Human ApoA-I Transfer Attenuates Transplant Arteriosclerosis via Enhanced Incorporation of Bone marrow–derived Endothelial Progenitor Cells

Yingmei Feng, Frank Jacobs, Eline Van Craeyveld, Christine Brunaud, Jan Snoeys, Marc Tjwa, Sophie Van Linthout, Bart De Geest

Objective—Transplant arteriosclerosis is the leading cause of graft failure and death in patients with heart transplantation. Endothelial progenitor cells (EPCs) contribute to endothelial regeneration in allografts. We investigated whether increased HDL cholesterol induced by adenoviral human apoA-I (AdA-I) transfer increases number and function of EPCs, promotes incorporation of EPCs in Balb/c allografts transplanted paratopically in C57BL/6 ApoE−/− mice, and attenuates transplant arteriosclerosis.

Methods and Results—EPC number in ApoE−/− mice was increased after AdA-I transfer as evidenced by 1.5-fold (P<0.01) higher Flk-1 Sca-1–positive cells and 1.4-fold (P<0.01) higher Dil-aclDL isolectin-positive spleen cells. In addition, HDL enhanced EPC function in vitro. Incorporation of bone marrow–derived EPCs was 5.8-fold (P<0.01) higher at day 21 after transplantation in AdA-I-treated apoE−/− mice compared with control mice. Enhanced endothelial regeneration in AdA-I-treated apoE−/− mice as evidenced by a 2.6-fold (P<0.01) increase of CD31-positive endothelial cells resulted in a 1.4-fold (P=0.059) reduction of neointima and a 3.9-fold (P<0.01) increase of luminal area.

Conclusion—Human apoA-I transfer increases the number of circulating EPCs, enhances their incorporation into allografts, promotes endothelial regeneration, and attenuates neointima formation in a murine model of transplant arteriosclerosis. (Arterioscler Thromb Vasc Biol. 2008;28:278-283)

Key Words: high density lipoproteins ■ apolipoprotein A-I ■ hypercholesterolemia ■ endothelial progenitor cells ■ transplant arteriosclerosis

Plasma levels of high-density lipoprotein (HDL) cholesterol and its major apolipoprotein (apo), apoA-I, are inversely correlated with the incidence of ischemic cardiovascular diseases. A meta-analysis of 4 prospective studies indicated that a 1 mg/dL increase of HDL cholesterol is associated with a 2% risk reduction in men and a 3% risk reduction in women. Reverse cholesterol transport is considered to be the principal mechanism underlying the beneficial effects of HDL. Additional protective mechanisms include the inhibition of low-density lipoprotein (LDL) oxidation, of cellular adhesion molecule expression, and of platelet activation and aggregation. HDL may also contribute to the maintenance of the integrity of the vascular endothelium by stimulating endothelial repair mechanisms. HDL has been shown to enhance endothelial cell migration in vitro and to promote reendothelialisation in vivo. Recent reports have demonstrated that administration of reconstituted HDL increases the recruitment of endothelial progenitor cells (EPCs) into the aortic endothelium of apoE-deficient mice and enhances the contribution of bone marrow–derived cells to neovascularization in a mouse hindlimb ischemia model.

Allograft-accelerated transplantation arteriosclerosis is the leading cause of graft failure and death in patients with heart transplantation. Hu et al have demonstrated that EPCs contribute to endothelial regeneration and to microvessel formation in allografts. Hypercholesterolemia is associated with impaired number and function of EPCs in patients with coronary artery disease and in apoE-deficient mice. Here, we investigated whether increased HDL cholesterol after adenoviral human apoA-I transfer increases number and function of EPCs in hypercholesterolemic apoE−/− mice. Using a paratopic model of transplant arteriosclerosis in apoE−/− mice, we evaluated the hypothesis that human apoA-I transfer may increase number and function of EPCs, enhance incorporation of EPCs in Balb/c allografts, stimulate endothelial repair, and reduce neointima formation. We show that apoA-I transfer increases the circulating number of EPCs, improves EPC function in vitro, enhances EPC incor-
poration in allografts, stimulates reendothelialization, and attenuates transplant arteriosclerosis.

Materials and Methods
For detailed methodology, please see http://atvb.ahajournals.org. Briefly, 2 weeks after gene transfer or saline injection, a common carotid artery of a female Balb/c donor mouse was transplanted paratopically into recipient C57BL/6 apoE−/− mice in an end-to-side anastomosis on the left common carotid artery as described previously by Shi et al.13 C57BL/6 apoE−/− mice artery transplant recipients were euthanized for histological analysis of the allografts 21 days or 56 days after artery transplantation. To quantify EPC incorporation in allografts, C57BL/6 apoE−/− mice were lethally irradiated with 9.0 Gy at the age of 9 weeks. Transplantation of 6.7×10⁶ bone marrow cells obtained from C57BL/6 apoE−/− β-actin GFP mice was performed 24 hours after irradiation. Gene transfer and artery transplantation were subsequently performed in chimeric mice at the age of 13 and 15 weeks, respectively. Cryosections obtained at day 21 after artery transplantation were incubated with rabbit anti-mouse GFP (Molecular Probes) and rat anti-mouse CD31 (BD) and then labeled with goat anti-rabbit Alexa Fluor 488 and goat anti-rat Alexa Fluor 568 (Molecular Probes) to detect bone marrow–derived endothelial cells.

