Supplemental Material

Donor simvastatin treatment abolishes rat cardiac allograft ischemia-reperfusion injury and chronic rejection through microvascular protection

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Tuuminen: Vascular protection by donor simvastatin treatment

Expanded Methods

Heterotopic Rat Heart Transplantations

Intra-abdominal heterotopic heart transplantations were performed from specific pathogen-free fully MHC-mismatched male Dark Agouti (DA, RT1\textsuperscript{av1}) to male Wistar Furth (WF, RT1\textsubscript{a}) rats (Scanburg, Göteborg, Sweden) weighing 300-350 g. After infusion of 300 IU heparin in 10 ml ice-cold PBS or Plegisol cardioplegia solution (Hospira, Inc., Lake Forest, Il; used only in Supplemental Figure 4C experiments) depending on the study model into the inferior vena cava of the heart donor, the vena cava and pulmonary veins were ligated with 6-0 silk and the pulmonary artery and aorta were cut 2 to 3 mm above their origin in the heart. After removal, allografts were left without hypothermic preservation or were preserved either in PBS or in Plegisol cardioplegia solution at +4 °C for 2 or 4 hours depending
on the study model. Cardiac allograft recipients were anesthetized with isoflurane anesthesia (2-5%/l \text{O}_2), and received buprenorphine 0.15 mg/kg s.c. (Temgesic 0.3 mg/ml, Schering-Plough, Kenilworth, NJ) for peri- and postoperative analgesia. A midline incision was made, and the aorta and pulmonary artery of the allograft were anastomosed to the abdominal aorta and inferior vena cava of the recipient, respectively. Warm ischemia occurring during heart transplantation was standardized to one hour. Permission for animal experimentation was obtained from the State Provincial Office of Southern Finland. The animals received good care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Academy Press (ISBN 0-309-05377-3, revised 1996).

**Drug Administration**

Peroral simvastatin (Merck Research Laboratories, Whitehouse Station, NJ) was given either to the donor as a single dose (5 mg/kg) 2 hours before graft removal, or to the recipient (2 mg/kg) 2 hours before reperfusion and daily thereafter (2 mg/kg/d), or to both. In dose-response analysis (Supplemental Figure 3A) simvastatin 0.5, 2.0 or 5.0 mg/kg was given to the donor 2 hours before graft removal. Simvastatin was diluted in polyethylene glycol (molecular weight 300) (Sigma-Aldrich, St. Louis, MO) to a concentration of 1.5 mg/ml, and the controls received polyethylene glycol vehicle daily p.o. Peroral pravastatin (P4498 pravastatin sodium salt, Sigma-Aldrich) was given to the donor as a single dose (5 mg/kg) 2 hours before graft removal. To prevent severe episodes of acute rejection and to allow the development of an alloimmune response and moderate chronic rejection, allograft recipients with 10-day and 56-day follow-up were given cyclosporine A (CsA, Novartis, Basel, Switzerland) diluted in Intralipid (Fresenius Kabi, Bad Homburg, Germany) 2.0 mg/kg/d s.c. for the first 7 days and 1.0 mg/kg/d thereafter. To inhibit NOS activity, \text{N}-\text{nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich)} was dissolved in drinking water which was changed every other day. Donor animals received the orally active L-NAME in a dose of 50 mg/kg/day for 4 days before allograft removal. To block HO-1 activity, zinc protoporphyrin (ZnPP; Porphyrin Products Inc., Logan, UT) was dissolved in 0.2M \text{NaOH}, adjusted to pH of 7.4, and diluted in 0.9% \text{NaCl} to a concentration of 1 mg/ml. ZnPP was administered i.p. (20 mg/kg) to donor rats 2
hours before allograft removal. To supplement RhoA GTPase prenylation pathway, geranylgeranyl pyrophosphate (GGPP; Sigma-Aldrich) at a concentration ~1 mg/ml in methanol was administered i.p. (1 mg/kg) to donor rats 2 hours before allograft removal.

