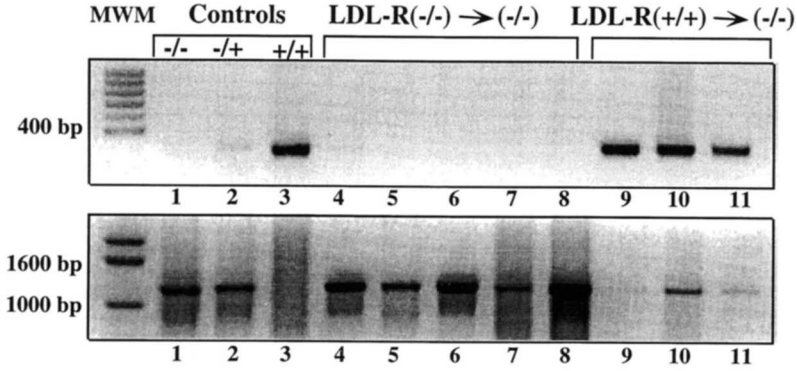

Fig. 2. PCR of DNA extracted from either the tail tissues (C57BL/6) or peripheral blood cells (LDL-R(+/+)→LDL-R(−/−) treated mice, and LDL-R(−/−)→LDL-R(−/−) control mice) using oligo-primers to amplify the normal LDL-R allele to give rise to a 383-bp band (top panel), or the LDL-R allele disrupted by homologous recombination to detect a 1127-bp band (bottom panel). After PCR, samples were separated on a 1% agarose gel containing ethidium bromide to visualize the DNA bands upon UV illumination.

92.4 ± 2.2 in LDL-R(+/+)→LDL-R(−/−) mice (n = 4), and 92.7 ± 6.6 in LDL-R(−/−)→LDL-R(−/−) mice (n = 4). These results demonstrate that the percentage of β-gal expressing cells in bone marrow of the experimental and control mice was essentially identical with that of ROSA 26 marrow, indicating the complete reconstitution of the recipient marrow with lymphoid cells of donor origin.

To prove that the circulating blood cells in LDL-R(+/+)→LDL-R(−/−) mice were carrying the normal LDL-R gene, we performed PCR tests to amplify either the intact or the disrupted LDL-R allele in separate reactions using DNA isolated from blood (transplanted mice) or from tail tissues (normal and LDL-R(−/−) controls). **Figure 2,** panel A, shows the amplification of a 383-bp fragment of the normal LDL-R allele in C57BL/6 DNA, but no product for LDL-R(−/−) DNA or DNA extracted from LDL-R(−/−)→LDL-R(−/−) control mice. The LDL-R(+/+)→LDL-R(−/−)-treated mice show the 383-bp band, indicating that the blood cells from which the DNA was extracted did contain the normal LDL-R gene, and were therefore capable of LDL-R expression in vivo. In contrast, amplification with a set of primers that only hybridize to the disrupted allele showed that the expected 1127-bp band was present in LDL-R(−/−) and LDL-R(−/−)→LDL-R(−/−) control mice, but was either absent or significantly reduced in LDL-R(+/+)→LDL-R(−/−) mice, suggesting that the vast majority of blood cells in the mice of the latter group contained the normal LDL-R gene.

Examination of the plasma cholesterol levels after BMT revealed no differences between experimental and control transplanted mice. **Table 1** shows data from 12 experimental and 10 control mice at 11 and 15 weeks after BMT. In this experiment, mice were fed a diet containing 11% fat. There were no differences in serum cholesterol levels between the two groups at either time point. In a separate experiment, mice were fed a diet with a lower fat content (4.5%) and followed for 6 weeks after BMT. At the end of the study period, total cholesterol was 260 ± 35 mg/dl in LDL-R(−/−)→LDL-R(−/−) mice and 253 ± 19 in LDL-R(+/+)→LDL-R(−/−) mice. Neither of these values was significantly different from the pre-BMT values (−3.6% in controls and −6.2% in experimental mice). Triglyceride levels also showed no differences between groups either on the low or high fat diet (not shown). These data prove that the reconstitution of LDL-R(−/−) mice with LDL-R(+/+) hematopoietic cells did not alter total plasma lipid levels. To determine whether more subtle changes in lipid parameters occurred in LDL-R(+/+)→LDL-R(−/−) mice compared with control LDL-R(−/−)→LDL-R(−/−) mice, the distribution of plasma cholesterol among the different plasma lipoproteins was assessed by size-exclusion chromatography. A Su-

