ONLINE SUPPLEMENTARY INFORMATION

Down-regulation of endothelial TLR4 signalling after apo A-I gene transfer contributes to improved survival in an experimental model of lipopolysaccharide - induced inflammation

RUNNING TITLE: HDL and endothelial TLR4

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Supplemental Materials and Methods

Animals and study design

Eight week old male C57BL/6 mice were intravenously injected with 5 x 10^{10} particles of the E1E3E4-deleted adenoviral vector *Ad.hapoA-I*, expressing human apo A-I [1]. As controls, age-matched C57BL/6 mice were injected with the same dose of *Ad.Null*, containing no expression cassette [1]. Fourteen days hereafter, endotoxic shock was induced by intraperitoneal injection of lipopolysaccharide (LPS) from *Escherichia coli*, serotype 055:B5 (Sigma, Steinheim, Germany), at a dose of 80 mg/kg. Mice were sacrified 20 hours after LPS injection. For survival studies, endotoxic shock was induced in a total of 60 mice of which 20 mice were injected with *Ad.hapoA-I*, 20 with *Ad.Null*, and 20 with saline. Blood was withdrawn from the retro-orbital plexus at day 6 after gene transfer and 20 hours after LPS or saline injection for determination of human apo A-I concentrations. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Human apo A-I ELISA

Human apo A-I levels were determined by sandwich ELISA as described previously [2].

Quantification of murine apo A-I plasma levels

Murine apo A-I plasma levels were determined by Western blot as described before [3]. After separation of 1 µl plasma in a 12% SDS-PAGE system, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) 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, Santa Cruz, CA, USA) 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).

Plasma lipid analysis

Mouse lipoproteins were separated by density gradient ultracentrifugation as described previously [4]. Fractions were stored at -20 °C until analysis. Cholesterol in lipoprotein fractions was determined with commercially available enzymes (Roche Diagnostics, Basel, Switzerland). Precipath L (Roche Diagnostics) was used as a standard.

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) [5]. 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 (33-

45) fractions were determined by the Amplex™ Red kit (Molecular Probes, Carlsbad, CA, USA) as described [4].

Determination of sphingosine-1-phosphate in HDL

S1P levels were determined as described [6]. Briefly, 1 ml methanol containing 2.5 µl concentrated HCl was added to 100 µl of HDL suspension (12.6 mg HDL per 1 ml buffer). Dihydro-S1P (50 pmol) was added, and lipids were extracted by addition of 1 ml chloroform and 200 µl NaCl (4 mol/l). For alkalization, 100 µl NaOH (3 mol/l) was added. The alkaline aqueous phase was transferred into a siliconized glass tube, and the organic phase was re-extracted with 0.5 ml methanol, 0.5 ml NaCl (1 mol/l), and 50 µl NaOH (3 mol/l). The aqueous phases were combined, acidified with 100 µl concentrated HCl, and extracted twice with 1.5 ml chloroform. The organic phases were evaporated, and the dried lipids were dissolved in 275 µl of a mixture of methanol 0.07 mol/l K_2HPO_4 (9:1). A derivatization mixture of 10 mg o-phthaldialdehyde, 200 µl ethanol, 10 µl 2-mercaptoethanol, and 10 ml boric acid (3%, vol/wt) was prepared and adjusted to pH 10.5 with KOH. Twenty-five µl of the derivatization mixture was added to the resolved lipids and this was incubated for 15 minutes at room temperature. The derivatives were analyzed with a Merck-Hitachi LaChrom HPLC system (Merck-Hitachi, Darmstadt, Germany) using an RP 18 Kromasil column (Chromatographie Service GmbH, Langerwehe, Germany). Separation was done with a gradient of methanol and K_2HPO_4 (0.07 mol/l) [6]. The recovery of S1P was calculated using dihydro-S1P as a standard [6]. To verify that no other substances were coincidentally overlapping the S1P peak, samples were incubated with alkaline phosphatase, which effectively cleaved the phosphate

groups from S1P and dihydro-S1P. Treatment of standard and HDL samples with this enzyme resulted in an almost 90% decrease of S1P and dihydro-S1P peaks (data not shown). For digestion experiments with alkaline phosphatase, 50 units of enzyme in 450 µl of buffer containing 200 mmol/l Tris-HCl, pH 4.5, and 75 mmol/l $MgCl_2$ in glycine (2 mol/l, pH> 9.0) were added to the combined alkaline aqueous phases of the lipid extraction and incubated for 30 minutes at 37 °C. Then, 100 µl of concentrated HCl was added and lipids were extracted twice with 1.5 ml chloroform.