Results
Human ApoA-I Gene Transfer Induces a Persistent Elevation of HDL Cholesterol in C57BL/6 ApoE−/− Mice
Supplemental Figure IA (please see http://atvb.ahajournals.org) shows human apoA-I plasma levels after transfer with 5×10⁹ particles of AdA-I in male C57BL/6 apoE−/− mice. AdA-I is an E1E3E4-deleted adenoviral vector containing a hepatocyte-specific expression cassette. Human apoA-I expression was sustained for the entire duration of the experiment and was 140±11 mg/dL at day 70 after transfer. Quantification of murine apoA-I levels by Western blot in AdA-I (n=5)-treated mice showed that, compared with baseline levels before gene transfer, murine apoA-I levels were reduced to 34±9.0% at day 14, 16±0.93% at day 35, 21±3.2% at day 56, and 35±7.7% at day 70 after transfer (data not shown). No alteration of murine apoA-I levels was observed after Adnull (n=5) transfer. To evaluate the effect of AdA-I transfer on lipoprotein levels, lipoproteins were fractionated by gel filtration. The lipoprotein profiles at day 0, day 14, day 35, and day 70 after transfer are shown in supplemental Figure IIA and IIB (please see http://atvb.ahajournals.org) for AdA-I and Adnull-treated mice, respectively. Table I shows total cholesterol levels, non-HDL cholesterol levels, and HDL cholesterol levels at baseline and at different time points after gene transfer with AdA-I or the control vector Adnull in male C57BL/6 apoE−/− mice (n=10 for each experimental condition). Compared with baseline, AdA-I transfer resulted in a 2.2-fold (P<0.01) increase of HDL cholesterol levels at day 14, 1.5-fold (P<0.01) increase of HDL cholesterol levels at day 14, day 35, and day 70 after transfer, respectively. Non-HDL cholesterol levels were unaltered after AdA-I transfer. Gene transfer with the control vector Adnull did not induce a significant alteration of non-HDL cholesterol levels or HDL cholesterol levels at any time-point (Table 1; supplemental Figure IIB).

AdA-I Transfer Reduces Oxidative Stress in C57BL/6 ApoE−/− Mice
The concentration of 8-isoprostanes was determined as a biomarker of oxidative stress. Compared with baseline, the concentration of 8-isoprostanes was 1.6-fold (P<0.05) lower at day 35 (130±18 pg/mL) after AdA-I transfer compared with baseline values (200±24 pg/mL). After transfer with Adnull, the concentration of 8-isoprostanes at day 35 (290±41 pg/mL) was not significantly different compared with baseline (200±32 pg/mL) and 2.2-fold (P<0.01) higher than 35 days after transfer with AdA-I.

Human ApoA-I Gene Transfer Increases the Number of Endothelial Progenitor Cells (EPCs) in the Circulation and in Bone Marrow
The number of Flk-1 Sca-1 double positive cells in the peripheral blood at different time points after AdA-I transfer, Adnull transfer, or saline injection in male C57BL/6 apoE−/− mice is shown in supplemental Figure IB. Because neither Adnull gene transfer (n=5) nor saline injection (n=5) had a significant effect on the number of Flk-1 Sca-1 double positive cells at any time point, the data of both groups were pooled in 1 control group. AdA-I transfer (n=10) resulted in a 1.5-fold increase (P<0.01) of Flk-1 Sca-1 double positive cells at day 10, day 21, and day 35 and a 1.4-fold (P<0.05) increase at day 56 after transfer compared with baseline (supplemental Figure IB). This effect was independent of the presence or absence of an allograft (data not shown). The increase of circulating EPCs was confirmed by quantification of the number of DiI-acLDL fluorescein isothiocyanate (FITC)-isolectin double positive cells after 4 days of ex vivo culture of spleen mononuclear cells isolated at day 10 after transfer. The number of EPCs in the spleen was 1.4-fold (P<0.01) higher after AdA-I transfer (n=6) than after Adnull transfer (n=6) (supplemental Figure IC).

To evaluate whether human apoA-I transfer increases EPC number in bone marrow, bone marrow mononuclear cells were isolated from saline (n=4) and Adnull (n=4) control mice and AdA-I gene transfer mice (n=8) 35 days after injection and cultured for 7 days. The number of DiI-acLDL FITC-isolectin double positive cells per field was 24% (P<0.01) higher in AdA-I treated mice than in control mice (data not shown).

Table 1. Total Cholesterol, Non-HDL Cholesterol and HDL Cholesterol Plasma Levels at Baseline and at Different Time-Points After Gene Transfer With 5×10⁹ Particles of AdA-I or Adnull in C57BL/6 ApoE−/− Mice

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day 14</th>
<th>Day 35</th>
<th>Day 70</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdA-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>380±14</td>
<td>470±33*</td>
<td>470±22*</td>
<td>430±18</td>
</tr>
<tr>
<td>Non-HDL cholesterol</td>
<td>350±12</td>
<td>370±29</td>
<td>380±18</td>
<td>360±16</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>34±1.3</td>
<td>100±4.7**</td>
<td>85±4.1**</td>
<td>76±3.5**</td>
</tr>
<tr>
<td>Adnull</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>390±11</td>
<td>410±21</td>
<td>400±8.7</td>
<td>400±15</td>
</tr>
<tr>
<td>Non-HDL cholesterol</td>
<td>360±11</td>
<td>380±20</td>
<td>370±8.5</td>
<td>360±16</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>34±1.4</td>
<td>36±1.0</td>
<td>37±0.82</td>
<td>37±0.91</td>
</tr>
</tbody>
</table>

Non-HDL and HDL particles were separated by fast performance liquid chromatography gel filtration. Data are expressed in mg/dl and represent means±SEM of 10 mice for each experimental condition. *P<0.05; **P<0.01 for comparison of different time points after transfer with baseline values.
Human ApoA-I Transfer Enhances EPC Function in Hypercholesterolemic ApoE−/− Mice

To investigate the effect of human apoA-I transfer and HDL cholesterol on the function of EPCs, EPC migration, adhesion, and invasion assays were performed. The number of migrated EPCs isolated from Adnull treated mice (n=6) and AdA-I–treated mice (n=6) was increased 1.5-fold (P<0.001) and 1.9-fold (P<0.001), respectively, by addition of 100 μg/mL HDL to the lower chamber (supplemental Figure ID). Migration in the presence of 100 μg/mL HDL in the lower chamber was 1.4-fold (P<0.001) higher for cells isolated from AdA-I–treated mice than for cells isolated from Adnull treated mice (supplemental Figure ID). The number of EPCs isolated from Adnull treated mice (n=5) and AdA-I–treated mice (n=5) that adhered to fibronectin coated plates was increased 1.6-fold (P<0.05) and 2.1-fold (P<0.001), respectively, by addition of 100 μg/mL HDL (supplemental Figure IE). Quantification of the number of EPCs invaded through solidified Matrigel showed that the number of invaded EPCs isolated from Adnull treated mice (n=5) and AdA-I–treated mice (n=5) was increased 1.7-fold (P<0.05) and 2.1-fold (P<0.001), respectively, by addition of 100 μg/mL HDL (supplemental Figure IF). Taken together, these data indicate that HDL improves EPC function in vitro.