**Drug Assay**

After blood samples were collected, they were immediately centrifuged at 2000 rpm for 10 min at +4 °C and stored at -80 °C until further analysis. The blood concentrations of simvastatin lactone and simvastatin β-hydroxy acid were measured by liquid chromatography-ionspray tandem mass spectrometry.¹

**Lipid Analysis**

Serum samples were collected during the harvest at 8 weeks and stored at -20°C until further analysis. The concentration of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured using the enzymatic colorimetric test (Roche Diagnostics Cholesterol CHOD-PAP method; Cat. no. 1491458, Basel, Switzerland) and a biochemical analyzer (Hitachi Modular PP-analyzer; Hitachi Ltd., Tokyo, Japan).

**Allograft Survival**

The allograft function was estimated by daily abdominal palpation, and graded according to heart beat from 0 to 4: 0 minimal or no contractility (heart beat <30 bpm); 1, poor; 2, moderate; 3, impaired; and 4 normal. Allografts were removed if the grade fell to 0.

**Histology**

Cardiac fibrosis was determined in a blinded review by two observers from paraformaldehyde fixed paraffin sections stained with Masson’s trichrome and scored semiquantitatively (0 to 3) as follows: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; and 3, severe myocardial fibrosis. Cardiac allograft vasculopathy was
determined from sections stained with hematoxylin-eosin and Resorcin-Fuchsin for internal elastic lamina using computer-assisted image processing (Zeiss Axiovision 4.4, Carl Zeiss International, Oberkochen, Germany) and measuring the area between the internal elastic lamina and the vessel lumen. The percentage of arterial occlusion was determined as the ratio of neointimal area to internal elastic lamina area.

**Immunohistochemistry and Immunofluorescence Stainings**

Cryostat sections were stained using the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and the reaction was revealed by 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). Counterstaining was performed using Mayer’s hemalum. Immunofluorescent stainings were performed using Alexa 568 red and Alexa 488 green (Promega, Madison, WI) secondary antibodies and DAPI nuclear staining (VECTASHIELD Mounting Medium with DAPI; Vector Laboratories). Antibodies and dilutions used were: CD4 (5 μg/ml, 22021D), CD8 (5 μg/ml, 22071D), ED1 (5 μg/ml, 22451D), CD161a to detect NK cells (5 μg/ml, 555006, BD Pharamingen, Franklin Lakes, NJ); CD42b (GPIb) (NCL-CD42b, dilution 1:100), CD61 (GPIIIa) (NCL-CD61-308, dilution 1:100, Novoceastra, Newcastle upon Tyne, United Kingdom); myeloperoxidase to detect neutrophil granulocytes (20μg/ml, ab9535), Tropomyosin (7 μg/ml, ab7785), phospho-Adducin (phospho T445, 10 μg/ml, ab58485), NG2 (15 μg/ml, ab50009, Abcam, Cambridge, United Kingdom); OX-62 (10 μg/ml, MCA 1029G), RECA-1 (50 μg/ml, MCA97, AbD Serotec, Dusseldorf, Germany); HMG-CoA Reductase (10 μg/ml, 07-457, Upstate, Ballerica, MA); α-SMA (1:5000, A2547, Sigma-Aldrich); VCAM (10μg/ml, MMS-141P, Covance, Princeton, New Jersey); S100A4 (FSP-1) (1:100, A5114, DakoCytomation, Glostrup, Denmark); HIF-1α (13 μg/ml, IMG629, Imgenex, San Diego, CA); phospho-Smad2 (Ser465/467, 2.5 μg/ml, AB3849, Millipore, Ballerica, MA) and Prolyl-4-Hydroxylase beta (2 μg/ml, AF 5110-1, Acris antibodies GmbH, Herford, Germany). The number of inflammatory cells and RECA1+ capillaries was determined by counting positive cells and capillaries from four random fields of each quadrant of the cardiac cross section with 40x magnification, and are given as a total for 1 mm².
**RNA Isolation and Reverse Transcription**

Total RNA was extracted from five random samples per group using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of mRNA was carried out from 100 ng total RNA using High-RNA-to-cDNA kit (Applied Biosystems Inc., Carlsbad, CA) in a total volume of 20 μl. After completed reverse transcription, 40 μl of PCR-grade water was added to each cDNA sample. Three μl of each sample (corresponding to 5 ng total RNA) were used in each subsequent PCR reaction.

