Lentiviral Transduction of ApoAI Into Hematopoietic Progenitor Cells and Macrophages

Applications to Cell Therapy of Atherosclerosis

Yan Ru Su, John L. Blakemore, Youmin Zhang, MacRae F. Linton, Sergio Fazio

Objective—We used genetically engineered mouse hematopoietic progenitor cells (HPCs) to investigate the therapeutic effects of human apoAI on atherosclerosis in apoE−/− mice.

Methods and Results—Lentiviral constructs expressing either human apoAI (LV-apoAI) or green fluorescent protein (LV-GFP) cDNA under a macrophage specific promoter (CD68) were generated and used for ex vivo transduction of mouse HPCs and macrophages. The transduction efficiency was >25% for HPCs and >70% for macrophages. ApoAI was found in the macrophage culture media, mostly associated with the HDL fraction. Interestingly, a significant increase in mRNA and protein levels for ATP binding cassette A1 (ABCA1) and ABCG1 were found in apoAI-expressing macrophages after acLDL loading. Expression of apoAI significantly increased cholesterol efflux in wild-type and apoE−/− macrophages. HPCs transduced with LV-apoAI ex vivo and then transplanted into apoE−/− mice caused a 50% reduction in atherosclerotic lesion area compared to GFP controls, without influencing plasma HDL-C levels.

Conclusions—Lentiviral transduction of apoAI into HPCs reduces atherosclerosis in apoE−/− mice. Expression of apoAI in macrophages improves cholesterol trafficking in wild-type apoE-producing macrophages and causes upregulation of ABCA1 and ABCG1. These novel observations set the stage for a cell therapy approach to atherosclerosis regression, exploiting the cooperation between apoE and apoAI to maximize cholesterol exit from the plaque. (Arterioscler Thromb Vasc Biol. 2008;28:1439-1446)

Key Words: macrophages ■ hematopoietic progenitor cells ■ lentiviral vector ■ apolipoprotein AI ■ cholesterol efflux ■ cell therapy of atherosclerosis

Monocytes and macrophages play a crucial role in all stages of atherosclerosis.1–2 Among their many functions, regulation of intracellular cholesterol homeostasis is an essential determinant of foam cell transformation and downstream events, including inflammatory changes and apoptosis. In addition to internalization of modified lipoproteins via scavenger receptors, macrophages have developed sophisticated mechanisms to ensure prompt cholesterol exit into the HDL pathway, also known as reverse cholesterol transport (RCT).

At least 3 pathways are involved in cholesterol export from macrophages: the ATP binding cassette A1 (ABCA1) pathway, which exports cholesterol and phospholipids to lipid-free apoAI; the ABCG1 pathway, which mediates cholesterol efflux to larger HDL particles; and the scavenger receptor class B type I (SRB-I) pathway, which promotes bidirectional cholesterol exchange between cells and lipoproteins, including HDL.3–4 Recent studies have suggested that ABCA1 and ABCG1 work synergistically in this process. ABCA1 carries out the initial transfer of free cholesterol and phospholipids to lipid-poor apoAI to generate nascent pre-β HDL particles, which are then used as acceptors of cholesterol exported by ABCG1.5–6

Lipid poor apoAI is not synthesized by macrophages. Thus, activation of RCT in the artery wall depends on the availability of apoAI that has infiltrated from the plasma compartment. Because of the obstacles to free diffusion presented by the growing atheroma, the concentration of apoAI in the artery wall may be reduced below a physiological threshold required for cholesterol extraction, possibly leading to impaired cholesterol exit and expansion of the foam cell lesion. We previously used a retroviral approach to transduce human apoAI into bone marrow cells, and showed significant reduction of atherosclerotic lesions in recipient apoE−/− mice without changes in HDL levels.7–9 However, apoAI expression was very low because of the inefficiency of retroviral transduction of quiescent bone marrow cells. Thus, the effect of apoAI on cholesterol efflux was only seen in apoE−/− cells, given the dominant position of locally produced apoE in regulating cholesterol homeostasis in the atheroma. This drawback obviously reduced the therapeutic implications of our earlier studies.
Hematopoietic progenitor cells (HPCs) possess 2 characteristics that make them ideal targets for gene transfer: self-renewal capacity and pluripotentiality. Using HPCs instead of total bone marrow cells as the delivery vehicle increases transduction efficiency and specificity. The human immunodeficiency virus type 1 (HIV-1)–based lentiviral vectors are capable of transducing both dividing and nondividing cells and have become the tool of choice because of long-term stable gene transfer both in vitro and in vivo.