Real-time RT-PCR Quantification

Quantitative real-time reverse transcriptase (RT)-PCR (ABI PRISM® 7900 HT Sequence Detection System software version 2.2.2., Perkin Elmer, Waltham, MA, USA) was used to quantify lung mouse *TLR4, myeloid differentiation factor 88 (MyD88), Toll/Il-1R-containing adaptor inducing interferon β (TRIF)* and *ribosomal protein L32* cDNA levels (n=6 per group) and human *MyD88, TRIF*, and *GAPDH* cDNA levels in HMEC-1 (n=4 per group). The mouse and human cDNA expression levels were normalized to *L32* and *GAPDH* cDNA, respectively. The sequences of the primer sets used in this study are represented in Supplemental Table 1.

Histology and immunohistology

Hematoxylin and eosin staining was performed on 5 µm thick paraffin sections. Immunohistological stainings were carried out on 5 µm cryosections with goat polyclonal anti-TLR4 antibody (1:50; Santa Cruz Biotechnology Inc). Sections were stained with rabbit polyclonal anti-von Willebrand Factor (vWF) antibody (1:20; DAKO) followed by anti-rabbit TRITC-conjugated secondary antibody for the identification of endothelial

cells and next exposed to goat polyclonal anti-TLR4 antibody (1:50; Santa Cruz Biotechnology Inc) followed by a FITC-conjugated anti-goat secondary antibody. Other sections were stained with rabbit polyclonal myeloperoxidase antibody (1:50; Lab Vision Products Thermo Fisher Scientific, Fremont, CA, USA), followed by a TRITC-conjugated anti-rabbit secondary antibody, for the identification of neutrophils. The same sections were then exposed to anti-TLR4 antibody (1:50; Santa Cruz Biotechnology Inc) followed by a FITC-conjugated anti-goat secondary antibody. vWF^+ cells, $TLR4^+$-vWF^+ double positive cells, $myeloperoxidase^+$ cells and $TLR4^+$-$myeloperoxidase^+$ cells were counted at 1000x magnification by using fluorescent microscopy. vWF^+ cells and $myeloperoxidase^+$ cells were expressed as positive cell density per mm^2 of tissue. The percentage (%) of endothelial cells or neutrophils expressing TLR4 was calculated by dividing the amount of $TLR4^+$-vWF^+ or $TLR4^+$-$myeloperoxidase^+$ cells towards total vWF^+ cells or $myeloperoxidase^+$ cells, respectively, followed by multiplying by 100.

Cells

HMEC-1, generously provided by Prof. Dr. U. Rauch (Charité, Berlin, Germany), were cultured in 6-well tissue culture plates at a density of 175 000 cells/well. After reaching 80% of confluence, cells were incubated for 24 h in the presence or absence of human HDL (50 µg protein/ml; MP Biomedicals, Solon, OH, USA) or human apo A-I (35 µg/ml; Sigma). Following the pre-incubation period, all LPS supplementations were performed in the absence of HDL or apo A-I to ensure that effects were independent of LPS-binding. Cells were incubated with LPS (100 ng/ml) for 2 h for FACS and mRNA expression analysis or for 4 h for NF-κB activity quantification. For all experiments, 50

µg of HDL protein/ml was used, since we previously showed that HDL exerts endothelial-protective effects at this concentration in *vitro* [7]. Since apo A-I comprises 70% of HDL protein content, we therefore chose for 35 µg/ml of apo A-I.

Flow cytometric analysis

Cells were washed with PBS and stained with FITC-conjugated mouse monoclonal anti-human TLR4 antibody (Imgenex, San diego, CA, USA). TLR4 expression was analyzed by fluorescence-activated cell sorting (FACS) using a FACSScan flow cytometer and Cell Quest software (BD Biosciences, San Jose, CA, USA).

Nuclear endothelial NF-κB p65 activity

Cells were washed with PBS and scraped in PBS supplemented with phosphatase inhibitor (from nuclear extract kit; Active Motif, Carlsbad, CA, USA). After centrifugation, nuclear proteins were extracted according to the manufacturer's protocol (nuclear extract kit; Active Motif). NF-κB p65 activity of 20 µg of nuclear protein was determined by TransAM™ NF-κB p65 according to the manufacturers protocol (Active Motif).