**Real-time PCR**

Real-time PCR reactions were carried out on a RotorGene-6000 (Corbett Research, Hilden, Germany) using 2X DyNAmo Flash SYBR Green Master mix (Finnzymes, Espoo, Finland). Measurement of the PCR product was performed at the end of each extension period. Amplification specificity was checked using melting curve analysis. The number of mRNA copies of each gene of interest was calculated from a corresponding standard curve using the RotorGene software. The results are given in relation to 18S rRNA molecule numbers. Please see Supplemental Table 2 for primer information.

**Transmission Electron Microscopy**

For transmission electron microscopy, cardiac mid-axial cross-sectional samples from left ventricle (2 samples) and septum (1 sample) were microdissected, fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.4, incubated for 2 hours at +25 °C and post-fixed with 1% buffered osmium tetroxide for 1 hour, dehydrated and embedded in epon at room temperature. Sections were post-stained with uranyl acetate and lead and examined with a Jeol EX1200 II transmission electron microscope (Jeol) operating at 60kV. EC-EC junctions were analyzed from 10-15 representative longitudinally cut microvascular vessels per each graft sample using x50,000 magnification and the incidence of EC-EC gaps per graft was quantitated. Images were acquired with an ES500W CCD camera (Gatan Corp., Pleasanton, CA).

**Microvascular Leakage and Perfusion**
A modified Miles Assay was used to measure extravasation of plasma proteins from the microvasculature into the interstitial space of cardiac allografts. Immediately after reperfusion, recipients were injected i.v. with Evans blue (Sigma-Aldrich) diluted in 0.9% NaCl, at 30 mg/ml concentration, which was allowed to circulate for 30 minutes, whereafter the cardiac allograft coronary network was flushed with 5 ml of 1% PFA in 0.05M citrate buffer, pH 3.5. For quantification of extravasated Evans blue, 100 mg of apical myocardium was dissolved in 500 μl of formamide on a shaker at +60 °C for 24 hours. One hundred μl of formamide containing dissolved Evans blue dye was pipetted to cuvettes and the absorbance was measured by a spectrophotometer at 610 nm wavelength. FITC-labeled *Lycopersicon esculentum* (Tomato) lectin (Vector Laboratories) was used to detect vessels 30 minutes after re-establishment of circulation. Coronary arteries were perfused with 50 μl FITC-labeled Tomato lectin diluted in 150 μl of 0.9% NaCl. The number of FITC⁺ microvascular vessels from mid-axial cryostat cross sections was analyzed by fluorescence microscopy by counting positive capillaries from four random fields of each quadrant of the cardiac cross section with 40x magnification, and is given as a total for 1 mm².

**Microvascular Interendothelial Gap Detection**

After heart removal, the coronary arteries were perfused with 50 μl of rhodamine-labeled Concavalin A lectin (red) (Vector Laboratories) that binds to the vascular endothelium and was dissolved in 100 μl of NaCl. Thereafter the hearts were predisposed to cold preservation (4 hours), subsequent warm ischemia (1 hour) and then the cardiac allograft coronary network was perfused with 50 μl of biotinylated *Ricinus communis* lectin (Vector Laboratories) that binds to the vascular basement membrane, and flushed with 200 μl 1% PFA in 0.05M citrate buffer, pH 3.5. Cryostat sections were stained with streptavidin and fluorescent-dye conjugate Avidin 350 nm (blue) (Promega Corp.) to detect biotinylated *Ricinus communis* lectin in order to identify endothelial-endothelial barrier disruption.

