Distinct roles of apolipoprotein components within the trypanosome lytic factor complex revealed in a novel transgenic mouse model

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Humans express a unique subset of high-density lipoproteins (HDLs) called trypanosome lytic factors (TLFs) that kill many Trypanosoma parasite species. The proteins apolipoprotein (apo) A-I, apoL-I, and haptoglobin-related protein, which are involved in TLF structure and function, were expressed through the introduction of transgenes in mice to explore their physiological roles in vivo. Transgenic expression of human apolipoprotein L-I alone conferred trypanolytic activity in vivo. Coexpression of human apolipoprotein A-I and haptoglobin-related protein (Hpr) had an effect on the integration of apolipoprotein L-I into HDL, and both proteins were required to increase the specific activity of TLF, which was measurable in vitro. Unexpectedly, truncated apolipoprotein L-I devoid of the serum resistance gene interacting domain, which was previously shown to kill human infective trypanosomes, was not trypanolytic in transgenic mice despite being coexpressed with human apolipoprotein A-I and Hpr and incorporated into HDLs. We conclude that all three human apolipoproteins act cooperatively to achieve maximal killing capacity and that truncated apolipoprotein L-I does not function in transgenic animals.

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purified Hpr was trypanolytic in vitro (11), but more recent data indicate that Hpr cooperates to increase the lytic activity of TLF1 (12) or to increase the uptake of TLF1 (13) via an Hp/Hpr receptor on trypanosomes (11, 14). Both mechanisms are currently proposed to be instrumental in the level of TLF activity in vitro, with the finding that hemoglobin must be bound to Hpr to generate an increase in both lytic capacity and the receptor-mediated uptake of TLF (15).

RESULTS AND DISCUSSION
Creation of TLF mice using hydrodynamic-based gene delivery
To elaborate on the role of Hpr, apoL-I, and Tr-apoL-I and to explore reconstitution of TLF activity in vivo, we created a physiologically relevant system that allows transgenic expression of individual or multiple TLF components in a mouse. Using a hydrodynamic gene-based delivery approach (16), we generated Tg mice that express native human Hpr, apoL-I or Tr-apoL-I, or both components predominantly from the liver (17), which is the normal site where TLF components are synthesized and assembled. Expression plasmids that encode Hpr and/or apoL-I or Tr-apoL-I are expressed and proteins are secreted into the circulation within 24 h.

Human TLF has several well-characterized properties that define it as a specific subset of HDL. We used the following criteria as a benchmark for comparing the activity of reconstituted TLFs from Tg mice: (a) coimmunoprecipitation experiments have revealed that Hpr and apoL-I are coexpressed in the same particle despite constituting only 1% of HDL molecules, suggesting that their distribution in HDL particles is nonrandom (12, 15). (b) Trypanolytic activity of human plasma or purified TLF against T. b. brucei can be demonstrated in an in vitro killing assay during which the trypanosomes swell and burst. Alternatively, human plasma can be i.v. injected into a mouse to provide protection against T. b. brucei infection (18). (c) Trypanosomes that express SRA either naturally (T. b. rhodesiense) or artificially (T. brucei-SRA) are resistant to trypanolysis by TLF in vitro (3) or in vivo after transfer of human plasma or purified TLF i.v. to mice (18). (d) Trypanolytic activity in vitro can be blocked by addition of weak bases to prevent acidification of internal vesicles, which is required to activate TLF after uptake and internalization of the lipoprotein particle by the parasite (19).

Apolipoprotein L-I protects mice from infection with T. b. brucei, T. evansi, and T. congolense
Tg mice expressing genes that encode for Hpr and apoL-I individually reveal that only mice expressing the apoL-I transgene (Tg-apoL-I) are protected from challenge with T. b. brucei. In contrast, mice that express the Hpr transgene (Tg-Hpr) are not protected and succumb to infection with the same kinetics as mice given the empty vector (Fig. 1 A). We then evaluated whether the expression of Hpr and apoL-I together in vivo would give additional protection beyond that seen with apoL-I. There is no increase in protection when the mice express both Hpr and apoL-I either from individual plasmids (Tg-Hpr/apoL-I) or coexpress them from a single plasmid (Tg-Hpr:apoL-I), which allows protein synthesis in the same transfected cell. These results suggest that in vivo apoL-I is the main lytic component of TLF.