Stimulation of EPC Migration and EPC Survival by HDL Is Abrogated in the Presence of Wortmannin

EPCs were isolated from spleens of control C57BL/6 apoE−/− mice. After culture for 7 days, EPC migration was evaluated in the presence of 100 μg/mL bovine serum albumin (control; n=4), HDL (100 μg/mL; n=4), or HDL (100 μg/mL; n=4) plus wortmannin (200 nmol/L). The 1.6-fold (P<0.01) increase of EPC migration in the presence of HDL compared with controls was completely abrogated when wortmannin was added to HDL (supplemental Figure IIIA). These observations were confirmed in experiments using human EPCs isolated from 4 healthy female volunteers and human HDL (supplemental Figure IIIB). Taken together, these data suggest that improved EPC function by HDL is dependent on phosphatidylinositol 3-kinase signal transduction.

Under conditions of serum and growth factor deprivation, HDL (100 μg/mL; n=6) increased the number of surviving EPCs 1.6-fold (P<0.001) compared with bovine serum albumin (100 μg/mL; n=6). In the presence of wortmannin (200 nmol/L; n=6), enhanced survival induced by HDL was completely abrogated (supplemental Figure IIIC).

Human ApoA-I Gene Transfer Reduces Intimal Area in Allografts

The common carotid artery of female Balb/c mice (H2d) was transplanted paratopically to male C57BL/6 apoE−/− mice (H2b) 2 weeks after gene transfer or saline injection. Because Adnull transfer did not induce a change of lipoprotein levels (supplemental Figure IIIB) nor significant differences in morphometric parameters compared with saline injected mice, morphometric data of saline and Adnull-treated mice were pooled in 1 control group (Table 2). At day 21, the intimal area was 83 000±20 000 μm² in Adnull treated mice (n=7) and 93 000±13 000 μm² in saline mice (n=8). At day 56, the intimal area was 87 000±83 000 μm² in Adnull-treated mice (n=13) and 97 000±20 000 μm² (n=13) in saline-treated mice. Compared with control mice, intimal area was reduced 1.4-fold (P=0.059) and luminal area was increased 3.9-fold (P<0.01) in AdA-I–treated mice at day 21 after transplantation. At day 56 after transplantation, no significant reduction of neointima was observed in AdA-I–treated mice but luminal area was 16-fold (P<0.001) higher compared with control mice (Table 2). The increase in luminal area is explained by more pronounced expansive remodeling because the area within the internal elastic lamina multiplied by 100 (‰). *P<0.05; **P<0.01; ***P<0.001 vs controls.

### Table 2. Morphometric Analysis of Allografts at Day 21 and Day 56 After Transplantation

<table>
<thead>
<tr>
<th></th>
<th>Day 21</th>
<th>Day 56</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>AdA-I</td>
</tr>
<tr>
<td>No. of mice</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>Area within the external elastical lamina</td>
<td>150±1.1</td>
<td>150±8.1</td>
</tr>
<tr>
<td>Area within the internal elastical lamina</td>
<td>97±9.5</td>
<td>95±6.4</td>
</tr>
<tr>
<td>Medial area</td>
<td>54±2.5</td>
<td>55±3.5</td>
</tr>
<tr>
<td>Intimal area</td>
<td>88±11</td>
<td>61±8.4</td>
</tr>
<tr>
<td>Luminal area</td>
<td>8.7±2.5</td>
<td>34±6.6**</td>
</tr>
<tr>
<td>% stenosis</td>
<td>87±4.5</td>
<td>62±6.8**</td>
</tr>
</tbody>
</table>

Morphometric data are expressed in 10⁵ μm² and represent means±SEM. The percentage stenosis is defined as the intimal area divided by the area within the internal elastic lamina multiplied by 100 (‰). *P<0.05; **P<0.01; ***P<0.001 vs controls.

Human ApoA-I Transfer Reduces Allograft Inflammation

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### Significant loss of endothelial cells in allografts at day 1 and day 3 after transplantation

To evaluate the degree of endothelial denudation after transplantation, the number of CD31-positive endothelial cells was quantified in allografts at day 1 (n=5) and day 3 (n=5) after transplantation in C57BL/6 apoE−/− mice injected 14 days before with 5×10¹⁰ particles of Adnull or AdA-I. Compared with the number of CD31-positive endothelial cells in the common carotid artery of nontransplanted Balb/c mice (48±4.5; n=5), the number of CD31-positive endothelial cells in allografts was 3.4-fold (P<0.01) reduced at day 1 after transplantation of mice pretreated with Adnull (14±0.51) or AdA-I (14±1.5). At day 3, the number of CD31-positive endothelial cells was 2.6-fold (P<0.01) and 2.1-fold (P<0.01) lower in allografts of Adnull (18±3.4) and AdA-I (23±2.4)-treated mice, respectively, than in the nontransplanted common carotid artery of Balb/c mice. The degree of endothelial cell loss was not significantly different between AdA-I and Adnull treated mice at day 1 or day 3.
after transfer. As an index of the number of CD31-positive endothelial cells per unit of surface lining the arterial lumen, the number of endothelial cells per section was divided by the circumference of the artery lumen in each section. This index was 0.031 ± 0.0030 in the common carotid artery of nontransplanted Balb/c mice, 0.011 ± 0.0013 at day 1 and 0.013 ± 0.0025 at day 3 after transplantation in Adnull pre-treated mice, and 0.010 ± 0.0013 at day 1 and 0.015 ± 0.0021 at day 3 after transplantation in AdA-I–treated mice.

**Figure 1.** Representative sections of transplant arteriosclerosis at day 21 (A, B) and day 56 (C, D) after transfer in control (A, C) and AdA-I (B, D)-treated C57BL/6 apoE−/− mice.