TABLE 1. Serum cholesterol levels in LDL-receptor negative (LDL-R−/−) mice after transplantation with either LDL-R−/− (control group) or LDL-R+/+ marrow (experimental group)

				Weeks Post BMT	
Donor	Recipient	N	Baseline	11	15
LDL-R(+/+)	LDL-R(−/−)	12	324 ± 91	407 ± 52	436 ± 86
LDL-R(−/−)	LDL-R(−/−)	10	332 ± 105	451 ± 110	435 ± 109

The diet contained 11% fat and no cholesterol. At the indicated time points, mice were anesthetized with methoxyflurane and blood was collected from the retro-orbital venous plexus. Serum cholesterol determinations were performed using the Sigma Kit #352 adapted for a microplate assay (see Methods). Values are in mg/dl (mean ± SD). N is the number of mice in each group. No differences between groups reached statistical significance.

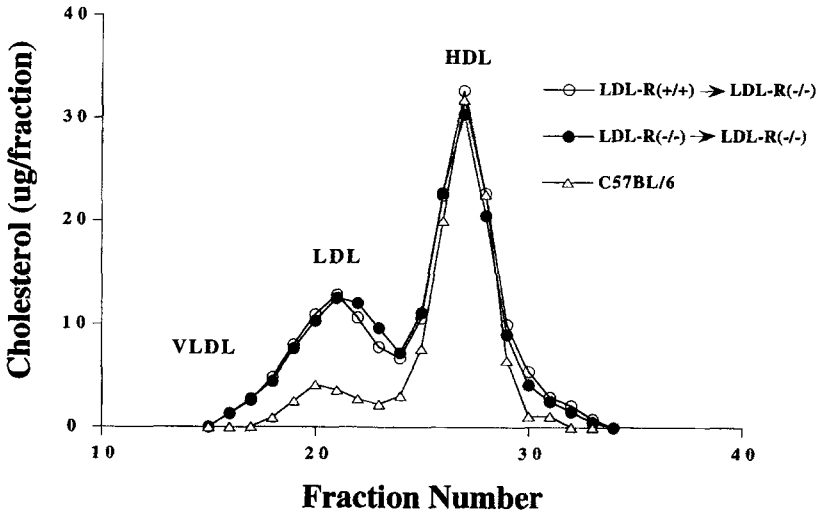

Fig. 3. Distribution of serum lipoprotein cholesterol in LDL-R(+/+)→LDL-R(−/−) mice. One hundred-μl aliquots of serum from individual mice were injected onto a Superose 6 column, in a Waters 600 HPLC, and separated in 0.15 M NaCl, 0.01 M Na_2HPO_4, 0.1 mM EDTA, pH 7.5, at a flow rate of 0.5 ml/min. The lipoproteins were contained within tubes 15–33. Fractions 15–19 = VLDL and chylomicrons; fractions 20–24 = IDL, LDL, and large HDL; fractions 25–33 = HDL. Cholesterol determinations were performed as described in the legend to Table 1, except that 100 μl from each tube was loaded directly to the microplate well and mixed 1:1 with the cholesterol reagent. Data points are mean of 15 individual mouse plasma samples in the control group and 12 in the experimental group. Standard deviations (not shown) were similar in the two groups. No differences between groups reached statistical significance.

perose 6 column in a HPLC setting was used to separate the major lipoprotein classes from aliquots of individual mouse plasma collected from the mice on the 4.5% fat diet. **Figure 3** illustrates that in both groups of mice the cholesterol distribution was identical to that originally reported for LDL-R(−/−) mice (28). The most represented lipoprotein class was the HDL, and the LDL peak was significantly increased relative to the distribution in normal mice. Moreover, the profile was remarkably similar in both groups, indicating that the reconstitution of leukocytes and macrophages with the ability to express the LDL-R did not have a measurable effect on the steady state levels of lipids and lipoproteins in LDL-R(−/−) mice.