Tg-apoL-I mice mimic humans in their resistance and sensitivity to infection with a variety of trypanosome species (Table I). Nonprimate mammals do not have the genes that encode for Hpr and apoL-I, and therefore they do not produce TLF. They are thus susceptible to a broader range of trypanosome species than humans, such as T. congolense, T. evansi,
Transgenic mice that express the apoL-I gene ameliorate infection with *T. brucei*, *T. evansi*, and *T. congolense*

| Transgenic DNA | Trypanosome species (1.78 × 10^6 injected i.p.) | Number mice alive/number mice infected |
|---------------|-----------------------------------------------|---------------------------------------|
| ApoL-I Vector  | *T. brucei*                                    | 5/5                                   |
| ApoL-I Vector  | *T. brucei* SRA                                | 0/5*                                  |
| ApoL-I Vector  | *T. evansi*                                    | 5/5                                   |
| ApoL-I Vector  | *T. congolense*                                | 5/5                                   |
| ApoL-I Vector  |                                               | 2/5                                   |

Mice expressing the gene for apoL-I were infected with 1.78 × 10^6 *T. b. brucei* (Lister 427-derived line), *T. b. brucei-SRA* (Lister 427-derived line expressing the SRA gene), *T. evansi* (AnTat 3), or *T. congolense* (STIB 680); *n* = 5 per group.

*Parasitemia ≥ 1 × 10^6* before mice were killed.

*No parasites detectable.

*Died of unknown cause, did not have detectable parasites.

AapoL-I (median survival 201 h) and Tg-Hpr:apoL-I (median survival 101 h; Fig. 1 B). These parasites that recrudesce are not resistant to TLF, but the trypanosome infection and protected the mice indefinitely. To gauge the relative amount of lytic units within the plasma from different Tg mice, we serially diluted human plasma until survival time was ~300 h (1:8, 37 μl; Fig. 1 B). In this plasma transfer assay, we find that Tg mouse plasma has ~10% the lytic capacity of normal human plasma. In contrast to human plasma, Tg-apoL-I plasma or Tg-Hpr:apoL-I plasma showed no trypanolytic activity in vitro within the time frame of our standard assay (unpublished data), which suggests that the Tg plasma is missing some additional human component that augments or stabilizes activity in vitro or in vivo.

**Human apoA-I and haptoglobin-related protein increase the specific activity of HDL synthesized by Tg mice**

We next evaluated whether human apoA-I could augment or stabilize the Tg-TLF activity in vitro or in vivo. In contrast to mice, humans have three major HDL subfractions designated HDL1 ~11.4 nm, HDL2 ~10.2 nm, and HDL3 ~8.7 nm. Mice containing a stably integrated human apoA-I transgene (Huapo-A-I) express ~2 mg/ml human apoA-I and 0.1 mg/ml murine apoA-I and exhibit a humanlike HDL profile of HDL2 and HDL3, indicating that the amino acid sequence of apoA-I is a determinant of the HDL distribution (20). Plasma from Huapo-A-I mice transfected with Hpr and apoL-I (Hpr:apoL-I) display similar levels of protein either compared with plasma from Huapo-A-I mice transfected with Hpr and apoL-I (Fig. 1 C) or compared with human plasma (Fig. 2 A), corresponding to ~5–10 μg/ml of apoA-I (21) and ~20–30 μg/ml of Hpr (22).

In contrast to the murine apoA-I plasma transfer experiments, we find that expression of apoA-I in the presence of human apoA-I results in plasma that is less lytic than that obtained when both TLF components (Hpr:apoL-I) are expressed. Plasma transfer from Huapo-A-I mice transfected with Hpr to naive mice infected with *T. b. brucei* showed no protection by Hpr (median survival 32 h), equivalent to vector alone (median survival 39.5 h; Fig. 2 B).

Plasma from Huapo-A-I:Hpr:apoL-I transferred to naive mice infected with *T. b. brucei* has an increase in lytic capacity (median survival time 288 h) compared with that observed with Huapo-A-I:apoL-I (median survival 101 h; Fig. 2 B). These data reveal that coexpression of Hpr in the context of human apoA-I increases lytic activity, which suggests that human apoA-I may augment or stabilize Tg-TLF (Hpr:apoL-I) activity in vivo. Despite an increase in activity, the triple Tg mouse plasma (Huapo-A-I:Hpr:apoL-I) is not as potent as human plasma in transfer experiments. Additional factors present in human plasma, such as TLF2, which is an immune complex of polyclonal IgM and lipid-poor TLF1 that circulates in plasma at ~10 μg/ml (4), could explain the difference in lytic capacity.

