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Simvastatin pretreatment reduces caspase-9 and RIPK1 protein activity in rat cardiac allograft ischemia-reperfusion☆

Raimo Tuuminen [a,*,1], Emil Holmström [a,1], Alireza Raissadati [a], Pipsa Saharinen [b], Eeva Rouvinen [a], Rainer Krebs [a], Karl B. Lemström [a]

[a] Transplantation Laboratory, University of Helsinki and Cardiac Surgery, Heart and Lung Center, Helsinki University Hospital, Helsinki, Finland
[b] Molecular Cancer Biology Program, Research Programs Unit and Haartman Institute, Department of Virology, University of Helsinki, Helsinki, Finland

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ABSTRACT

Background: In transplantation-associated ischemia/reperfusion injury (Tx-IRI), tumor necrosis factor alpha and damage-associated molecular patterns promote caspase-8 and -9 apoptotic and receptor-interacting protein kinase-1 and -3 (RIPK1/3) necroptotic pathway activation. The extent of cell death and the counterbalance between apoptosis and regulated necrosis eventually determine the immune response of the allograft. Although simvastatin prevents Tx-IRI, its role in apoptotic and necroptotic activity remains unsolved.
Methods: Rat allograft donors and recipients were treated with a single-dose of simvastatin 2 h prior to allograft procurement and reperfusion, respectively. Intragraft caspase-3, -8, and -9 and RIPK1 and -3 mRNA expression was analysed by quantitative RT-PCR and protein activity measured by immunohistochemistry and luminescent assays 6 h after reperfusion. Lactate and lactate dehydrogenase (LDH) levels were analysed from allograft recipient and from hypoxic endothelial cell cultures having treated with activated simvastatin.
Results: When compared to without cold ischemia, prolonged 4-hour cold ischemia significantly enhanced intragraft mRNA expression of caspase-3 and -9, and RIPK1 and -3, and elevated protein activity of caspase-9 and RIPK1 in the allografts. Simvastatin pretreatment decreased mRNA expression of caspase-3 and -9, and RIPK1 and -3 and protein activity of caspase-9 and RIPK1 in the allografts. Intragraft caspase-8 mRNA expression remained constant regardless of cold ischemia or simvastatin pretreatment. Simvastatin pretreatment attenuated lactate and LDH levels, both in the allograft recipients and in hypoxic endothelial cell cultures.
Conclusions: The beneficial effects of simvastatin pretreatment in cardiac allograft IRI may involve prevention of apoptosis and necroptosis.

1. Introduction

Heart transplantation is the only curative treatment for many end-stage heart diseases. The steadily declining number of brain dead organ donors due to advances in trauma-care, and increasing numbers of heart recipient candidates has drawn the focus on efforts to identify suitable donors with extended criteria and cardiac donation after cardiocirculatory death [1,2]. However, these conditions predispose the heart transplant to early donor induced insults, leading to a low utilization proportion and fairly poor long-term survival [3]. Based on these aspects, management of organ donors is a topical issue [4].

Transplant ischemia/reperfusion injury (Tx-IRI) predisposes cardiac allografts to rapid alloantigen-independent injuries, and remains a major obstacle for both early and late survival of cardiac allografts. These innate immune injuries include a cardiotoxic pro-inflammatory cytokine storm, microvascular instability, metabolic changes at loss of oxygen and nutrient supply, and finally reoxygenation that increases the myocardial injury caused by ischemia alone [5].

The well-described pathway leading to controlled cell death – apoptosis – can be induced by many signals, among them death receptor and apoptosome mediated caspases-8 and -9, respectively. These inactive initiator-procaspases are dimerized into active forms, which in turn activate the downstream effector-caspases, such as caspase-3, by

Abbreviations: APC, antigen-presenting cell; c-FLIP, cellular FLICE-inhibitory protein; DAMP, damage-associated molecular pattern; EC, endothelial cell; IRI, ischemia/reperfusion injury; LDH, lactate dehydrogenase; PGD, primary graft dysfunction; RIPK1/3, receptor-interacting protein kinase-1 and -3; TNF, tumor necrosis factor; Tx-IRI, transplant ischemia/reperfusion injury.

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* Corresponding author at: Transplantation Laboratory, University of Helsinki, Helsinki, Finland.
E-mail address: raimo.tuuminen@helsinki.fi (R. Tuuminen).

[1] Equal contribution.

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cleaving their prodomains [6]. Due to controlled degradation of target cells and phagocytosis by adjacent cells, inflammation and damage in surrounding environment remain low [7].