We have generated a lentiviral vector-based human apoAI cDNA expression construct (LV-apoAI) driven by a macrophage specific promoter, CD68. With this construct, we have transduced HPCs ex vivo for transplantation into apoE−/− mice and demonstrated its value as a cell-mediated gene therapy for atherosclerosis. We also show that expression of apoAI increases cholesterol efflux in macrophages also expressing apoE, suggesting that cooperation between these 2 apolipoproteins can be exploited for cell-based approaches to plaque regulation.

Methods

Generation of a Lentiviral Human apoAI cDNA Expression Construct

The self-inactivating (SIN) lentiviral vector (pWPT-WRPE) was a gift from Dr Trono (University of Geneva). The human CD68 promoter along with the first intron were amplified from human genomic DNA by polymerase chain reaction (PCR). The human apoAI cDNA with growth hormone (GH) poly-A signal sequence were verified via DNA sequencing to ensure accuracy.

Five-micrometer frozen sections were fixed in cold acetone, blocked with 4% BSA in PBS, then incubated with anti-human apoAI antibody (Biodesign) or anti-mouse CD68-Alexa Fluor 488 (Sero-tec). The slides were then washed with PBS and incubated with Cy3-conjugated donkey anti-goat Ig-G (Jackson Immuno Research Laboratories Inc). Vectashield mounting medium with DAPI (Vector Laboratories Inc) was used to stain nuclei. Images were captured using an Olympus DP71 microscope and Olympus DP controller image software.

Cholesterol Efflux Studies

Cholesterol efflux from murine peritoneal macrophages was measured by a modified procedure from Mazzone and Yancey. Briefly, macrophages were transduced with LV-apoAI or LV-GFP, cultured in DMEM/10% FBS for 24 hours, and then incubated with 100 μg/mL acLDL and 5 μCi/mL [3H]-cholesterol (NEN) in DMEM/0.1% FBS for 72 hours. Cells in control wells (C) were washed with DPBS, air dried, and extracted with isopropanol to provide a baseline value for total [3H] cholesterol content. Cells were incubated with efflux media (DMEM with or without 10 μg/mL apoAI) at 37°C for 48 hours. At the end of the efflux period, the media were collected, the cells were incubated with isopropanol at room temperature for 4 hours, and radioactivity in the media and cell extract was measured by scintillation counting. Three independent experiments were conducted.

Transplantation of HPCs into ApoE−/− Mice and Quantitation of Atherosclerotic Lesions

HPCs were isolated from male apoE−/− mice (B6.129P2-Apoetm1Unc) at 12 to 13 weeks of age, and then transduced with LV-GFP or LV-apoAI at an MOI of 30 and cultured 22 to 24 hours in Stemspan media (Stem Cell Technologies Inc) overnight before being transplanted into recipient mice at 50,000 HPCs per mouse. The transduction efficiency of both macrophages and HPCs were measured by the GFP expression levels using flow cytometry.

Western Blot Analysis of ABCA1, ABCG1, and SR-BI mRNA

Total RNA was collected using the Purescript kit (Genetra System). The cDNAs were reverse-transcribed using the High Capacity cDNA Archive kit (ABI, Applied Biosystems). Primers and probes were purchased from ABI, and the real-time RT-PCR reactions were carried out on a Taqman 7900 (ABI) according to a modified method described previously.

Enzyme-Linked Immunosorbent Assay for Detection of Human apoAI

Human apoAI protein levels from macrophage culture media as well as serum from mice receiving apoAI transduced HPCs were determined by enzyme-linked immunosorbent assay (ELISA) as previously described.

Immunofluorescent Staining of Human ApoAI and CD68 in the Proximal Aortic Lesions

Briefly, mice were transduced with LV-apoAI or LV-GFP, cultured in DMEM/10% FBS for 24 hours, and then incubated with 100 μg/mL acLDL and 5 μCi/mL [3H]-cholesterol (NEN) in DMEM/0.1% FBS for 72 hours. Cells in control wells (C) were washed with DPBS, air dried, and extracted with isopropanol to provide a baseline value for total [3H] cholesterol content. Cells were incubated with efflux media (DMEM with or without 10 μg/mL apoAI) at 37°C for 48 hours. At the end of the efflux period, the media were collected, the cells were incubated with isopropanol at room temperature for 4 hours, and radioactivity in the media and cell extract was measured by scintillation counting. Three independent experiments were conducted.