To evaluate the assembly of Hpr and apoL-I into HDLs and the lytic capacity of our various Tg mice, we purified all of the different Tg-HDLs containing human apoA-I by density

**Table I.** Transgenic mice that express the apoL-I gene ameliorate infection with *T. brucei*, *T. evansi*, and *T. congolense*
gradient ultracentrifugation followed by size fractionation and analyzed each fraction by Western blot for localization of Hpr, apoL-I, and apoA-I. Size fractionation of lipoproteins of the HuapoA-I mice show a broad HDL peak (plain lines, #10-14; Fig. 2 C), which matches the distribution of human HDL (dashed line). When mice were transfected with a plasmid that encodes for Hpr, we find that Hpr-HDL is enriched in fractions 11 and 12 (Fig. 2 D). When mice were transfected with a plasmid that encodes for apoL-I, we find that apoL-I-HDL is enriched in fractions 12 and 13 (Fig. 2 D). When we transfected a single plasmid that encodes both genes under the control of individual promoters to drive expression in the same cell, we find that apoL-I has been “redistributed” upon coexpression with Hpr predominantly into fractions 11 and 12 (Fig. 2 D). This suggests that when coexpressed, Hpr and apoL-I localize to the same HDL particle (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071463/DC1). We observe a similar redistribution of apoL-I upon coexpression of Hpr in murine apoA-I HDLs (unpublished data).

Purified human TLFs containing either Hpr or apoL-I have low specific activities for in vitro trypanolysis relative to TLFs that contain both Hpr and apoL-I in the same particle (12). Notably, in our experimental system, only the purified triple Tg-HDL, which is composed of HuapoA-I:Hpr:apoL-I, showed trypanolytic activity in vitro within the time frame of our standard assay (Fig. 2 E). The lytic capacity of the triple Tg-HDL was equivalent to that of human TLF-enriched HDL (both isolated by the same method). In addition, trypanosome lysis by both HDLs was inhibited by incubation of trypanosomes with ammonium chloride (10 mM), a lysosomotropic agent that blocks the acidification of internal organelles (Fig. 2 E). This indicates that we have created a functional Tg TLF (HuapoA-I:Hpr:apoL-I) that mimics the properties of human TLF. These data reveal that human apoA-I contributes to an increase in activity of Tg-Hpr:apoL-I HDLs (but not of Tg-apoL-I) that is measurable in plasma transfer experiments in vivo (Fig. 2 B), and more so in lytic assays in vitro (Fig. 2 E). There was no measurable activity in mouse apoA-I:Hpr:apoL-I HDL in vitro (unpublished data). Therefore, we conclude that the increase in specific activity observed previously in vitro (13) is caused not only by the presence of just Hpr and apoL-I, but by an interaction between the three human proteins apoA-I, Hpr, and apoL-I (Fig. 2, B and E).

The difference in the specific activity of the Tg-HDLs could arise from changes in the assembly and stability of the
HDL complex or changes in the trafficking of Tg-HDLs in the trypanosome. We do not detect any differences in the assembly of TLFs generated with mouse or human apoA-I based on the following observations. Hpr and apoL-I are exclusively found associated with HDL. We consistently find redistribution, and sometimes an increase, in concentration of apoL-I upon coexpression with Hpr. This suggests that there is an interaction of Hpr with apoL-I. Purification of the Tg-Hpr:apoL-I HDLs by KBr ultracentrifugation and size chromatography gave equivalent yields of Hpr and apoL-I (unpublished data). This suggests that there was no loss of protein components during the purification of TLFs generated with mouse or human apoA-I. Given that no difference in the relative binding to trypanosomes of purified human HDL subclasses containing either Hpr or apoL-I or both Hpr and apoL-I has been reported (12), we hypothesize that human apoA-I in the presence of Hpr and apoL-I effects either the trafficking of the purified triple Tg HDL or the resistance to trypanosomal proteases within the lysosome in agreement with others (23).