Fluorometric dequenching assay for LPS-FITC

To study the LPS-binding capacity of HDL and apo A-I, 100 ng/ml of LPS-FITC (Sigma) was incubated in the presence of saline, HDL (50 µg/ml) or apo A-I (35 µg/ml) in a 96-well plate at 37 °C for 1 h. Then, fluorescence was measured in a fluorometer (SpectraMax Gemini microplate reader, Molecular Device GmbH, Munich, Germany) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. The increase in LPS-FITC fluorescence is due to dequenching of LPS-FITC, indicating LPS-binding [8].

Wet lung weight to body weight ratio

Mouse wet lung weight to body weight ratio was used as a parameter of pulmonary vascular dysfunction after LPS injection. Wet lung weight was measured immediately after removal of the lungs. The wet lung weight to body weight ratio was calculated by dividing the wet weight of both lungs by the body weight.

Lung myeloperoxidase activity

Lung tissue was homogenized in 300 µL of 50 mM potassium phosphate (pH 6.0; working buffer). Next, 700 µl of working buffer was added to the homogenate, followed by centrifugation at 2500 rpm for 15 minutes at 4 °C. Then, the pellet was resuspended in 300 µl of working buffer containing 50 mM of hexadecyl-trimethylammonium bromide, followed by homogenization for 30 seconds. Hereafter, 700 µl of working buffer was added. Samples were subsequently sonicated for 20 seconds, snap frozen, and thawed to room temperature. This extraction procedure was repeated 3 times, and the supernatants

were collected. The supernatants were mixed 1:30 (vol/vol) with 50 mmol/L PBS at pH 6.0 containing 0.167 mg/mL o-dianisidine (Sigma) and 0.0005% hydrogen peroxide. The absorbance was read at 450 nm corrected to 650 nm. Myeloperoxidase activity was then calculated as the absorbance over time normalized by wet lung weight.

Statistical analysis

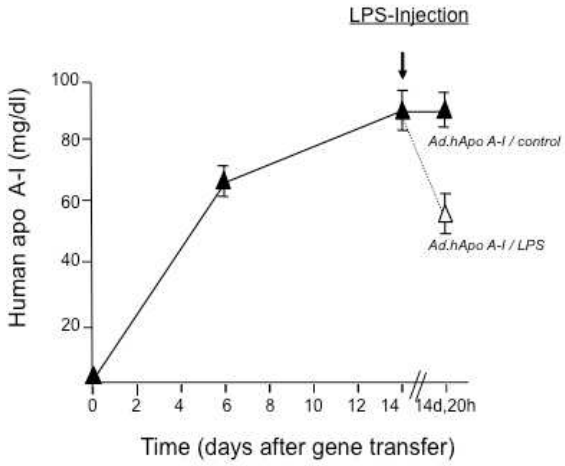
Data are presented as mean ± SEM. Paired and unpaired Student's t tests were used for statistical analysis. Survival analysis was performed with Prism4 (GraphPad Software, San Diego, CA, USA). Differences were considered to be significant at $p<0.05$.

Supplemental Table 1. Primers.

Gene	Primers
Mouse TLR4	FOR: 5’-CATGGAACACATGGCTGCTAA-3’ REV: 5’-GGAAAGGAAGGCATCAGTGCTA-3’
Mouse MyD88	FOR: 5´-TGGACTCCTT CATGTTCTCCATAC-3´ REV: 5´-GATAGGCGGCGCCTCACT-3
Mouse Trif	FOR 5’-TCGCCATGTCCGAAGAACTT-3’ REV 5’-ACAGACAGGGCAGTAGAAATCATCT-3’
Mouse L32	FOR: 5'-TGCCCACGGAGGACTGACA-3' REV: 5'-AGGTGCTGGGAGCTGCTACA-3'
Human MyD88	FOR: 5’-AGCTCCACCTGGCATGAGAA-3’ REV: 5’-TGACTCATCCCCAGAACTCATACTT-3’
Human TRIF	FOR: 5’-CTCCAGAAACCAGCACCAACTAC-3’ REV: 5’-GTTTTTGACCGGCTCCAGAA-3’
Human GAPDH	FOR: 5'-CCACCCATGGCAAATTCC-3' REV: 5'-TGGGATTTCCATTGATGACAAG-3'