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Statistical Analysis

Statistical analysis was performed with GraphPad PRISM software (GraphPad Software Inc). Results were expressed as the mean ± SE intragroup and between-group comparisons were achieved using either 1-way ANOVA followed by the Bonferroni’s post test when statistical significance was detected, or the unpaired 2-tailed t test when appropriate.

Results

High-Level Lentiviral-Mediated ApoAI Expression in HPCs and Mouse Peritoneal Macrophages

We have generated a lentiviral apoAI expressing construct and a GFP expressing construct driven by a macrophage specific promoter, CD68. Supplemental Figure IA (available online at http://atvb.ahajournals.org) shows diagrams of these constructs. Using the conditions described above, we were able to achieve greater than 70% and 25% transduction efficiency in primary cultured macrophages and HPCs, respectively, at an MOI of 50 (supplemental Figure IB).

ApoAI was efficiently secreted from macrophages, as it was detected in the culture media and was not found in the cell lysate (Figure 1A). The distribution of apoAI in the media was analyzed by FPLC in wild-type and apoE−/− macrophages transduced with LV-apoAI. Endogenously synthesized apoAI was primarily detected in the HDL range in both cell types, but was associated with larger particles in apoE−/− macrophages, and relegated to smaller particles in the presence of apoE (Figure 1B). Similar patterns were detected by ELISA in FPLC fractions (Figure 1D).

ApoAI was detected in the culture media of mouse peritoneal macrophages up to 4 weeks after transduction, indicating long-term stable expression of the apoAI transgene. The concentration of apoAI in the media ranged from 440 to 510 ng/mL/10^6 cells in 24-hour cultures. When the apoAI-expressing HPCs were transplanted into lethally irradiated apoE−/− recipient mice, human apoAI was detected in plasma 12 weeks post-HPC transplantation at a concentration of 8.4 ± 1.2 g/dL. This represents a nearly 50-fold improvement over our previous results with retroviral apoAI–mediated bone marrow transplantation, which produced plasma apoAI at concentrations of 100 to 200 ng/dL 12 weeks after BMT.

The distribution of endogenous apoE in the media of LV-apoAI–transduced wild-type macrophages was also analyzed in the FPLC fractions. In complete agreement with the data reported above for apoAI, human apoAI was detected in plasma 12 weeks post-HPC transplantation at a concentration of 8.4 ± 1.2 μg/dL. This represents a nearly 50-fold improvement over our previous results with retroviral apoAI–mediated bone marrow transplantation, which produced plasma apoAI at concentrations of 100 to 200 ng/dL 12 weeks after BMT.

Cholesterol Efflux in Macrophages Transduced With Lentiviral ApoAI

LV-apoAI transduction increased cholesterol efflux by 2.5-fold in wild-type macrophages (Figure 2A) and by 3.5-fold in
apoE<sup>-/-</sup> macrophages (Figure 2B) compared to the LV-GFP transduced controls. Our observation suggests that apoAI produced in the apoE<sup>-/-</sup> macrophages is as efficient as apoE in mediating cholesterol efflux. Interestingly, the expression of apoAI completely corrected the defective efflux caused by apoE deficiency (from 1.6 to 5.7%) and reinstated the same level of efflux seen in wild-type macrophages (Figure 2A). The addition of exogenous apoAI in the efflux media (10 μg/mL) resulted in a further increase in cholesterol efflux in both wild-type (from 11% to 21%) and apoE<sup>-/-</sup> (from 5.7% to 14%) macrophages compared to DMEM alone, indicating that endogenous and exogenous apoAI have a synergistic effect on cholesterol efflux.