### Human ApoA-I Transfer Increases Endothelial Regeneration and EPC Incorporation in Allografts

The number of CD31–positive endothelial cells was 2.6-fold (P < 0.01) and 13-fold (P < 0.0001) higher at day 21 (supplemental Figure IVA) and day 56 (supplemental Figure IVB) after transplantation, respectively, in AdA-I–treated mice compared with control mice. This was confirmed by a significant increase of the number of Tie2-positive endothelial cells at day 56 after transplantation (data not shown). CD31-positive cells were lining the allograft lumen at day 21 after transplantation whereas at day 56, we also observed CD31 positive microvessels within the neointima, particularly in AdA-I–treated mice (supplemental Figure V). At day 21, no microvessels were observed in the neointima. The number of CD31–positive endothelial cells per unit of surface lining the arterial lumen was 1.5-fold (P < 0.05) higher at day 21 after transplantation in AdA-I–treated mice (0.027 ± 0.0036) compared with control mice (0.018 ± 0.0024) and not significantly different compared with nontransplanted common carotid arteries of Balb/c mice (0.031 ± 0.0030). To quantify the incorporation of bone marrow–derived EPCs in allografts at day 21 after transplantation, bone marrow transplantation with bone marrow of C57BL/6 apoE−/− β-actin GFP mice was performed 4 weeks before gene transfer or saline injection. Supplemental Figure IVC shows that the number of incorporated CD31 GFP double positive cells was 5.8-fold (P < 0.01) higher at day 21 after transplantation in AdA-I–treated chimeric mice (n = 10) compared with control chimeric mice (n = 10). The ratio of the number of CD31 GFP double positive cells versus the total number of CD31-positive cells increased from 0.064 ± 0.023 in control mice to 0.22 ± 0.040 (P < 0.01) in AdA-I–treated mice. Double positive staining for CD31 and GFP is shown in Figure 2.

### Discussion

The main findings of the present study are that (1) increased HDL cholesterol after human apoA-I gene transfer increases the number of endothelial progenitor cells (EPCs) in the circulation and in bone marrow in apoE−/− mice; (2) HDL improves EPC function in vitro; (3) human apoA-I transfer stimulates incorporation of bone marrow–derived EPCs in the regenerating endothelium of allografts and enhances regeneration of the endothelium; (4) neointima formation is attenuated after human apoA-I transfer in this murine model of transplant arteriosclerosis.

The loss of endothelial function and integrity initiates a cascade of events that may lead to native atherosclerosis, vein graft atherosclerosis, restenosis after percutaneous revascularization, and transplant arteriosclerosis. EPCs have been shown to contribute to reendothelialisation and neovascularization and may play an essential role in endothelial maintenance and repair. Hypercholesterolemia is associated with impaired number and function of EPCs in apoE-deficient mice. Epidemiological studies show reduced number and function of EPCs in the presence of cardiovascular risk markers such as hypercholesterolemia, hyperton, diabetes, and hyperhomocysteinemia. In contrast, a positive correlation between HDL cholesterol plasma levels and circulating EPCs has been observed in cross-sectional studies. Tso et al recently reported that a single administration of reconstituted HDL 18 hours before euthanasia of apoE-deficient mice enhanced the incorporation of Sca-1–positive cells in the thoracic aortic endothelium. In another study, injection of recombinant HDL twice per week was shown to augment collateral development in a murine model of hindlimb ischemia and doubled EPC incorporation in the process of neovascularization. Here, we show for the
first time that a persistent increase of HDL cholesterol after human apoA-I gene transfer in hypercholesterolemic apoE−/− mice results in a sustained increase of the number of EPCs in the circulation and in bone marrow. HDL enhances EPC function in vitro as evidenced by stimulation of EPC migration, adhesion, and invasion. The enhanced EPC incorporation in the regenerating endothelium of allografts in the current study is in line with the increase of EPC incorporation in the process of neovascularization in the study of Sumi et al. The 2.5-fold to 3.0-fold increase of HDL cholesterol in apoE−/− mice after human apoA-I transfer in the current study is similar as in C57BL/6 apoE−/− human apoA-I transgenic mice that were characterized by potent inhibition of progression of native atherosclerosis.

Recently, it was demonstrated that reconstituted HDL stimulates the phosphatidylinositol 3-kinase/Akt signaling pathway to regulate human EPC differentiation. Here we show that the stimulation of murine and human EPC migration by HDL is abrogated in the presence of wortmannin, suggesting that the enhancement of EPC function in vitro by HDL is mediated at least in part via a phosphatidylinositol 3-kinase signal transduction pathway. In addition, the current study demonstrates that HDL isolated from AdA-I–treated mice enhances survival of EPCs under conditions of serum and growth factor deprivation. The effect of HDL on EPC survival is inhibited by wortmannin, consistent with phosphatidylinositol 3-kinase/Akt signaling.

Oxidative stress is likely to be an important mediator of reduced number and function of EPCs in the presence of hypercholesterolemia. Human apoA-I transfer resulted in a reduction of oxidative stress as evidenced by a decrease of the concentration of 8-isoprostanes. This may also have contributed to increased number and function of EPCs in apoE−/− mice after human apoA-I transfer.

The impact of bone marrow progenitor cells as a source of endothelial cells has been shown by Hu et al who provided evidence that more than 30% of regenerated endothelial cells on the surface of large vessel allografts originated from bone marrow progenitor cells. The data of the current study show that human apoA-I transfer not only increases the absolute number of CD31-positive endothelial cells but also the ratio of the number of CD31 GFP double positive cells versus the total number of CD31 positive cells, indicating an enhanced contribution of bone marrow–derived cells to endothelial regeneration in the allograft at day 21 after transplantation. Therefore, the effect of increased HDL cholesterol on incorporation of EPCs is likely to be critical in promoting reendothelialization.