Apolipoprotein L-I devoid of the “SRA-interacting domain” does not protect mice from trypanosome infection

Having established that transient transgenesis provides the first animal model capable of validating reconstituted TLF activity in vivo, we next assessed the trypanolytic potential of a truncated apoL-I devoid of the SRA-interacting domain (Tr-apoL-I). Full-length apoL-I can kill *T. brucei*, but not *T. brucei* that expresses SRA. Tr-apoL-I, which is constructed by deletion of the C-terminal \(^{340}\)-helix starting at amino acid \(^{350}\), has been synthesized in bacteria and cell-free systems and is reported to kill SRA-expressing trypanosomes both in vitro (3) and in vivo when conjugated to nanobodies directed against VSG carbohydrate epitopes and injected into *T. brucei*-infected mice (8). Transgenic expression of Tr-apoL-I in livestock could therefore conceivably create animals that are resistant to infection by all species of trypanosomes. Tr-apoL-I–transfected mice revealed robust expression of Tr-apoL-I that readily assembled into HDL particles in a manner similar to full-length apoL-I (Fig. 3 A). To our surprise, we were unable to detect any lytic activity in vivo against SRA-expressing trypanosomes, or even against the susceptible *T. b. brucei* (Table II). To improve the potential activity of Tr-apoL-I, we generated a double plasmid (Hpr:Tr-apoL-I) and transfected Tg-HuapoA-I mice, thereby creating HuapoA-I:Hpr:Tr-apoL-I mice. Serial dilution of Tg mice plasma revealed robust expression of both proteins (Fig. 3 B), which could be coimmunoprecipitated indicating their localization in the same HDL (Fig. S1). However, inoculation of mice with a
The discrepancy between active Tr-apoL-I generated in vitro and inactive Tr-apoL-I generated in vivo could be caused by potential differences in conformation, binding, trafficking, and accumulation within trypanosomes. It is unknown how apoL-I folds in solution when synthesized in bacteria in vitro compared with that present in HDL molecules when synthesized in transgenic mice in vivo, although it is likely that the structures are different. Lipid-poor apolipoproteins, depending on the concentration, will form oligomers in vivo, by plasma transfer (Fig. 2B), and in vitro (Fig. 2E). Despite the lack of activity of Tr-apoL-I, full-length apoL-I bound to HDL is completely effective in vivo (Table I and Fig.1). Coexpression with human apoA-I and Hpr increases the lytic activity of full-length apoL-I that is measurable in vitro (27), which is in agreement with our data. We find that the addition of Hpr does not enhance the in vivo protection directly within the Tg mice, over and above apoL-I. Despite the observation that Hpr redistributes apoL-I into higher molecular mass HDLs, Hpr in combination with apoL-I is not sufficient to generate TLF with lytic activity that is measurable in vitro within the time frame of our assay. It is the combination of human apoA-I, Hpr, and apoL-I that recreates a fully functional “human TLF” in vitro, with equivalent lytic capacity to purified human TLF. These data lead us to conclude that all three human proteins are necessary to effect maximal killing and emphasize the importance of Tg mice to validate hypotheses generated through in vitro experimentation. Further use of these Tg mice, coupled with the development of newly engineered lines of mice, should help unravel many questions associated with the in vivo biological properties of TLF.

**Table II.** Transgenic mice that express the truncated apoL-I devoid of the SRA-interacting domain do not protect from infection with *T. b. brucei* or *T. b. brucei*-SRA

| Transgenic DNA | Trypanosome species | Number mice alive/number mice infected |
|---------------|---------------------|---------------------------------------|
| ApoL-I        | *T. b. brucei*      | 5/5                                   |
|               |                     | 3/5*                                  |
| Tr-apoL-I     | *T. b. brucei*      | 0/5*                                  |
| Vector        |                     | 0/5*                                  |
| ApoL-I        | *T. b. brucei SRA*  | 0/5*                                  |
| Tr-ApoL-I     |                     | 0/5*                                  |
| Vector        |                     | 0/5*                                  |

Tg mice expressing full-length apoL-I or truncated apoL-I infected with 1.78 x 10^6 *T. b. brucei* (Lister 427-derived line) or *T. b. brucei*-SRA (Lister 427-derived line expressing the SRA gene). n = 5 per group.

*No parasites detectable.