Effects of ApoAI Expression on Cholesterol Transporters in Macrophages

ABCA1 and ABCG1 mRNA levels were significantly increased in both wild-type and apoE<sup>-/-</sup> macrophages after acLDL loading when compared to GFP-transduced macrophages (Figure 3A and 3B, lower panel). These were accompanied by an increase in ABCA1 and ABCG1 protein levels (Figure 3A and 3B, upper panel). Interestingly, SR-BI mRNA levels were decreased by 20% in LV-apoAI–transduced wild-type macrophages (but not in apoE<sup>-/-</sup> macrophages) compared to GFP expressing cells (Figure 3C, lower panel). However, SR-BI protein levels were not affected (Figure 3C, upper panel). In addition, transduction of wild-type macrophages with LV-GFP reduced apoE mRNA levels by 50%, whereas LV-apoAI–transduced macrophages showed no significant changes in apoE secretion (Figure 3D).

Transplantation of ApoAI-Producing HPCs into ApoE<sup>-/-</sup> Mice Reduces Atherosclerosis Without Changing Plasma Lipid Profiles

Twelve weeks after transplant, atherosclerotic lesions in apoAI-HPC recipient apoE<sup>-/-</sup> mice were 50% smaller than in GFP-HPC recipient controls. No significant differences were seen in the aortic sinus (Figure 4A and 4B). Human apoAI was identified in the proximal aortic area by immunofluorescence staining in LV-apoAI HPC recipients (Figure 4C and 4D). No differences were detected between groups in total cholesterol, HDL-C, and lipoprotein profiles during the course of study (Figure 5A, 5B, and 5C). HPC transplantation into lethally irradiated apoE<sup>-/-</sup> mice reconstituted the normal lineage of hematopoietic cells. Flow cytometric analysis of splenocytes collected from recipients of LV-GFP–transduced HPCs showed similar percentages of B-cells, T-cells, and monocytes as compared to nontransplanted age-matched apoE<sup>-/-</sup> mice (supplemental Figure II).<sup>20</sup> The use of the CD68 promoter resulted in a significantly higher percentage of GFP expression among CD11b<sup>+</sup> or CD11c<sup>+</sup> cells (macrophages) relative to B-cells (B-220<sup>+</sup>) and T-cells (CD4<sup>+</sup> and CD8<sup>+</sup>).
Discussion

We used a lentiviral-based transduction approach to engineer HPCs and macrophages to secrete high levels of human apoAI, the main driver of cholesterol efflux into the RCT system. HPCs are quiescent in nature and thus difficult to transduce. Lentiviral vectors have the highest efficiency in transducing stem cells among available vectors. We were able to achieve an average of 25% transduction efficiency in stem cells, a dramatic improvement compared to the 1% to 2% we reported using retrovirus-mediated transduction.7,9,21,22 Similar results were reported in lentivirally transduced human CD34+/H11011 hematopoietic stem cells23 and in primary blood-derived murine monocytes/macrophages,24 where transduction efficiency was also higher in primary macrophages than in HPCs. Although the difference in transduction efficiency between primary macrophages and HPCs was not directly investigated, we speculate that it may be attributable to: (1) The slow cell cycle of the stem cells compared to macrophages; (2) The use of cytokine-free and serum-free medium (StemSpan) for transduction and culture of HPCs, which does not stimulate cell differentiation; and (3) The macrophage phagocytosis activity may help increasing uptake of viral particles.

Our results show that transduced macrophages efficiently secrete apoAI and export more free cholesterol. The secreted apoAI was fully lipidated, associated with HDL-sized particles, and competed with apoE for HDL surface space. Transplantation of transduced HPCs into apoE−/− mice resulted in high level expression of apoAI, with plasma concentrations reaching levels over 8 µg/dL, ≈50 times the levels we previously reported using a retroviral vector under the control of the scavenger receptor A promoter.9 However, the amount of plasma human apoAI produced by macrophages was still extremely low compared to the amount of apoAI in human plasma (>100 mg/dL) and did not affect HDL-C levels. However, human apoAI was clearly detectable in the artery wall and was associated with a significant reduction in plaque size and number in the aorta, with a trend toward less atherosclerosis in cross-sections of the aortic sinus.

The lack of statistical difference in the aortic sinus lesions was likely attributable to volume saturation of advanced plaques at this anatomic site25 and perhaps a consequence of reduced macrophage content in advanced lesions.26

Purified HPCs allow the use of fewer transduced cells in transplantation (50 000 HPCs versus 3 to 5 × 10⁶ bone marrow cells) while still achieving complete reconstitution of the hematopoietic system in the recipient mice. In addition, the use of the CD68 promoter increased specificity for monocytes/macrophages.12,27 Because these cells are key components of the plaque,28 they can be used to deliver apoAI for therapeutic effects on atherosclerosis plaque formation or regression.