**Laser Doppler Monitoring**

Cardiac allograft tissue perfusion was analyzed one hour after reperfusion by dynamic
measurement with laser doppler monitor, Transonic BLF21-Series (Transonic Systems Inc., Ithaca, NY). The moving doppler effect was calculated via a 18 gauge (1.2mm) probe head receiving fiber optic light reflected by stationary structures within the tissue as well as by moving particles (red blood cells). A low intensity beam of monochromatic light was emitted to the portion of apical myocardium, to 1mm depth, and the tissue volume under laser doppler monitoring was approximately 1mm$^3$. Flow signal was sampled 200 s$^{-1}$ from transmitted 19200 baud (=pulses per second) and is represented as tissue perfusion units (TPUs) that are relatively proportional to ml/min/100g of tissue.$^2$

**Measurement of Cardiac Troponin T**

The rat serum levels of cTnT were analyzed with the third generation troponin T test (Troponin T STAT, Roche Diagnostics), which shows cross-reactivity of 0.001% with TnT originating in skeletal muscle at a concentration of 2.000 ng/ml. The functional sensitivity is 0.03 μg/l and the lower detection limit 0.01 μg/l. The cTnT was measured by electrochemiluminescence immunoassay (ECLIA) on the Elecsys 2010 immunoassay analyser (Roche Diagnostics).

**Magnetic Resonance Imaging**

MRI studies were performed with a 4.7 T scanner (PharmaScan, Bruker BioSpin, Billerica, MA) using a 90 mm shielded gradient that is capable of producing maximum gradient amplitude of 300 mT/m with 80-μs rise time. A linear birceage RF coil with an inner diameter of 60 mm was used. After a scout, T2-weighted fast spin echo [rapid acquisition with relaxation enhancement (RARE)] sequence was used (TR/TEeff = 4200/55 ms, rare factor = 8, matrix size = 256 x 256, field-of-view = 30 × 30 mm, 15 slices, slice thickness = 2 mm). The multi-spin multi-echo sequence (MSME), based on CPMG (Carr-Purcell Meiboom-Gill) spin echoes was used for the determination of T2 [repetition time (TR) = 1500 ms, echo time (TE) = 11 - 220 ms and 20 echoes, number of averages = 2, matrix size = 256 x 192, field of-view = 70 x 70 mm, single slice with slice thickness = 2.0 mm, acquisition time = 9 min 36s]. The value of T2 was calculated by fitting the measured intensities to the exponential relaxation curve using the least-squares method.
TGF-β1-induced Endothelial-to-mesenchymal Transition of Human Cardiac Microvascular Endothelial Cells in vitro

TGF-β1 was used to induce EndMT in HMVEC-C at a concentration of 10 ng/ml. Simvastatin was dissolved in EtOH and activated by treatment with NaOH followed by neutralization to pH 7 and concentrations of 0.1, 0.5 and 1.0 μM were used to inhibit EndMT. Antibodies used in in vitro studies were CD31 (ab9498, Abcam), ZO-1 (61-7300, Invitrogen, Carlsbad, CA) and Calponin (C2687, Sigma-Aldrich). HMVEC-C and EGM-2 MV growth medium were from Lonza, Basel, Switzerland. The cells used for the experiments were between passages 4-8. For immunofluorescence microscopy the cells were grown on glass coverslips for the indicated times. Coverslips were then washed three times with PBS, and the cells were fixed in ice-cold methanol at -20 °C. After washing three times with PBS, the cells were incubated in Dulbecco's PBS containing 3% BSA to prevent nonspecific binding of the antibodies. The cells were then incubated with the primary antibody in Dulbecco's PBS for 1 hour. The bound antibodies were detected using Alexa Fluor-594 secondary antibodies (Molecular Probes, Invitrogen). The coverslips were finally washed in water, mounted on glass slides using Vectashield anti-fading reagent (Vector Laboratories) and examined under an Axioplan 2 imaging microscope (Zeiss) using a 40x objective. Images were acquired with an AxioCamHRm camera and Axiovision 4.6 software (Zeiss) at the Molecular Imaging Unit of the University of Helsinki. For RNA isolation and real-time RT-PCR analysis, RNeasy mini kit (Qiagen) was used to isolate total cellular RNA. Reverse transcription was carried out with random hexamer primers (Invitrogen) and Superscript III reverse transcriptase (Invitrogen) using 1.0 μg of total RNA according to the manufacturer's instructions. The cDNAs were amplified using TaqMan Assays-on-Demand gene expression products (Applied Biosystems) and GeneAmp 7500 Sequence Detector thermal cycler (Applied Biosystems). Control amplifications directly from RNA were performed in order to rule out DNA contamination. The levels of gene expression were determined using the Ct method and the results are shown as mRNA expression levels normalized to the levels of a gene with a constant expression (TBP, tata binding protein). SDS-PAGE and immunoblotting were performed after the cells were lysed in RIPA buffer. Equal amounts of protein were separated by SDS-PAGE under reducing conditions.
using 4-20% gradient Tris-glycine gels (Lonza). The proteins were transferred to Protran nitrocellulose membranes (Whatman plc., Kent, United Kingdom) using a semi-dry blotting system (BioRad, Hercules, CA).