*Parasitemia ≥ 1 x 10^9/ml before mice were killed.

low dose of 5,000 parasites did not reveal any significant trypanolytic activity (P > 0.05) of HuapoA-I:Hpr:Tr-apoL-I against *T. b. brucei* (Fig. 3C) or *T. b. brucei*-SRA (Fig. 3D).

The discrepancy between active Tr-apoL-I generated in vitro and inactive Tr-apoL-I generated in vivo could be caused by potential differences in conformation, binding, trafficking, and accumulation within trypanosomes. It is unknown how apoL-I folds in solution when synthesized in bacteria in vitro compared with that present in HDL molecules when synthesized in transgenic mice in vivo, but it is likely that the structures are different. Lipid-poor apolipoproteins, depending on the concentration, will form oligomers in physiological media, whereas apolipoproteins bound to lipid-rich HDL particles do not (24). The recombinant “lipid-free” Tr-apoL-I–nanobody binds to VSG all over the surface of the parasite, whereas HDL and TLF bind to specific receptors in the flagellar pocket (11, 15). TLF is trafficked to lysosomes, wherein it is activated (7). It is unknown where the “lipid free” Tr-apoL-I nanobody traffic, accumulates, or acts. Despite the lack of activity of Tr-apoL-I, full-length apoL-I bound to HDL is completely effective in vivo (Table I and Fig.1). Coexpression with human apoA-I and Hpr increases the lytic activity of full-length apoL-I that is measurable in vivo, by plasma transfer (Fig. 2B), and in vitro (Fig. 2E). In contrast, there is no significant activity of Tr-apoL-I bound to HDL, even when coexpressed with human apoA-I and Hpr (Fig. 3, C and D). Therefore, the data indicate that the C terminus of apoL-I clearly contributes to lytic activity and is absolutely required when associated with HDL.

An individual from India was diagnosed with *T. evansi*, which is not normally infective for humans (25). He had two mutated apoL-I alleles that prevented the production of functional apoL-I protein. It was speculated that the lack of the apoL-I pore-forming domain (stop codon at aa 149) or membrane-addressing domain (stop codon at 268) were key to his susceptibility to *T. evansi* (26). In contrast, our Tg-Tr-apoL-I mice data show that deletion of the last α-helix at the C-terminus (stop codon at 342) is sufficient to eliminate TLF activity in vivo, even though Tr-apoL-I is synthesized and incorporated into HDL and contains both the pore-forming and membrane-addressing domains. Therefore, it is unlikely that a transgene that encodes for this Tr-apoL-I will lead to the production of trypanosome-resistant transgenic animals.

**Conclusion**

Overall, our in vivo data show that apoL-I is necessary and sufficient to kill trypanosomes. Conversely, Tr-apoL-I, which was predicted to be lytic for *T. b. brucei*-SRA, and therefore *T. b. rhodesiense*, is unable to kill any trypanosomes in vivo or in vitro, thereby underscoring the importance of the C-terminal α-helix of apoL-I in the context of HDL. Hpr expressed in vivo does not cause the lysis of African trypanosomes. Although Tg-Hpr mice have been previously described, no lytic activity was detected in purified HDL in vitro (27), which is in agreement with our data. We find that the addition of Hpr does not enhance the in vivo protection directly within the Tg mice, over and above apoL-I. Despite the observation that Hpr redistributes apoL-I into higher molecular mass HDLs, Hpr in combination with apoL-I is not sufficient to generate TLF with lytic activity that is measurable in vitro within the time frame of our assay. It is the combination of human apoA-I, Hpr, and apoL-I that recreates a fully functional “human TLF” in vitro, with equivalent lytic capacity to purified human TLF. These data lead us to conclude that all three human proteins are necessary to effect maximal killing and emphasize the importance of Tg mice to validate hypotheses generated through in vitro experimentation. Further use of these Tg mice, coupled with the development of newly engineered lines of mice, should help unravel many questions associated with the in vivo biological properties of TLF.

**MATERIALS AND METHODS**

**Cloning and expression of apolipoprotein L-I and haptoglobin-related protein in individual plasmids and together in one plasmid.**

*pRGG77* plasmid was obtained from Regeneron Pharmaceuticals, Inc. Human haptoglobin-related protein encoding the full-length signal peptide (accession no. NM_020905), a gift from M. Redpath (New York University School of Medicine, New York, NY), was cloned into pRGG77 plasmid. A Kozac sequence CCACC was introduced by site-directed mutagenesis (Stratagene; P1).