Supplemental Figures

A



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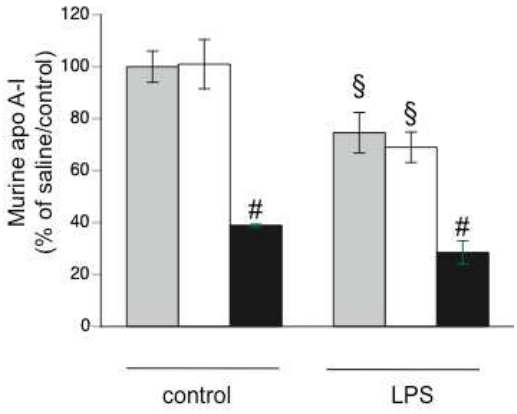

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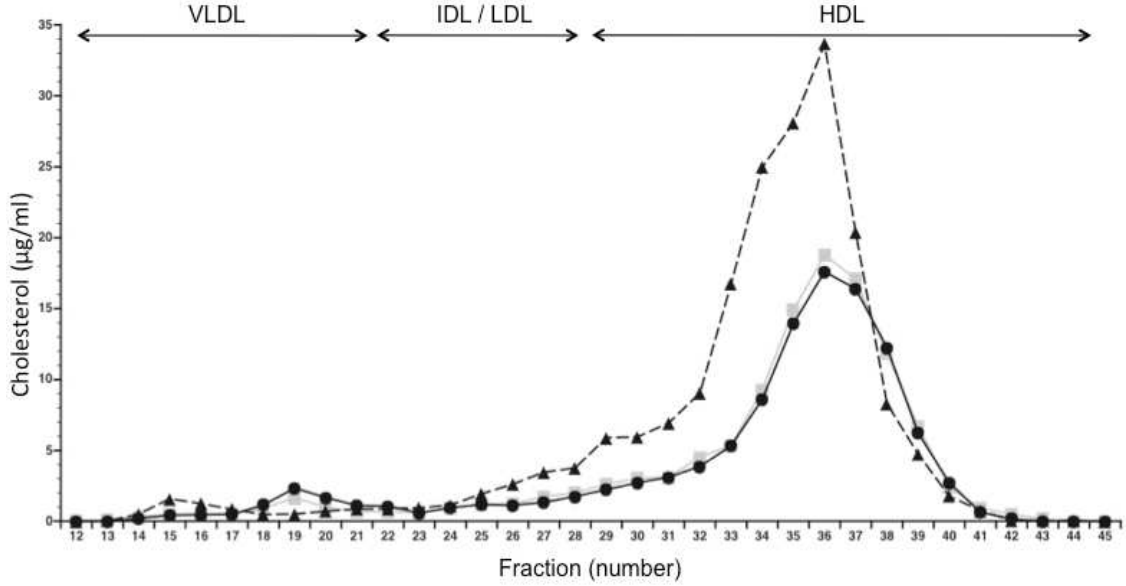