**Effect of Simvastatin on EC and SMC Gene Expression in vitro**

Human cardiac microvascular endothelial cells (HMVEC-C) were cultured in EGM-2 MV growth medium. The cells used for the experiments were between passages 4-8. Rat coronary artery SMC (kindly provided by Dariusz Leszczynski; Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland) were cultured in 5% fetal calf serum. HMVEC-C and rat coronary artery SMC were supplemented with activated simvastatin at a concentration of 1.0 μM for 72 hours. RNA isolation and real-time RT-PCR analysis were performed as described above.

**Statistics**

Mann-Whitney U test was used for two-group comparison and Kruskall-Wallis with Dunn test when multiple groups were compared to control. Dunn post hoc test was applied only if Kruskall-Wallis test demonstrated an overall statistically significant difference. For comparison in a longitudinal study, data was analyzed by repeated-measures ANOVA. For survival Kaplan-Meier with Log-rank (Mantel-Cox) was applied. Linear regression analysis was applied to evaluate the relation of different simvastatin concentrations to expression of mesenchymal genes in vitro. Data is given as mean±SEM for longitudinal analysis and for discrete variables and by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) for continuous variables (SPSS Statistics 15.0, SPSS Inc., Somers, NY). The outliers are shown as circles outside the box. P<0.05 was regarded as statistically significant.
Expanded Results

Simvastatin Lactone and β-hydroxy Acid Blood Levels in Rat and Human

Pharmacokinetic analysis with liquid chromatography-ionspray tandem mass spectrometry showed that after single-dose peroral simvastatin 5.0 mg/kg administration, simvastatin lactone was absorbed rapidly within hours (Supplemental Figure 1A). Simvastatin lactone was also absorbed within 2 hours in human organ donors after clinically-approved simvastatin 80 mg administration via nasogastric tube (Supplemental Figure 1B) indicating the applicability of donor simvastatin treatment in a clinical situation. Simvastatin lactone was hydroxylated to its active β-hydroxy acid form and was eliminated much more rapidly in the rat (Supplemental Figure 1A) than in human organ donors (Supplemental Figure 1B).

Simvastatin Treatment Modulates mRNA Levels of Genes Involved in Microvascular Homeostasis Through Endothelial but not of Smooth Muscle Cells

HMVEC-C and rat coronary artery SMC were cultured in vitro with or without simvastatin for 72 hours. Quantitative real time RT-PCR analysis showed that simvastatin resulted in a 50 % reduction in HMVEC-C HIF-1α and ET-1 mRNA levels (Supplemental Table 1). In contrast, in similar in vitro experiments with rat coronary artery SMC simvastatin did not alter the mRNA levels of the investigated genes (Supplemental Table 2).

Low Presence of Intravascular Thrombosis After Cardiac Allograft Reperfusion

The no-reflow phenomenon observed after reperfusion of cardiac allografts with 4-hour cold ischemia (Figure 3) could result from intravascular thrombosis. Immunohistochemical platelet stainings showed only a few CD42b⁺ and CD61⁺ platelet aggregates in allograft capillaries (Supplemental Figure 2) indicating that wide-spread thrombosis was not the reason for compromised microvascular perfusion.