A TopoTA-PCR product of apolipoprotein L-I from human liver (accession no. O14791), a gift of E. Lugli (New York University School of Medicine), was cloned into pRGG77 plasmid (P2). The DNA sequence encoding the full-length signal peptide of apol-I and a Kozac sequence CCACC was introduced by PCR amplification with Pfu Ultra (Stratagene) and cloned into pRGG77 (P3). A dual construct containing apoL-I and Hpr (each with their own promoter, and poly[A] tail) was cloned into pRGG77 (P5). For expression of apoL-I, Hpr, or both in HDL particles containing human apoA-I, high-volume
injections were performed in C57BL/6-Tg(APOA1)1Rub/J (The Jackson Laboratory). Control mice were C57BL/6, which express murine apoA-I. 3 d after injections and every other day thereafter blood samples (20 μl) were taken from the animals via tail bleeds for evaluation of secreted human proteins. All procedures were approved by the Institutional Animal Care and Use Committee of the New York University Langone Medical Center.

Purification of lipoproteins. HDL was purified from the plasma of two mice by adjusting the density to 1.25 g/ml with KBr and centrifuged for 16 h at 49,000 rpm at 10°C in NVTl65 rotor. The lipoprotein fractions were collected, concentrated, and fractionated on a Superdex 200 HR 10/30 column (GE Healthcare) (5).

Electrophoresis and immunoblotting. Plasma samples or lipoprotein fractions were separated on 7.5% Tris-glycine PAGE Gold precast gels (Cambrex), transferred onto PVDF membranes (GE Healthcare), and probed with the following: polyclonal rabbit anti-human haptoglobin (1:20,000; Sigma-Aldrich); mouse monoclonal anti-Hpr (1:5,000); mouse monoclonal anti-apoL-I (1:10,000), polyclonal rabbit anti-human apoL-I (1:10,000; Proteintech Group, Inc.), polyclonal goat anti-human apoL-I (N20, 1:100; Santa Cruz Biotechnology), polyclonal sheep anti-human apoA-I (1:10,000; AbD Serotec), and polyclonal rabbit anti mouse apoA-I (1:10,000; Abcam). Secondary antibodies conjugated to horseradish peroxidase used were as follows: anti-rabbit IgG (1:10,000), anti-mouse IgG (1:5,000), anti-goat IgG (1:10,000; all Promega), and anti-sheep IgG (1:10,000; Roche).

Trypanosomes. The following trypanosomes were used: serum-sensitive T. b. brucei ILTat1.25 and T. b. brucei Lister 427-derived cell line, the serum-resistant strain T. b. brucei 427-derived cell line expressing the serum resistance-associated (SRA) gene (28), serum-sensitive T. evansi (Antat 3), and T. congolense (STIB68) (6).

In vivo experiments. For survival experiments mice were infected i.p. with 1.78 × 10^6 or 5,000 trypanosomes on day 3 after high-volume injection of the human genes. For plasma transfer experiments, on day 3 after transfection with human transgenes, mice were killed. Aliquots of 300 μl of plasma were injected i.v. into naive mice that were infected with T. b. brucei (0.5–2.5 × 10^6/ml). Parasitemia was followed, and mice that reached parasitemia of 10^7/ml were killed and their time of death was recorded.

In vitro experiments. Trypanosomes (T. b. brucei ILTat 1.25; 10^6) were incubated for 150 min at 37°C in DME, 0.2% BSA with aliquots of sized fractionated lipoproteins. Where indicated, parasites were incubated with anti-apoL-I (1:10,000), polyclonal rabbit anti-human apoL-I (1:10,000; Sigma-Aldrich); mouse monoclonal anti-Hpr (1:5,000); mouse monoclonal anti-apoL-I (1:10,000), polyclonal goat anti-human apoL-I (N20, 1:100; Santa Cruz Biotechnology), polyclonal sheep anti-human apoA-I (1:10,000; AbD Serotec), and polyclonal rabbit anti mouse apoA-I (1:10,000; Abcam). Secondary antibodies conjugated to horseradish peroxidase used were as follows: anti-rabbit IgG (1:10,000), anti-mouse IgG (1:5,000), anti-goat IgG (1:10,000; all Promega), and anti-sheep IgG (1:10,000; Roche).

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