To determine whether the presence of leukocyte and macrophage LDL-R in an otherwise LDL-R(−/−) organism would have any measurable effect on clearance of plasma LDL, we performed turnover studies to determine the rate of disappearance of injected ^{125}I-labeled LDL from plasma of LDL-R(+/+)→LDL-R(−/−), LDL-R(−/−)→LDL-R(−/−), and C57BL/6 mice. For these experiments, LDL were isolated as d 1.019–1.040 g/ml lipoproteins. This density cut provides the separation of a LDL particle that contains mostly apoB-100, smaller amounts of apoB-48, traces of apoE, and no detectable apoA-I (not shown). Due to the unique distribution of lipoproteins in the mouse, the classical density cut (d 1.019–1.063 g/ml) used for human plasma results in the collection of significant amounts of large HDL, rich in apoCs, apoE, and apoA-I (29). The results of the turnover experiment are reported in **Fig. 4.** As expected from normal mice, the clearance of LDL in C57BL/6 mice was very fast, with about 50% of the radiotracer cleared in the first 10 min, and only 20% remaining in plasma after 3.5 h. By contrast, clearance of LDL in control LDL-R(−/−)→LDL-R(−/−) mice was slow, with only 20% of the label cleared from plasma 1 h after injection, and 60% remaining in plasma after

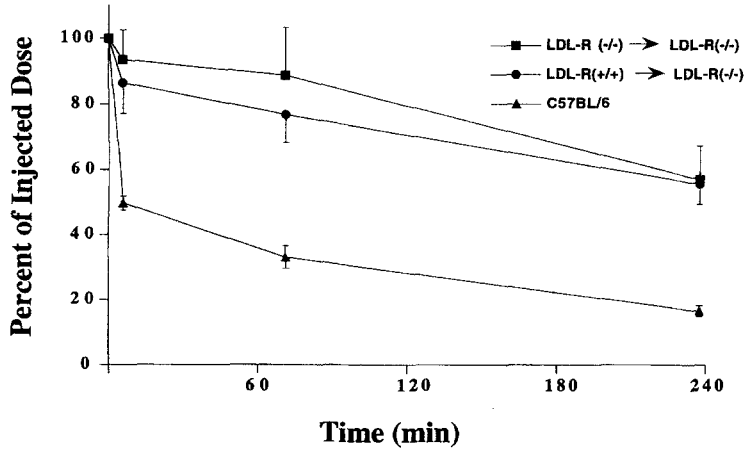

Fig. 4. Turnover of ^{125}I-labeled d 1.019–1.40 g/ml lipoproteins (LDL) in LDL-R(+/+)→LDL-R(−/−) and LDL-R(−/−)→LDL-R(−/−) mice. Lipoproteins were prepared from LDL-R(−/−) mice on a normal chow diet. After iodination with $Na^{125}I$, 5 μg of lipoprotein protein was injected, in a 300 μl volume, into mice of three groups: 1. LDL-R(+/+)→LDL-R(−/−) mice (experimental group); 2. LDL-R(−/−)→LDL-R(−/−) mice (slow-turnover controls); 3. C57BL/6 mice (fast-turnover controls). The radiotracer was injected into the jugular vein of mice anesthetized through brief exposure to metofane. We used 5 mice in the C57BL/6 group, and 4 mice in the other groups. At the indicated time point, blood was collected by retrorbital bleeding, and a 10-μl aliquot of plasma was measured on a γ-counter. Bars represent the standard deviation.

TABLE 2. Binding of ^{125}I-labeled mouse LDL to peritoneal macrophages from C57BL/6, LDL-R(−/−) → LDL-R(−/−), and LDL-R(+/+) → LDL-R(−/−) mice

Macrophages	N	Specific Binding
		ng LDL/mg cell protein
1. C57BL/6 [LDL-R(+/+)]	9	79.8 ± 41.4
2. LDL-R(−/−) → LDL-R(−/−)	6	13.6 ± 18.2
3. LDL-R(+/+) → LDL-R(−/−)	12	23.2 ± 12.7