The Range of Donor Simvastatin Treatment Doses Induces Protection Against Cardiac Allograft IRI
To determine the range of effective donor simvastatin doses and possible dose-response on IRI, we administered simvastatin 0.5, 2 or 5 mg/kg p.o. to donor rats 2 hours before allograft removal (Supplemental Figure 3A). All these simvastatin doses resulted in comparable reduction in serum TnT levels and allograft ED1+ macrophage and MPO+ neutrophil infiltration (Supplemental Figure 3A) 6 hours after reperfusion of cardiac allografts with 4-hour cold ischemia. A dose-response effect was noted in allograft CD4+ and CD8+ cell densities with the highest simvastatin 5 mg/kg dose resulting in lowest T cell infiltration (Supplemental Figure 3A).

**Donor Pravastatin Treatment Protects Against Cardiac Allograft IRI**

To determine whether other HMG-CoA reductase inhibitors protect against cardiac allograft IRI, donor rats were treated with pravastatin 5 mg/kg p.o. 2 hours before allograft removal. Donor pravastatin treatment halved serum TnT levels (Supplemental Figure 3B) and reduced allograft ED1+ macrophage and MPO+ neutrophil infiltration ($P < 0.05$; Supplemental Figure 3B) 6 hours after reperfusion of cardiac allografts with 4-hour cold ischemia. These results indicate a class-effect for donor statin treatment in cardiac allograft IRI protection.

**Donor Simvastatin Treatment Has Cardioprotective Effects in Experimental Protocols with Concomitant Use of Clinically-relevant Cardioplegia Solution**

Different cardioplegia solutions are used in the clinic to promote cardiac allograft viability and to reduce the early IRI-related complications. As we used heparinized +4 °C PBS for allograft perfusion and preservation so far, we next performed additional experiments using heparinized +4 °C Plegisol cardioplegia solution for allograft perfusion and preservation (Supplemental Figure 3C). In groups with Plegisol cardioplegia solution, concomitant donor simvastatin 5 mg/kg treatment 2 hours before allograft removal decreased allograft MPO+ neutrophil infiltration ($P < 0.01$; Supplemental Figure 3C) 6 hours after reperfusion of cardiac allografts with 4-hour cold ischemia. These results indicate that donor simvastatin treatment effectively protects against IRI also in experimental protocols with concomitant use of a clinically-relevant cardioplegia solution.
Effect of Simvastatin on Cholesterol Levels of Rats with Low-Cholesterol Diet

Serum cholesterol levels were analyzed from cardiac allograft recipients without simvastatin treatment and from recipients with daily simvastatin 2.0 mg/kg p.o. treatment at 56 days. Serum total cholesterol (1.2±0.3 vs. 1.4±0.3; mean±SEM), HDL (0.5±0.2 vs. 0.7±0.2) and LDL fractions (0.5±0.2 vs. 0.4±0.3), and triglyceride levels (0.7±0.1 vs. 0.7±0.2) were low in recipient rats with low-cholesterol diet without simvastatin treatment, and the cholesterol levels were not affected by daily simvastatin treatment.
Supplemental Figure Legends

Supplemental Figure 1. Simvastatin is absorbed rapidly after peroral administration in rats and human organ donors. Inactive simvastatin lactone form and its active β-hydroxy acid form were analyzed with liquid chromatography-ionspray tandem mass spectrometry. A, Simvastatin blood levels in rats after peroral simvastatin 5 mg/kg administration. B, Simvastatin blood levels in human organ donors after peroral simvastatin 80 mg administration (B). n=3 per group. Data is given as mean±SEM.

Supplemental Figure 2. Microvascular platelet aggregates in rat cardiac allografts at 6 hours after reperfusion. Very few CD42b\(^+\) (A) or CD6\(^+\) (B) platelet aggregates were found in non-treated allografts subjected to 4-hour cold ischemia and 1-hour warm ischemia 6 hours after reperfusion determined by immunohistochemistry. Scale bars = 50 μm.