Because most retroviruses require cell division and nuclear membrane dissolution to integrate into the target cell genome, the use of these vectors for delivery of genes to the relatively

Figure 3. Expression of ABCA1, ABCG1, SR-BI, and apoE in LV-apoAI–transduced macrophages. Western blot (upper panel) and real-time RT-PCR (lower panel) analysis of ABCA1 (A), ABCG1 (B), and SRBI (C) in LV-GFP and LV-apoAI–transduced macrophages after 72 hours of acLDL loading. Equal amount of protein were loaded on to the gel, and blotting for β-actin showed no difference in loading (data not shown). ApoE mRNA levels were decreased in the wt macrophages transduced with GFP. The secretion of apoE to the media was similar between untransduced and LV-apoAI–transduced macrophages (D).
quiescent HPCs and nondividing macrophages has proven difficult, and expression levels are very low.\textsuperscript{29} The lentiviral vectors based on human immunodeficiency virus type 1 (HIV-1) have the ability to transduce both dividing and nondividing cells,\textsuperscript{10} as they actively enter the cell nucleus without requiring cell division and therefore can effectively deliver genes to progenitor cells and provide sustained transgene expression (\textsuperscript{7} months in mice).\textsuperscript{30} Recent reports have shown this technique to be successful in introducing the normal human \(\alpha\)-globin gene into HPCs to correct sickle cell disease in a mouse model.\textsuperscript{30} Furthermore, a complete correction of murine artemis immunodeficiency was achieved through transplantation of purified HPCs transduced by a lentivirus expressing human artemis gene.\textsuperscript{31}

Macrophages constitute approximately one-half of the cellular mass of atherosclerotic lesions. Therefore, promoting macrophage cholesterol export is a key objective in the treatment of atherosclerosis. Cholesterol efflux in macrophages is finely regulated and requires a series of coordinated and interconnected events, including the upregulation of membrane transporters and the functional presence of extracellular acceptors such as lipid-poor apoAI, apoE, or HDL particles.\textsuperscript{5,32,33} There are at least 3 key transporters involved: ABCA1, ABCG1, and SR-BI. Recent studies suggest that ABCA1 and ABCG1 work synergistically in this process. ABCA1 transfers free cholesterol and phospholipids to lipid-poor apoAI to form small HDL particles, which are acceptors for cholesterol exported by ABCG1. SR-BI–mediated cholesterol efflux is bidirectional and dependent on the choles-

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**Figure 4.** Quantification of atherosclerotic lesions and immunofluorescent staining of apoAI in the lesions. A, Mean lesion area in the proximal aorta (\(n=7\) in GFP group and \(n=10\) in apoAI group). B, Quantification of en face lesions. C and D, Immunofluorescent staining of apoAI (red) and CD68 (green) in proximal aorta. A representative section from the LV-apoAI group (\(\times 200\); C and D) and the LV-GFP group (E). F, A negative control without addition of Cy3-conjugated secondary antibody.
terol gradient across the cell membrane. Our LV-apoAI–transduced macrophages showed an upregulation of ABCA1 and ABCG1 expression after acLDL loading and, as a result, a substantial increase in cholesterol efflux.

It is worth noting that expression of apoAI increased cholesterol efflux in wild-type macrophages, suggesting a cooperative effect of apoAI and apoE on cholesterol efflux. To our knowledge, this is the first report showing additive effects of endogenously produced cholesterol acceptors (apoAI and apoE) on increasing cholesterol exit from macrophage. This supports the contention that genetically engineered macrophages can serve as a useful tool for promoting RCT in the plaque. In addition, the expression of apoAI in apoE−/− macrophages completely normalized cholesterol efflux to the level of wild-type macrophages, suggesting that apoAI can compensate and rescue reduced cholesterol efflux attributable to the loss of apoE.