Myointimal hyperplasia and extracellular matrix accumulation in allografts occur as a consequence of endothelial injury and dysfunction caused by immunologic and nonimmunologic factors. Increased HDL cholesterol after human apoA-I transfer may have altered the development of transplant vasculopathy by accelerated regeneration of the endothelium, by reducing inflammation, or by inhibiting vascular smooth muscle migration and proliferation. Whereas effects on inflammation and vascular smooth muscle cells may be partially endothelium independent, accelerated endothelial regeneration after human apoA-I transfer as a result of increased EPC incorporation or effects on local endothelial cells likely is a key factor for the observed attenuation of transplant arteriosclerosis at day 21 after transfer. Reduced inflammation after human apoA-I transfer may have contributed to the reduction of transplant arteriosclerosis. HDL is known to inhibit the expression of E- and P-selectin as well as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule-1 (ICAM-1) stimulated by either cytokines, oxidized LDL, or C-reactive protein. Antiangiogenic therapy limits experimental atherogenesis of allografts. Increased microvessel formation induced by HDL may have promoted neointima formation between day 21 and day 56 after transfer. Neointima formation in this murine model of transplant arteriosclerosis is unopposed by immunosuppressive drugs. A protective intervention can therefore be expected to retard the progression of neointima formation, but not to prevent extension of the neointima to nearly obliteration of the lumen at later time points.

Notwithstanding the absence of an effect of human apoA-I transfer on the extent of neointima formation at day 56 after transplantation, luminal size was significantly increased as a result of more pronounced expansive remodeling. Higher HDL cholesterol levels were also associated with expansive remodeling in the left coronary system in humans. Whereas expansive remodeling is often associated with increased inflammation, expansive remodeling in the current study occurs in the setting of reduced inflammation. Several animal studies have previously shown that flow-induced expansive remodeling does not depend on a local inflammatory response. Flow-related remodeling depends on the presence of endothelium and nitric oxide is an essential intermediate in the shear-induced remodeling response. We speculate that the increased expansive remodeling of allografts between day 21 and day 56 in AdA-I–treated mice may have been mediated at least in part by accelerated regeneration of the endothelium and by enhanced endothelial NO production induced by HDL.

In conclusion, human apoA-I transfer increases the number of EPCs in hypercholesterolemic apoE−/− mice, enhances the incorporation of bone marrow–derived EPCs into transplanted arteries in apoE−/− mice, promotes endothelial regeneration, and attenuates neointima formation in a murine model of transplantation arteriosclerosis.

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Disclosures

Frank Jacobs is a Research Assistant of the Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen. Elke Van Craeyveld is a Research Assistant of the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen. Marc Tjwa is a Postdoctoral Fellow of the Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen.

References

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SUPPLEMENTAL MATERIALS AND METHODS

Animals-C57BL/6 apo E<sup>−/−</sup> mice, originally developed by Piedrahita et al. in a mixed 129 and C57BL/6 background and backcrossed to C57BL/6 (H2<sup>b</sup>) background for at least 11 generations, were purchased from Taconic. Balb/c (H2<sup>d</sup>) mice were obtained from the Specific Pathogen Free Facility of the Center for Molecular and Vascular Biology. β-actin green fluorescent protein (GFP) mice, kindly provided by Dr. Nagy (Toronto, Canada), were backcrossed to C57BL/6 background for 8 generations and were crossed with C57BL/6 apo E<sup>−/−</sup> mice to generate C57BL/6 apo E<sup>−/−</sup> β-actin GFP mice. Mice were fed normal chow ad libitum.

Gene transfer and artery transplantations-Gene transfer was performed with 5 x 10<sup>10</sup> particles of AdA-I or Adnull at the age of 13 weeks in male C57BL/6 apo E<sup>−/−</sup> mice. AdA-I is an E1E3E4-deleted adenoviral vector containing the human α<sub>1</sub>-antitrypsin promoter upstream of the genomic human apo A-I sequence and 4 copies of the human hepatic control region-1. The E1E3E4-deleted control vector Adnull does not contain an expression cassette. Two weeks after gene transfer or after saline injection, a common carotid artery of a female Balb/c donor mouse was transplanted paratopically into the recipient C57BL/6 apo E<sup>−/−</sup> mice in an end-to-side anastomosis on the left common carotid artery of the recipient as described previously by Shi et al.

Bone marrow transplantations-C57BL/6 apo E<sup>−/−</sup> mice were lethally irradiated with 9.0 Gy at the age of 9 weeks. Transplantation of 6.7 x 10<sup>6</sup> bone marrow cells obtained from C57BL/6 apo E<sup>−/−</sup> β-actin GFP mice was performed 24 hours after irradiation. Gene transfer and artery transplantation were subsequently performed in chimeric mice at the age of 13 and 15 weeks, respectively.

Human apo A-I ELISA-Human apo A-I levels were determined by sandwich ELISA as described previously.
Quantification of murine apo A-I levels by Western blot—After separation of 1 μl plasma in a 12% SDS-PAGE system, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, New York, U.S.A.) by semi-dry blotting (LKB electroblot apparatus, Bromma, Sweden) in transfer buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol, 0.1% SDS, pH=7.5) for 75 min. Following overnight incubation with a 1:500 dilution of goat anti-mouse apo A-I antibodies (sc-23606, Santa Cruz Biotechnology Inc, CA, U.S.A.) and subsequent incubation with horseradish peroxidase-conjugated rabbit anti-goat antibodies (DAKO, Glostrup, Denmark) in a 1:1000 dilution, the membrane was developed using ECL detection reagent (Amersham Biosciences). Films were scanned together with a set of calibration slides with known OD and murine apo A-I levels were quantified by computer assisted image analysis using KS300 software (Zeiss, Zaventem, Belgium).

Determination of the concentration of 8-isoprostanes in plasma—8-isoprostanes in plasma were determined by 8-Isoprostane EIA Kit (Cayman Chemical Company, Ann Arbor, Michigan, U.S.A.) according to the instructions of the manufacturer.

Separation of lipoproteins by gel filtration—Mouse plasma lipoproteins were fractionated by fast performance liquid chromatography gel filtration of 100 μl plasma on a Superdex 200 HR column (Pharmacia, Uppsala, Sweden). Samples were eluted with phosphate buffered saline (37 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) (PBS) at a constant flow rate of 0.5 ml/min and fractions of 0.5 ml were collected. Cholesterol levels in non-HDL (12-32) and HDL fractions (33-45) were determined by Amplex™ Red kit (Molecular Probes, CA, U.S.A.).