Supplemental Figure 3. Effect of different donor simvastatin treatment doses, donor pravastatin treatment and concomitant use of cardioplegia solution on cardiac allograft IRI. A, Effect of a range of donor simvastatin doses (0.5, 2.0 and 5.0 mg/kg p.o. 2 hours before graft procurement) on cardiomyocyte injury and allograft inflammation 6 hours after reperfusion in allografts with 4-hour cold ischemia. B, Effect of donor pravastatin treatment (5.0 mg/kg p.o. 2 hours before graft procurement) on IRI. C, Effect of PBS and Plegisol cardioplegia solution on the cardiac allograft IRI. n = 6 (A) and 5 (B-C) per group. D, donor treatment; PBS, phosphate buffer solution, PLG, plegisol cardioplegia solution. Kruskall-Wallis with Dunn test was used to compare various donor simvastatin treatment doses to non-treated control (A). Mann-Whitney U test was applied for two-group comparison (B-C). Data is given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) and the outliers are shown as circles outside the box. *\(P < 0.05\), **\(P < 0.01\).

Supplemental Figure 4. mRNA levels of genes involved in innate and adaptive
immune responses in rat cardiac allografts 6 hours (A-C) and 10 days after reperfusion (D-F). mRNA expression of genes was measured by real time RT-PCR. The results were normalized to 18S rRNA and are given as the ratio to mRNA expressed in allografts without donor simvastatin treatment and without cold ischemia. n = 6-7 (A-C) and n = 5 (D-F) per group. D, donor; R, recipient; and D/R, donor and recipient simvastatin treatment. Mann-Whitney U test was used to compare prolonged cold ischemia to no cold ischemia. Kruskall-Wallis with Dunn test was used to compare donor, recipient or combined donor and recipient simvastatin to no treatment (A-F). Data is given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) and the outliers are shown as circles outside the box. *P < 0.05, **P < 0.01, ***P < 0.001.

**Supplemental Figure 5.** Effect of simvastatin on HIF-1α protein expression. A, quantification of allograft HIF-1α immunoreactive cells indicated that simvastatin treatment of donors and both donors and recipients decreases cardiac allograft HIF-1α protein expression 6 hours after reperfusion. B, Photomicrographs of non-treated and simvastatin-treated donors (arrows point to HIF-1α+ cells). n = 6-7 per group. D, donor; R, recipient; and D/R, donor and recipient simvastatin treatment. Mann-Whitney U test was used to compare prolonged cold ischemia to no cold ischemia. Kruskall-Wallis with Dunn test was used to compare donor, recipient or combined donor and recipient simvastatin to no treatment (A). Data is given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) and the outliers are shown as circles outside the box. **P < 0.01. Scale bar = 75 μm.

**Supplemental Figure 6.** Allograft infiltrating inflammatory cells at 8 weeks. Representative microphotographs of immunohistochemical stainings of the effect of simvastatin treatment allograft on ED1+ macrophages, MPO+ neutrophils, CD4+ and CD8+ T cells and OX62+ dendritic cells in a chronic rejection model. Please see the details and inflammatory cell quantification in Figure 8. D, donor; R, recipient; and D/R, donor and recipient simvastatin treatment. Scale bar = 50 μm.
References


Supplemental Table 1. Relative mRNA levels after simvastatin treatment in EC and SMC in vitro.

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HMVEC-C and rat coronary artery SMC were cultured with or without activated simvastatin at a concentration of 1.0 μM for 72 hours. The results were normalized to TBP and are given as a ratio to mRNA expressed in EC and SMC culture without simvastatin treatment. Data is given as mean±SEM. Mann-Whitney U test was used for two-group comparison. p-values were non-significant. Ang, angiopoiétin; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; HIF-1α, hypoxia inducible factor-1α; HMVEC-C, human cardiac microvascular endothelial cell; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; SMC, smooth muscle cell; TBP, tata binding protein.
Supplemental Table 2. RT-PCR primer list.