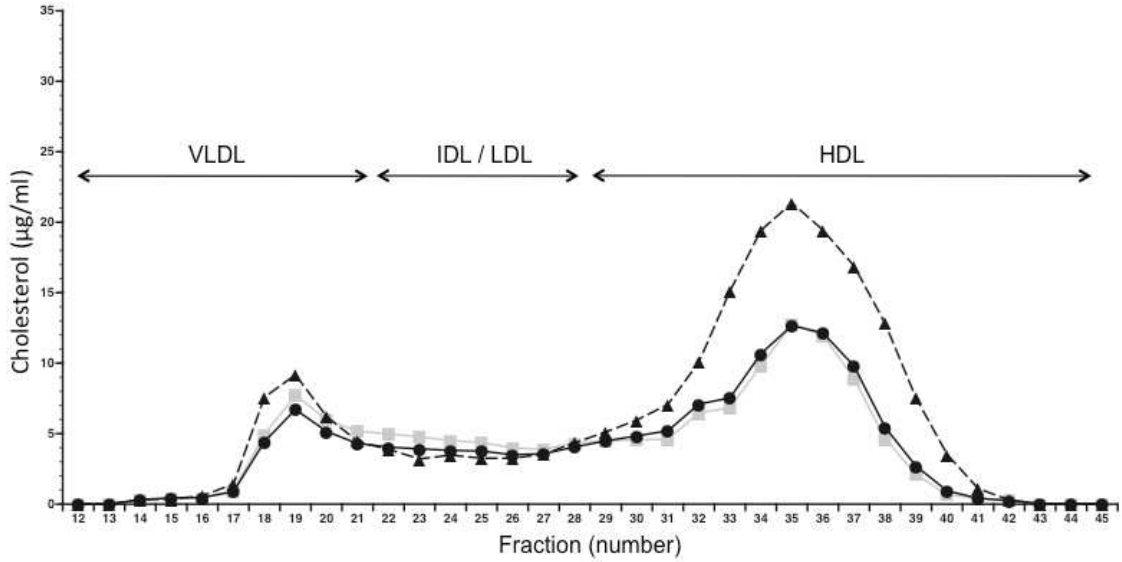

Figure S1. Effect of human apo A-I gene transfer and lipopolysaccharide injection on human apo A-I plasma concentrations, murine apo A-I plasma levels, and cholesterol lipoprotein profiles. A. Time-course of human apo A-I expression after *Ad.hapoA-I* gene transfer in C57BL/6 mice. Data represent mean ± SEM (n=6). B. Murine apo A-I plasma levels determined by Western Blot and represented as the % relative to saline control mice set as 100% with gray bars: saline, open bars: *Ad.Null,* and black bars: *Ad.hapoA-I.* # $p<0.005$ versus respective saline and *Ad.Null,* § $p<0.05$ versus respective controls (n=4-6/group). C. Cholesterol (µg/ml) profile in control (upper panel) and LPS-injected mice (lower panel), which underwent saline-injection (■), *Ad.Null* (●), or *Ad.hapoA-I* (▲) gene transfer. Cholesterol levels in non-HDL (12-28) and HDL fractions (29-45) were determined by the Amplex^{TM} Red kit as described.

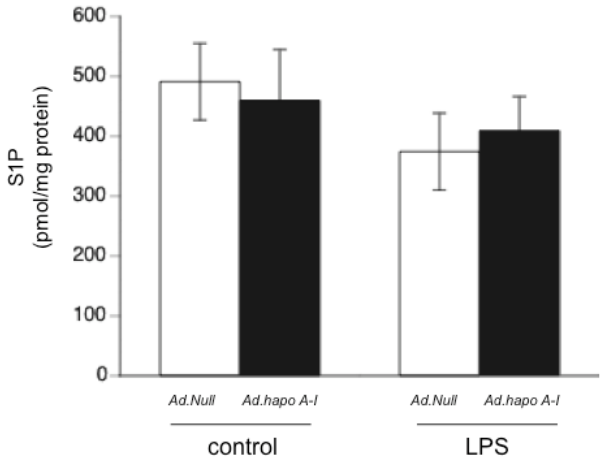

Figure S2. Effect of human apo A-I gene transfer and lipopolysaccharide injection on sphingosine-1-phosphate content in HDL. Data represent mean ± SEM (n=3) with open bars: *Ad.Null* and black bars: *Ad.hapoA-I*.

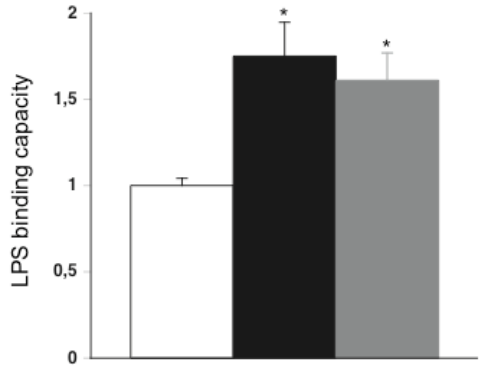

Figure S3. HDL and apo A-I bind LPS. The LPS-binding capacity of HDL and apo A-I was analyzed via a fluorometric LPS-FITC dequenching assay. In this assay, an increase in LPS-FITC fluorescence is due to dequenching of LPS-FITC and indicates LPS-binding [8]. Herefore, 100 ng/ml of LPS-FITC was incubated in the presence of saline, HDL (50 µg/ml) or apo A-I (35 µg/ml) in a 96-well plate at 37 °C for 1 h. Then, fluorescence was measured in a fluorometer at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Bar graphs represent the LPS-binding capacity of HDL (black bar) and apo A-I (gray bar), normalized to saline (open bar) set as 1. * $p<0.005$ versus saline (n=6/group).