Macrophages were collected and plated in 35-mm dishes as described under Experimental Procedures. Cells were incubated in DME with 10% FBS, and 6 h before the experiment were washed and incubated with DME with 10% murine LPDS. Incubation with 10 μg of ^{125}I-labeled LDL (specific activity, 124 cpm/ng) was carried out at 4°C for 4 h. Cells were extensively washed and then extracted from the plate using 0.1 N NaOH (mean concentration, 20.2 μg of cell protein per plate). Data are presented as ng of LDL bound per mg of cell protein (mean ± standard deviation). Specific binding is total binding minus nonspecific binding (that observed in 6 parallel plates incubated with 10 μg of ^{125}I-labeled LDL and 300 μg of cold LDL). N is the number of plates in this experiment. Statistical analysis was performed using the Student's *t* test. 1 vs. 2, $P = 0.003$; 1 vs. 3, $P < 0.001$; 2 vs. 3, $P = 0.208$.

TABLE 3. Serum cholesterol levels in C57BL/6 mice following transplantation with either LDL-R−/− (experimental group) or C57BL/6 marrow (control group)

Donor	Recipient	N	Baseline	8 Weeks Post BMT
LDL-R(−/−)	C57BL/6	12	99 ± 11	106 ± 12
LDL-R(+/+)	C57BL/6	12	95 ± 5	102 ± 7

Mice were fed a 5% fat diet for the 4 weeks after BMT. Blood collection and analysis of cholesterol levels were done as described in methods. N is the number of mice in each group. Values are mean and standard deviation in mg/dl. No differences between groups reached statistical significance.

3.5 h. These values are in line with those previously reported for LDL-R(−/−) mice (28). The turnover rate in LDL-R(+/+)→LDL-R(−/−) mice was not significantly different from that of the control LDL-R(−/−)→LDL-R(−/−) mice, providing definitive evidence that normal leukocytes and tissue macrophages do not contribute in a measurable way to LDL-R-mediated clearance of plasma LDL, at least in conditions of increased plasma LDL concentrations.

The lack of effect of LDL-R(+/+) leukocytes on plasma cholesterol levels could be explained either by the fact that the normal contribution of these cells to the receptor-mediated clearance of plasma LDL is minimal, and/or by the fact that the high plasma cholesterol concentration in LDL-R(−/−) mice induced down-regulation of the LDL-R in transplanted leukocytes. To discriminate between these two possibilities, we performed binding studies with peritoneal macrophages using mouse ^{125}I-labeled LDL as a ligand. As shown in **Table 2,** specific binding of LDL to macrophages from LDL-R(+/+)→LDL-R(−/−) mice was not significantly different than that of LDL-R(−/−)→LDL-R(−/−) control macrophages, and was 3.4-fold less ($P < 0.001$) than that of C57BL/6 mouse macrophages. This result strongly suggests that, in the presence of hypercholesterolemia, the expression of the LDL-R is inhibited. However, a functional role for the leukocyte LDL-R in the setting of normal plasma cholesterol levels could not be excluded. This experimental scenario was created by eliminating the LDL-R activity from leukocytes of normal C57BL/6 mice. We did so by performing the complementary experiment of transplanting C57BL/6 mice [LDL-R(+/+)] with marrow from LDL-R(−/−) mice [LDL-R(−/−)→LDL-R(+/+)], whereas mice of the control group received C57BL/6 marrow [LDL-R(+/+)→LDL-R(+/+)]. As in the original experiments, such intervention had no effect on plasma cholesterol levels **(Table 3)** or on the distribution of lipoprotein cholesterol in plasma **(Fig. 5),** demonstrating that in vivo the leukocyte LDL-R has no effect in determining plasma LDL levels even in the presence of low levels of LDL cholesterol.