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<td>fwd: TTTGGAATCCTTTTCTCGGTG</td>
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<tr>
<td></td>
<td>rev: TTTGAGCCTTTGTGAACGGG</td>
</tr>
<tr>
<td>eNOS</td>
<td>fwd: TAGGGCTATGCGGCAAGCAG</td>
</tr>
<tr>
<td></td>
<td>rev: CACTGAGGGTATCGTAGGTGATGC</td>
</tr>
<tr>
<td>ET-1</td>
<td>fwd: AGTGTTGCTATTTTCTACCGTCC</td>
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<tr>
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<td>rev: GCATCTGTTCCCTTGTTGCTTG</td>
</tr>
<tr>
<td>FoxP3</td>
<td>fwd: GGAAGATGGCCATTGCAAAGACG</td>
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<tr>
<td>GATA-3</td>
<td>fwd: CATTACCACCTATCCGCCTATG</td>
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<tr>
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<td>rev: TTCACACACTCCCTGCCTTCG</td>
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<tr>
<td>HAS1</td>
<td>fwd: TACACGGGTTTCAAGGCACTGG</td>
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<tr>
<td></td>
<td>rev: ACATCTCCTCAAACAGCACCTACC</td>
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HAS2
fwd    ACTGGGCAGAAGCGTGGATTATGT
rev    AACACCTCAACCATCGGGTCTTCTT

HIF-1α
fwd    GAACCCATTTCCATCCATCAAAC
rev    TCTTCTGGCTCATAACCCATCAAAC

HO-1
fwd    CGCCTTCTCCTCAACATTTG
rev    ATCTGGGATTTTCTCGGG

HSP27
fwd    ACTCAGCAGCGGTCTCAGAG
rev    CTGGAGGGAGCGTGTATTTCC

HSP70
fwd    TGGGACATTCTGCCTATCAAGTTTC
rev    TGAGAGAGTCGAATAAACGCAG

IFN-γ
fwd    GCCATCAGCAAACATAAGTGTC
rev    AGAATCAGCACCAGCTCCTTTTC

IL-1b
fwd    GCTATGGCAACTGTCCCTGAACTC
rev    CGAGATGCTGCTGTGAGATTTGAAG

IL-2
fwd    GCAGCGTGTGTTGGATTGACTC
rev    GAGATGATGCTTGGACAGATGGC

IL-6
fwd    TTTGACAGCCACTGACTTC
rev    GAATTCACCCAACACTTCTTTC

IL-17
fwd    CTCAGTTTCCTAATGGGATCTAC
rev    GACCTTGGGGATCACAACCATC

iNOS
fwd    GATTTTTTCAGCACCCTCACC
rev    GGTCTCTCTGCTCAACACTTTGGG

MCP-1
fwd    GCTGTTCAGCCAGATGCAGTT
rev    TTCCCTATTGGGGTCAAGGACAG

NF-κB
fwd    CTACACTTAGCCATCATCCACCTTC
rev    CTCCACCACATCTCTCTGGCTTG

RORc
fwd    ATCAATGCCAACCCTCCTTG
rev    TGGAGGTGCTGGAAAGTCTGTAG

T-box21
Bold = rat primers; Bold Italic = human primers; fwd = forward sequence; rev = reverse sequence; rev.compl. = reverse complement sequence
Supplement Figure 2

A. CD42b+ vessels

B. CD61+ vessels
Supplement Figure 4

A

Cold ischemia (h)
Simvastatin

B

Cold ischemia (h)
Simvastatin

C

Cold ischemia (h)
Simvastatin

D

Cold ischemia (h)
Simvastatin

E

Cold ischemia (h)
Simvastatin

F

Cold ischemia (h)
Simvastatin
Supplement Figure 5

A

![Bar chart showing HIF-1α+ cell counts with different treatments and cold ischemia times.](chart.png)

B

![Images showing HIF-1α+ cells in different treatments.](images.png)

**Cold ischemia (h)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HIF-1α+ cells/mm²</th>
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<tbody>
<tr>
<td>-</td>
<td>1000</td>
</tr>
<tr>
<td>4</td>
<td>2000</td>
</tr>
<tr>
<td>4</td>
<td>3000</td>
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</table>

**Simvastatin**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HIF-1α+ cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1000</td>
</tr>
<tr>
<td>D</td>
<td>2000</td>
</tr>
<tr>
<td>R</td>
<td>3000</td>
</tr>
<tr>
<td>D/R</td>
<td>4000</td>
</tr>
</tbody>
</table>

**Notes:**

- **NO TREATMENT (4h)**
- **SIMVASTATIN (DONOR)**