Determination of cytokines in plasma—Concentrations of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-2, IL-4 and IL-5 were determined by BD™ Cytometric Bead Array (BD Biosciences, San Jose, CA, U.S.A.) according to the instructions of the manufacturer.
Histological analysis-C57BL/6 apo E−/− mice artery transplant recipients were euthanised for histological analysis of the allografts 21 days or 56 days after artery transplantation. After perfusion fixation with 1% paraformaldehyde in PBS for 5 minutes and postfixation of the dissected allografts in 1% paraformaldehyde in PBS overnight, further processing was performed using routine histology and allografts were embedded in paraffin. Morphometric analysis of hematoxylin and eosin stained 7 μm thick sections was performed by computer assisted image analysis using KS300 software.

C57BL/6 apo E−/− mice artery transplant recipients with prior bone marrow transplantation were perfused with 4% paraformaldehyde in PBS for 5 minutes at day 21 after artery transplantation. After subsequent postfixation in 4% paraformaldehyde and overnight dehydration in 25% sucrose, allografts were embedded in OCT.

Immunohistochemistry-Paraffin sections were stained with rat anti-mouse CD45 (BD Biosciences), rat anti-mouse Mac-3 (BD), rat anti-mouse CD31 (BD) and rabbit anti-human CD3 (DAKO) to detect leukocytes, macrophages, endothelial cells and T-lymphocytes, respectively. Rabbit anti-mouse Tie2 antibodies (sc-324, Santa Cruz Biotechnology) were used to stain Tie2 positive endothelial cells. Inflammatory cells were quantified in a blinded fashion by computer assisted image analysis using KS300 software. The percentage of leukocytes, macrophages and T-lymphocytes was determined as the ratio of CD45, Mac-3 and CD3 positive cells, respectively, in the neointima divided by the total number of cells in the neointima and multiplied by 100. The absolute number of endothelial cells per section was determined by CD31 immunohistochemistry and quantification via computer assisted image analysis using KS300 software.

Cryosections were incubated with rabbit anti-mouse GFP (Molecular Probes) and rat anti-mouse CD31 (BD) and then labeled with goat anti-rabbit Alexa Fluor 488 and goat anti-rat Alexa Fluor 568 (Molecular Probes) to detect bone marrow-derived endothelial cells.
Negative controls were performed by omitting primary antibodies. Negative controls for GFP staining were also performed on sections of mice that did not undergo bone marrow transplantation with bone marrow of C57BL/6 apo E<sup>-/-</sup> GFP mice. Endothelial cells from bone marrow origin were quantified by counting CD31 GFP double positive cells via a computer assisted image analysis using KS300 software.

**HDL isolation by density gradient ultracentrifugation**—Plasma HDL (1.063 g/ml<d<1.21 g/ml) was isolated from plasma of C57BL/6 apo E<sup>-/-</sup> mice treated with AdA-I vector or from human plasma by density gradient ultracentrifugation in a swing-out rotor as described by Chapman et al.<sup>7</sup>. HDL was subsequently dialysed against PBS and concentrated. Protein concentration of HDL was determined using a bicinchoninic protein assay kit (Pierce Biotechnology Inc., Rockford, IL, U.S.A.).

**Fluorescence-activated cell sorting (FACS)**—Mononuclear cells were isolated from 200 µl of peripheral blood using Lymphoprep™ (Nycomed Pharma, Roskilde, Denmark). Cells were resuspended for 30 minutes at 4 °C in 160 µl PBS containing 2% rat serum to prevent nonspecific binding followed by incubation for 30 minutes at 4 °C with a 1:100 dilution of rat anti-mouse fetal liver kinase (Flk)-1 monoclonal antibodies (R-Phycoerythrin-conjugated, BD) and a 1:100 dilution of rat anti-mouse stem cell antigen (Sca)-1 monoclonal antibodies (Fluorescein isothiocyanate-conjugated, BD). After washing and pelleting, cells were resuspended in 300 µl 1 x Cellfix (BD) for FACS (FACS Calibur, BD) and quantified with CellQuest software (BD). As control, isotype-matched control antibodies (BD) were used. 10 000 events were acquired per sample. To calculate the number of Flk-1 Sca-1 positive cells per µl blood, the percentage of Flk-1 Sca-1 positive cells obtained by FACS was divided by 100 and multiplied by the concentration of mononuclear cells in the peripheral blood.

**Murine EPC culture assay**—Spleen mononuclear cells were cultivated and EPCs were quantified as described before by Dimmeler et al.<sup>8</sup>. Spleen mononuclear cells were isolated
10 days after gene transfer by Ficoll-based centrifugation and seeded onto fibronectin (40 µg/ml)-coated 24-well plates (Sigma, Steinheim, Germany) at a density of 8 x 10^6 cells/well in 0.5 ml EGM-2MV BulletKit medium (Cambrex, East Rutherford, NJ, U.S.A.) according to the instructions of the manufacturer. After 4 days of culture, medium was removed and adhered cells were stained for Dil-acLDL (Invitrogen, Carlsbad, CA, U.S.A.) (3.3 µg/ml) for 4 hours and then FITC-labeled isolectin (Invitrogen) (10 µg/ml) for 1 hour. The number of EPCs, identified as Dil-acLDL isolectin double positive cells, per microscopy field was quantified.

*Human EPC culture assay*-Mononuclear cells were isolated from 30 ml peripheral blood of healthy human volunteers by density gradient centrifugation using Histopaque-1077 (Sigma), as previously described^9, 10^. Immediately after isolation, 4 x 10^6 cells were plated onto 24-well culture dishes coated with human fibronectin (Sigma) and cultured in 500 µl EGM-2MV BulletKit (Cambrex) medium. EPC phenotype of cultured cells was confirmed by Dil-ac-LDL (Invitrogen) uptake and von Willebrand Factor (vWF) (DAKO) staining.