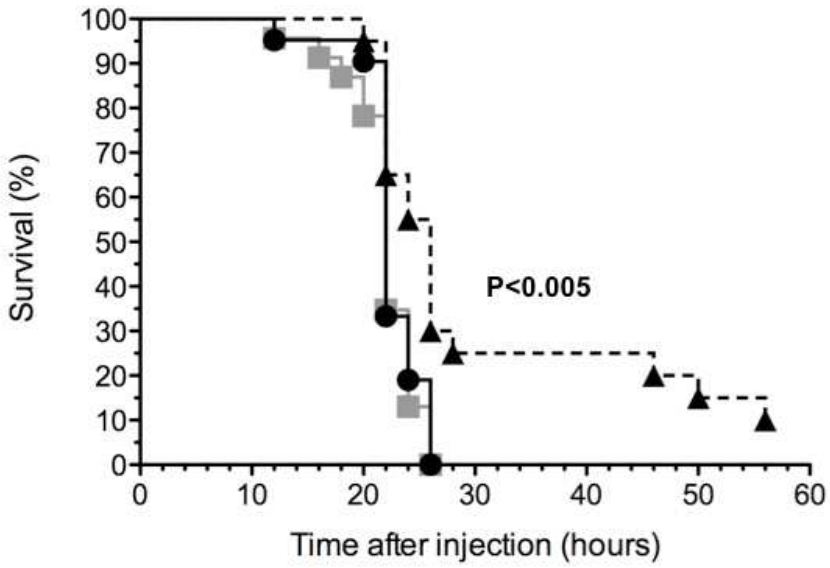

Figure S4. Kaplan-Meier survival analysis demonstrating that apo A-I transfer improves survival of mice in endotoxic shock. C57BL/6 mice were intravenously injected with saline, *Ad.Null*, or *Ad.hapoA-I*. Fourteen days afterwards, LPS was administered and the mortality of 20 saline-injected (■), 20 *Ad.Null*-LPS (●), and 20 *Ad.hapoA-I*-LPS (▲) was monitored for 60 hours. Survival was significantly ($p<0.005$) higher in *Ad.hapoA-I*-LPS mice compared to saline-LPS and *Ad.Null*-LPS mice. No significant differences were found between the survival rate of *Ad.Null*-LPS and saline-LPS mice.

Supplemental references

1. Van Linthout S, Lusky M, Collen D, De Geest B (2002) Persistent hepatic expression of human apo A-I after transfer with a helper-virus independent adenoviral vector. Gene Ther 9:1520-1528
2. Van Linthout S, Spillmann F, Riad A, Trimpert C, Lievens J, Meloni M, Escher F, Filenberg E, Demir O, Li J, Shakibaei M, Schimke I, Staudt A, Felix SB, Schultheiss HP, De Geest B, Tschope C (2008) Human apolipoprotein A-I gene transfer reduces the development of experimental diabetic cardiomyopathy. Circulation 117:1563-1573
3. Feng Y, Jacobs F, Van Craeyveld E, Brunaud C, Snoeys J, Tjwa M, Van Linthout S, De Geest B (2008) Human ApoA-I transfer attenuates transplant arteriosclerosis via enhanced incorporation of bone marrow-derived endothelial progenitor cells. Arterioscler Thromb Vasc Biol 28:278-283
4. Jacobs F, Van Craeyveld E, Feng Y, Snoeys J, De Geest B (2008) Adenoviral low density lipoprotein receptor attenuates progression of atherosclerosis and decreases tissue cholesterol levels in a murine model of familial hypercholesterolemia. Atherosclerosis 201:289-297
5. Van Linthout S, Collen D, De Geest B (2002) Effect of promoters and enhancers on expression, transgene DNA persistence, and hepatotoxicity after adenoviral gene transfer of human apolipoprotein a-I. Hum Gene Ther 13:829-840.
6. Ruwisch L, Schafer-Korting M, Kleuser B (2001) An improved high-performance liquid chromatographic method for the determination of sphingosine-1-phosphate in complex biological materials. Naunyn Schmiedebergs Arch Pharmacol 363:358-363
7. Van Linthout S, Spillmann F, Lorenz M, Meloni M, Jacobs F, Egorova M, Stangl V, De Geest B, Schultheiss HP, Tschope C (2009) Vascular-protective effects of high-density lipoprotein include the downregulation of the angiotensin II type 1 receptor. Hypertension 53:682-687
8. de Haas CJ, Poppelier MJ, van Kessel KP, van Strijp JA (2000) Serum amyloid P component prevents high-density lipoprotein-mediated neutralization of lipopolysaccharide. Infect Immun 68:4954-4960