Crucial aspects of this work include: (1) lentiviral apoAI was sufficient to completely normalize the impaired efflux in apoE−/− macrophages; (2) Lentiviral apoAI increased cholesterol efflux in normal macrophages, thus adding to the effect of apoE; (3) Adding exogenous apoAI caused a further increase in cholesterol efflux in both wild-type and apoE−/− macrophages, possibly because of the ability of exogenous apoAI to increase secretion of endogenous acceptors. Recent studies in our laboratory suggest that apoAI and apoE work cooperatively in the formation of buoyant HDL by cholesterol-enriched macrophages; (4) Lentivirally mediated apoAI transduction in HPCs revealed a long-term sustained expression of apoAI in transplanted recipient mice with reduction of aortic lesion size. One can anticipate that the concurrent production of both apoAI and apoE from macrophages in the artery wall will significantly promote cholesterol exit from these cells via both ABCA1-dependent and independent pathways.

It must be emphasized that with the current approach we are finally able to demonstrate an effect of macrophage apoAI in the presence of physiological amounts of apoE, and that apoAI and apoE produced by macrophages work cooperatively in promoting cholesterol efflux and maintaining cholesterol homeostasis. This is an essential milestone not achieved in previous studies where the effect of lower expression levels of apoAI was only detectable in the absence of apoE.

In wild-type macrophages, where apoAI and apoE compete for binding on the lipoprotein surface, apoE associates with larger HDL-size particles as compared to apoAI. Whether these large particles were generated as a result of enhanced cholesterol efflux through the ABCA1 pathway or due to an independent mechanism is not clear. It can be speculated that the larger apoE-containing particles are better acceptors for ABCG1-mediated cholesterol efflux.

The protective effects of apoAI can be attributable to anti-inflammatory and antioxidant properties not directly related to plasma HDL cholesterol levels. The recent negative outcomes of several trials of the CETP inhibitor torcetrapib are a reminder that plasma HDL may not be the sole target for therapeutic strategies directed at removing cholesterol from the arterial plaque. Our studies support the notion that increasing the concentration of apoAI in the lesion is a bona fide objective of gene therapy approaches to control the atheroma.

Acknowledgments

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Disclosures

None.

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**Su, Figure 2**

**A.** % cholesterol efflux

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**D.** % cholesterol efflux

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***: p < 0.001

*: p < 0.05

**: p < 0.01
Su, Figure 3
A. Proximal aortic lesions

Mean Lesion area /section µm²x1000

B. En-Face lesions

% of lesion/total aorta area

Su, Figure 4
A. The constructs

Su, Figure I
B. Expression of GFP with varying multiplicities of infection (MOI).

a. Macrophages

% GFP positive cells

b. HPCs

% of GFP positive Cells

Su, Figure. I
B-cells  T-cells  Monocytes

0  25  50  75  GFP+

Cell types

% of spleenocytes

Su, Figure II
Figure legends

Figure I. Lentiviral Expression constructs and LV-GFP expression in HPCs and macrophages. A. Schematic illustration of LV-GFP and LV-apoAI cDNA expression constructs. The human apoAI cDNA and Green Fluorescent Protein (GFP) are driven by a human macrophage specific promoter, CD68.$^{13}$ The HIV-1 3' long terminal repeat (LTR) is shown with a self inactivating (SIN) deletion. Ψ: Packaging signal. SD, SA: splicing donor and acceptor site. CPPT: central polypurine tract or DNA flap/central DNA termination sequence. HGH: human growth hormone poly-A signal sequence. WPRE: woodchuck hepatitis virus post-transcriptional regulatory element. B. Expression of GFP with varying multiplicities of infection (MOI). a. LV-GFP transduced macrophages. At an MOI of 50, more than 70% of the transduced macrophages express GFP three days after transduction. b. LV-GFP transduced HPCs. At an MOI of 50, more than 25% of the transduced HPCs expressed GFP three days after transduction.

Microscopic images of lentiviral-CD68-GFP transduced macrophages (c) and HPCs (d).

Figure II: Flow cytometric analysis of splenocytes from apoE-/--recipient mice transplanted with LV-GFP. About 50% of the splenocytes were positive for B-220 and among these cells less than 2% were GFP positive. There were less than 1% GFP positive cells that were CD4$^+$ or CD8a$^+$. Notably, 16% of the splenocytes were monocytes, positive for (CD11b$^+$, or CD11C$^+$) and among those 7% were positive for GFP.
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apoA-I 28 kd

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apoA-I 28Kd

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<th>b. Wt LV-apoA-I</th>
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apoE 34Kd

D.

![Graph showing ApoA-I and ApoE concentrations across fractions](Su, Figure 1)