*EPC migration assay*-EPC migration was studied by using modified Boyden chambers (Costar, Avon, France) as described^11^. After 7 days of culture, spleen EPCs were seeded in the upper chamber with a density of 2 x 10^4 cells per well in 200 µl EGM-2MV medium. 500 µl EGM-2MV medium supplemented with HDL (100 µg/ml) or an equivalent amount of bovine serum albumin (Roche, Mannheim, Germany) was placed in the lower chamber. Additional experiments were performed in the presence HDL (100 µg/ml) plus wortmannin (200 nM). EPCs were allowed to migrate for 5 hours at 37 °C^11^ and the number of migrated EPCs was quantified per microscopy field. To evaluate human EPC migration, human EPCs were harvested after 4 days of culture and migration was evaluated similarly as for murine EPCs except that human HDL was used instead of HDL isolated from AdA-I treated mice.

*EPC adhesion assay*-The *in vitro* adherence of EPCs to fibronectin coated plates was
performed as described\textsuperscript{12}. In brief, after 7 days of culture, spleen EPCs were collected and 2 x 10\textsuperscript{4} cells per well were allowed to adhere onto fibronectin (40 µg/ml)-coated 96-well plates for 30 min in the presence 100 µg/ml of HDL or an equivalent amount of bovine serum albumin. Subsequently, the plates were vigorously washed with PBS and the number of adherent cells was counted under the microscope.

\textit{EPC invasion assay}-An EPC invasion assay was performed as described\textsuperscript{13}. The transwells of the modified Boyden chamber used in migration assays were filled with Matrigel (4 mg/ml; 200 µl per well) (BD Biosciences). Spleen EPCs isolated after 7 days of culture were suspended in 200 µl EGM-2MV medium and 1 x 10\textsuperscript{5} cells per well were placed onto solidified Matrigel in the upper compartment. 500 µl EGM-2MV medium supplemented with 100 µg/ml HDL or an equivalent amount of bovine serum albumin was placed in the lower chamber. After 24 hours at 37 °C, the chambers were removed and cells migrated into the lower chamber were counted under the microscope.

\textit{EPC survival assay}-Murine spleen mononuclear cells were seeded onto fibronectin-coated (40 µg/ml) 48-well plates at a density of 1.5 x 10\textsuperscript{5} cells/well containing 200 µl EGM-2MV BulletKit medium. After 7 days of culture, medium was removed, cells were washed with PBS and subsequently cultured for 24 hours in 200 µl EGM-2MV medium or medium without serum and growth factors either supplemented with HDL (100 µg/ml), HDL (100 µg/ml) with wortmannin (200 nM) (Calbiochem, San Diego, CA, U.S.A.) or bovine serum albumin (100 µg/ml). Next, the medium was removed and cells were extensively washed. Adherent cells were fixed with 4% paraformaldehyde and stained with FITC-labeled isolectin (Invitrogen) (10 µg/ml) for 1 hour. The number of positive cells per microscopy field was quantified in a blinded fashion.

\textit{Bone marrow EPC isolation and quantification}-Bone marrow mononuclear cells from saline (n=4) and Adnull (n=4) control mice and AdA-I gene transfer mice (n=8) were isolated 35
days after injection by density gradient centrifugation using Histopaque-1077 (Sigma) as described\textsuperscript{14}. Immediately following isolation, cells were plated onto fibronectin-coated (40 µg/ml) 24-well plates at a density of 4 × 10⁶ cells/well and cultured in EGM-2MV BulletKit medium (Cambrex). After 7 days of culture, the number of EPCs per microscopy field, identified as Dil-ac-LDL isolectin double positive cells, was quantified in a blinded fashion.

\textit{Statistical Analysis}-Data are expressed as means ± standard error of the means (SEM). Cholesterol values or the number of Flk-1 Sca-1 double positive cells at different time-points after gene transfer were compared with baseline values by one-way analysis of variance (ANOVA) followed by Dunnett Multiple Comparisons Test. Data of EPC cell migration assay were compared by ANOVA followed by Tukey Multiple Comparisons Test. Differences of histological parameters between control mice and AdA-I treated mice were analysed by Student’s t-test. A two-sided p-value of less than 0.05 was considered statistically significant.
SUPPLEMENTAL RESULTS

*Human apo A-I transfer reduces allograft inflammation:* AdA-I transfer reduced the number of infiltrated leukocytes 1.3-fold (p<0.05) and 1.5-fold (p<0.0001) at day 21 and day 56 after transplantation, respectively, compared to control mice (Table I). The number of infiltrated macrophages was 1.4-fold (p<0.05) and 1.3-fold (p<0.001) lower in AdA-I treated mice at day 21 and day 56 after transplantation, respectively, than in control mice. No significant difference of T-lymphocyte infiltration was observed (Table I). Following transplantation, AdA-I transfer did not induce a significant alteration of leukocyte or mononuclear cell counts in the peripheral blood compared to Adnull or saline treated control mice or compared to baseline values. Analysis of the cytokine profile at day 3 after transplantation in AdA-I treated mice (n=5) and Adnull treated mice (n=5) showed that the concentration of TNF-α, IFN-γ, IL-2, and IL-5 was not significantly different between both groups and that IL-4 level was 1.2-fold (p<0.01) lower in AdA-I treated mice (12 ± 0.44 pg/ml) than in Adnull mice (14 ± 0.62 pg/ml). Taken together, these data suggest that reduced inflammation in allografts following AdA-I transfer is predominantly due to a local and not to a systemic anti-inflammatory effect.
SUPPLEMENTARY REFERENCES


### SUPPLEMENTARY TABLES

**Table I.** Immunohistochemical analysis of allograft inflammation at day 21 and day 56 after transplantation.

<table>
<thead>
<tr>
<th></th>
<th>Day 21</th>
<th>Day 56</th>
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<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>AdA-I</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>56 ± 3.1</td>
<td>44 ± 7.9*</td>
</tr>
<tr>
<td>Macrophages</td>
<td>46 ± 3.8</td>
<td>32 ± 3.1*</td>
</tr>
<tr>
<td>T-lymphocytes</td>
<td>5.7 ± 1.2</td>
<td>5.1 ± 1.4</td>
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Data are expressed in percentages and represent means ± SEM. *: p<0.05; ***: p<0.001; ****: p<0.0001. The number of mice in each group is identical as in Table 2.
LEGENDS TO THE SUPPLEMENTARY FIGURES

**Figure I.** Effect of adenoviral human apo A-I transfer on human apo A-I plasma levels and EPC number and function in C57BL/6 apo E\(^{-/-}\) mice. All data are expressed as means ± SEM.