DISCUSSION

The present studies had the objective of evaluating the role played in vivo by LDL-R expression in cells of hematopoietic origin, such as lymphocytes, monocytes, and tissue macrophages, on the plasma clearance of LDL. Mice carrying the homozygous disruption of the LDL-R gene were used as recipients of marrow either from LDL-R(+/+) mice (LDL-R(+/+)→LDL-R(−/−) mice; experimental group) or from LDL-R(−/−) mice (LDL-R(−/−)→LDL-R(−/−) mice; control group). However, the re-population with LDL-R(+/+) hematopoietic cells exerted no effect on plasma lipid levels, lipoprotein cholesterol distribution, or on the rate of LDL removal from plasma in the mice of the treated group, suggesting that cell types other than those derived from bone marrow are responsible for the extrahepatic LDL-R-mediated clearance that occurs in normal organisms (4). Flow cytometric analyses demonstrated that the myeloid and lymphoid cells of the bone marrow were essentially completely reconstituted by the donor bone marrow cells in both the experimental and control mice. To prove that the complete reconstitution observed in the bone marrow of the LDL-R(−/−) recipient mice was reflected in the presence of LDL-R(+/+) cells in the circulation, we performed PCR analyses that confirmed that the vast majority of blood nucleated cells were donor in origin, and thus carried two copies of the normal LDL-R allele. This allowed us to conclude that in the setting of high LDL concentration induced

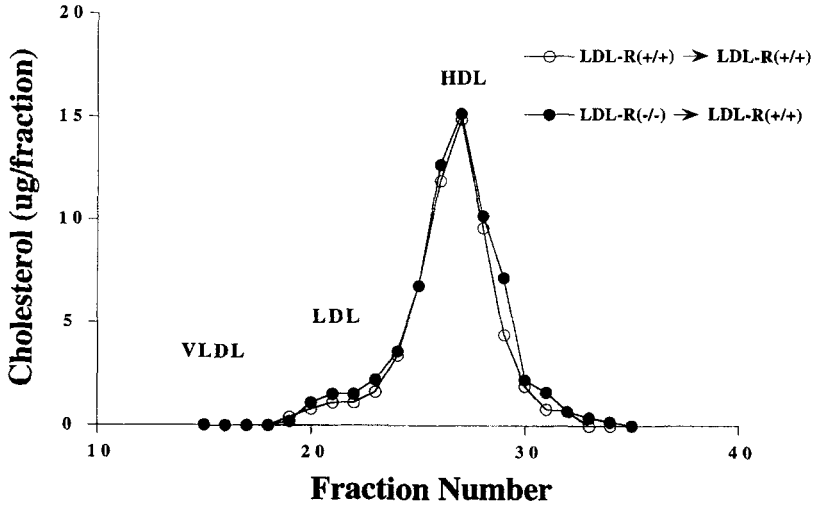

Fig. 5. Distribution of serum lipoprotein cholesterol in LDL-R(−/−)→LDL-R(+/+) mice. One-hundred-μl aliquots of mouse serum were injected onto a Superose 6 column, in a Waters 600 HPLC, and separated as explained in legend to Fig. 2. Cholesterol determinations were performed as described in the legend to Table 1, except that 100 μl from each tube were loaded directly to the microplate well and mixed 1:1 with the cholesterol reagent. Each data point represent the mean of four separate pools of plasma deriving from three different mice, and thus reflect the cholesterol distribution in the plasma of 12 mice in each of the two groups (experimental and control). Standard deviations (not shown) were similar in the two groups. No differences between groups reached statistical significance.

by the lack of the LDL-R, the reconstitution of leukocyte LDL-R has no detectable effect on plasma cholesterol and LDL clearance. The binding experiment presented in Table 2 suggests that the lack of effect of LDL-R(+/+) leukocytes on plasma lipids in vivo is explained by a down-regulation of the LDL-R induced by the hypercholesterolemic environment. However, when we reconstituted normal C57BL/6 mice with bone marrow from LDL-R(−/−), to determine whether the leukocyte LDL-R might have a functional role in LDL clearance in normocholesterolemic conditions, we again observed no effects on plasma cholesterol levels and lipoprotein distribution, strongly suggesting that cells of the hematopoietic system do not contribute significantly to the LDL-R-mediated clearance of LDL occurring in extrahepatic tissues (4).