(A) Human apo A-I expression levels after adenoviral gene transfer with \(5 \times 10^{10}\) particles of AdA-I in male C57BL/6 apo E\(^{-/-}\) mice (●; n=14). (B) Time-course of the number of Flk-1 Sca-1 double positive cells in control C57BL/6 apo E\(^{-/-}\) mice (▲; n=10) or AdA-I treated mice (●; n=10) at different time-points after saline injection or gene transfer. *: p<0.05; **: p<0.01 versus baseline. (C) Bar graph showing the number of DiI-acLDL FITC-isolectin double positive cells after 4 days of *ex vivo* culture of spleen mononuclear cells isolated at day 10 after Adnull transfer (n=6) or AdA-I transfer (n=6). (D) Bar graph showing the number of migrated EPCs in modified Boyden chambers. After 7 days of culture, spleen EPCs isolated from Adnull injected mice (n=6) or AdA-I treated mice (n=6) were seeded in the upper chamber. The lower chamber was supplemented with either HDL (100 µg/ml) or an equivalent amount of bovine serum albumin and the number of migrated cells per microscopy field was quantified after 5 hours. (E) Bar graph illustrating the number of EPCs adhered to fibronectin coated plates. After 7 days of culture, spleen EPCs isolated from Adnull injected mice (n=5) or AdA-I treated mice (n=5) were allowed to adhere onto fibronectin plates for 30 min in the presence of 100 µg/ml of HDL or an equivalent amount of bovine serum albumin. Following vigorously washing with PBS, the number of adherent cells was counted under the microscope. (F) Bar graph showing the number of EPCs invaded through solidified Matrigel. The transwells of the modified Boyden chamber used in migration assays were filled with Matrigel. After 7 days of culture, spleen EPCs isolated from Adnull injected mice (n=5) or AdA-I treated mice (n=5) were placed onto solidified Matrigel in the upper compartment. The lower chamber was supplemented with either HDL (100 µg/ml) or an equivalent amount of bovine serum albumin and the number of invaded cells per microscopy field was...
quantified after 24 hours.

**Figure II.** Cholesterol lipoprotein profiles obtained by fast performance liquid chromatography gel filtration of 100 µl plasma of AdA-I (A) or Adnull (B) treated mice at day 0 (■), day 14 (●), day 35 (▲) and day 70 (●●) after transfer. Fractions 12-32 correspond to non-HDL sized particles whereas fractions 33-45 correspond to HDL sized particles.

**Figure III.** Evaluation of the role of the phosphatidylinositol 3-kinase signal transduction pathway in mediating the effects of HDL on EPC migration and EPC survival. All data are expressed as means ± SEM. (A) Bar graph showing the number of migrated murine EPCs in modified Boyden chambers. After 7 days of culture, spleen EPCs isolated from control C57BL/6 apo E<sup>−/−</sup> mice (n=4) were seeded in the upper chamber. The lower chamber was supplemented with either bovine serum albumin (100 µg/ml), HDL (100 µg/ml) or HDL (100 µg/ml) plus wortmannin (200 nM) and the number of migrated cells per microscopy field was quantified after 5 hours. (B) Bar graph showing the number of migrated human EPCs in modified Boyden chambers. After 4 days of culture, human EPCs isolated from 4 healthy female human volunteers were seeded in the upper chamber. The lower chamber was supplemented with either bovine serum albumin (100 µg/ml), human HDL (100 µg/ml) or human HDL (100 µg/ml) plus wortmannin (200 nM) and the number of migrated cells per microscopy field was quantified after 5 hours. (C) Bar graph showing the number of surviving murine EPCs. After 7 days of culture, spleen EPCs isolated from control C57BL/6 apo E<sup>−/−</sup> mice (n=6) were cultured for 24 hours in EGM-2MV medium or medium without serum and growth factors either supplemented with HDL (100 µg/ml), HDL (100 µg/ml) with wortmannin (200 nM) or bovine serum albumin (100 µg/ml). The number of FITC-isolectin positive cells per microscopy field was quantified after 24 hours in a blinded fashion.
**Figure IV.** Effect of AdA-I transfer on endothelial cell regeneration. All data represent means ± SEM. (A) Bar graph showing the number of CD31 positive endothelial cells at day 21 after artery transplantation in control (n=15) and AdA-I (n=19) treated C57BL/6 apo E<sup>−/−</sup> mice; (B) Bar graph showing the number of CD31 positive endothelial cells at day 56 after artery transplantation in control (n=26) and AdA-I (n=14) treated C57BL/6 apo E<sup>−/−</sup> mice; (C) Bone marrow transplantation with bone marrow of C57BL/6 apo E<sup>−/−</sup> β-actin GFP mice was performed 4 weeks before gene transfer or saline injection. Comparison of the number of CD31 GFP double positive cells at day 21 after artery transplantation in control (n=10) and AdA-I (n=10) treated chimeric C57BL/6 apo E<sup>−/−</sup> mice.

**Figure V.** Immunostaining for CD31 showing microvessels (indicated by arrows) in the neointima at day 56 after transplantation in an AdA-I treated mouse.
SUPPLEMENTARY FIGURES

Figure I
Figure II
Figure III

(A) Migrated cells (number/field) for Control, HDL, and HDL + wortmannin, with p < 0.01 and p < 0.001.

(B) Migrated cells (number/field) for Control, HDL, and HDL + wortmannin, with p < 0.01 and p < 0.05.

(C) Di-l-Ac-LDL and cells (number/field) for Serum, No serum, No serum + HDL, and No serum + HDL + wortmannin, with p < 0.001 and p < 0.001.
Figure IV

A

\[ \text{CD31 + cells (number/section)} \]

\[ \text{Controls} \quad \text{AdA-I} \]

\[ p < 0.01 \]

B

\[ \text{CD31 + cells (number/section)} \]

\[ \text{Controls} \quad \text{AdA-I} \]

\[ p < 0.0001 \]

C

\[ \text{CD31 GFP + cells (number/section)} \]

\[ \text{Controls} \quad \text{AdA-I} \]

\[ p < 0.01 \]
Figure V