As only two thirds of the receptor-mediated clearance of LDL occurs in the liver (30), it was plausible to speculate that an important contributor to the remaining one third would be the hematopoietic system. Among the products of bone marrow progenitors are circulating leukocytes (such as lymphocytes and monocytes) and tissue macrophages. Lymphocytes are the predominant cell type in lymph nodes, spleen, and thymus, whereas resident macrophages are present in spleen, lung, skin, bone, liver, central nervous system, and serosal cavities. Lymphocytes and macrophages express functional LDL-R molecules in the circulation and within tissues and organs (11, 31). However, freshly isolated lymphocytes have a low number of LDL-R, which is increased by incubating the cells for 72 h in the absence of LDL (5). Although nondividing lymphocytes derive most of their cholesterol from de novo synthesis (7), exposure to high LDL cholesterol levels will result in LDL-R-mediated uptake and inhibition of cholesterol synthesis (5). Moreover, in proliferating cells the transcription of the LDL-R is enhanced even more than predicted by the increased cholesterol need, and is not completely down-regulated by LDL (32). Similarly, in monocyte/macrophages the activity of the LDL-R is lower than in hepatocytes or fibroblasts (12, 13), but is definitely measurable and can be modulated by exposure to LDL in the media (31). Expression of the LDL-R has also been demonstrated in macrophage/foam cells extracted from the artery wall (10). An argument in favor of a role of blood cells in the regulation of plasma cholesterol levels was also provided by the observation that patients with acute leukemia present with hypocholesterolemia, which is likely due to the high levels of LDL-R activity in tumor cells, and is correlated with the count of white cells in the blood (33). Moreover, in patients with hairy cell leukemia, a rare condition characterized by cytopenia, splenomegaly, and impaired immunity, the extent of hypocholesterolemia is correlated with the degree of spleen enlargement, and might be mediated by release of granulocyte-macrophage colony stimulating factor from the leukemia cells (34).

Our study clearly establishes that leukocytes and macrophages with a normal LDL-R gene are unable to alter the increased plasma LDL levels observed in LDL-R(−/−) mice. It is likely that the exposure to the high plasma LDL levels determines a rapid down-regulation of the LDL-R in the transplanted cells to levels that are too low to significantly influence plasma LDL clearance, as suggested by the binding experiment presented in Table 2. Our data are concordant with previous studies showing a very low LDL-R activity in mouse peritoneal macrophages (13). However, a reduction in binding of more than 70% was consistently observed in macrophages from LDL-R(+/+)→LDL-R(−/−) mice. From

these results, it appears evident that the approach of BMT does not represent a viable therapeutic strategy for familial hypercholesterolemia, which is characterized by reduced or absent LDL-R activity and increased plasma LDL levels (3). This study also establishes that the cells of the hematopoietic system do not contribute to the receptor-mediated clearance of LDL that occurs in extrahepatic tissues, suggesting that cells other than the resident macrophages (e.g., epithelial cells or fibroblasts) take up LDL in these tissues.

We and others have previously shown that BMT represents a very effective treatment intervention in the hypercholesterolemia induced by apolipoprotein (apo)E deficiency in mice (35, 36). Transplanted apoE(−/−) mice, which received marrow expressing the normal complement of apoE, showed low levels of apoE in plasma, a 75% reduction in plasma cholesterol, an enhanced clearance of remnant lipoproteins by the liver, and the complete prevention of aortic atherosclerosis which is rampant in apoE deficiency. In these studies, we were able to identify donor-derived macrophages in the artery wall of recipient mice on the basis of their ability to synthesize apoE (M. F. Linton and S. Fazio, unpublished results). Because murine BMT is performed in genetically identical organisms, transplant rejection or GVHD are not common occurrences. This lack of side effects makes it possible to use the BMT approach to study the biology of macrophage involvement in the different phases of atherogenesis or to use the macrophage as a vehicle for gene delivery to the atherosclerotic lesion (37).

The role of the LDL-R expression by the macrophage in the artery wall might go beyond its effects, or lack thereof, on plasma LDL concentration. Evidence suggests that the LDL-R is involved in the binding and internalization of β-VLDL by macrophages (38), and that this results in cholesteryl ester accumulation and transformation into foam cells (39). It is therefore possible that expression of the LDL-R by the macrophage in the artery wall might influence the progression of atherosclerosis. Studies to examine the role of the expression of the LDL-R by the macrophage in atherosclerosis are ongoing in our laboratory.

In conclusion, our study demonstrates that the LDL-R activity in leukocytes and macrophages does not play a significant role in LDL clearance under physiologic conditions, and is likely down-regulated in hypercholesterolemic states.

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