Alarm signals released by damaged cells (damage-associated molecular patterns; DAMPs) are fundamental for the activation of antigen-presenting cells (APC) that ultimately control the balance between host-versus-graft disease and tolerance [8]. The release of DAMPs caused by IRI may evoke a recently described form of regulated cell death called necroptosis. In necroptosis, intracellular contents are released into intercellular space to induce local inflammation [9]. The pathway first evolved to oppose viral infections [10]. The best-known pathway in necroptosis is activated by cytokines of the tumor necrosis factor (TNF) family, which leads to the formation of a complex consisting of receptor-interacting protein kinases 1 and 3 (RIPK1-RIPK3) [11,12]. The subsequent steps following the complex still remain unknown [10]. Interestingly, caspase-8 suppresses the formation of the RIPK1-RIPK3 complex diverting cell death to a more inflammation-limiting form [13]. Caspase-8, in turn, is regulated by a variety of factors, such as cellular FLICE-inhibitory protein (c-FLIP) and TNF signaling [14,15]. Accumulating evidence underscores the importance of necroptosis as a main mediator of a variety of pathophysiologic events, including IRI after interventions against myocardial and cerebrocortical ischemia [16,17], as well as in kidney and cardiac transplantation [18,19].

The vasculoprotective effects of simvastatin, an HMG-CoA reductase inhibitor, are independent of lipid lowering [20,21]. In rat cardiac allografts subjected to prolonged cold ischemia, simvastatin improved vascular EC and pericyte function, reduced inflammation, and inhibited tissue remodeling. In addition, simvastatin abolished Tx-IRI-induced myocardial injury when administered to donors and recipients prior to allograft procurement and reperfusion, respectively [5].

2. Objective

As an extension of analyses based on our samples and data from our previous experiment, we investigated here, whether the clinically relevant simvastatin pretreatment protocol affected the activation of cell death pathways - a link between cold ischemia with metabolic compromise and Tx-IRI.

3. Methods

3.1. Experimental design

To investigate the effect of cardiac TX-IRI on cell death pathways the control allografts without cold ischemia (group 1) and with clinically relevant 4-hour cold ischemia (group 2) received polyethylene glycol vehicle p.o. To investigate a clinically feasible time-window for a pretreatment protocol with simvastatin, the allografts were subjected to 4-hour cold ischemia and p.o. simvastatin was administered to the donors and recipients 2 h before graft removal and reperfusion (group 3), respectively. Allografts (n = 6/group) were removed at 6 h for determination of intragraft mRNA levels and protein activity or serum analysis of cell death. The chosen time-window for donor pretreatment is based on clinical experience, that the gap between declaration of brain death and explantation of the graft is 1–2 h.

3.2. Heterotopic rat heart transplantations

Intra-abdominal heterotopic heart transplantations were performed from specific pathogen-free fully MHC-mismatched inbred male Dark Agouti (DA, RT1av1) to male Wistar Furth (WF, RT1u) rats (Scanbur, Göteborg, Sweden) weighing 300–350 g. The donor heart was perfused with heparinized ice-cold PBS and after procurement were transplanted immediately or preserved in PBS solution at +4 °C for 4 h. Cardiac allograft recipients were anesthetized with isoflurane anesthesia (2–5%/l O_2), and they received buprenorphine 0.15 mg/kg s.c. (Temgesic 0.3 mg/ml, Schering-Plough, Kenilworth, NJ) for peri- and postoperative analgesia. The allografts (n = 6/group) were then transplanted into heterotopic position to the recipients' abdomen using a microsurgical technique. Warm ischemia occurring during heart transplantation was standardized to 1 h. For analysis of Tx-IRI cardiac allografts were harvested under isoflurane anesthesia at 6 h [5]. *Please see Supplementary methods for detailed information.* Permission for animal experimentation was obtained from the State Provincial Office of Southern Finland. The animals received 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 Academies Press (ISBN 0-309-15400-6, revised 2011).

3.3. Drug administration

Peroral simvastatin (Merck Research Laboratories, Whitehouse Station, NJ) was given as a single dose to the donor (5 mg/kg) 2 h before graft removal and to the recipient (2 mg/kg) 2 h before reperfusion. The timing of simvastatin pretreatment and doses selected were based on our previous pharmacokinetic and dose-response analysis in rats [5]. Simvastatin was diluted in polyethylene glycol (molecular weight 300) (Sigma-Aldrich, St. Louis, MO) to a concentration of 1.5 mg/ml. The control allografts received polyethylene glycol vehicle p.o.

3.4. RNA isolation and reverse transcription

Total RNA was extracted from myocardial samples using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of mRNA was carried out from 100 ng total RNA using the 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.

3.5. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) reactions were carried out on a RotorGene-6000 (Corbett Research, Hilden, Germany) using 2× Fast SYBER Green Master Mix (Applied Biosystems Inc., Carlsbad, CA). 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.

3.6. Endothelial cell culture

Human dermal blood endothelial cells (BEC, PromoCell GmbH, Heidelberg, Germany) were cultured on fibronectin coated plates for 24–48 h, changed to new growth media (PromoCell) and transferred to hypoxia (1% O_2). Simvastatin was dissolved in EtOH and activated by treatment with NaOH followed by neutralization to pH 7 at a concentration of 1.0 μM. The growth media was collected at 24 h (n = 6/group).

3.7. Immunohistochemistry

Cryostat sections were stained using the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA), and the reaction was revealed with 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). Counterstaining was performed using Mayer's hemalum. Following anti-rat antibodies were used for rat caspase-3 (1.0 mg/ml, Ab13847, Abcam, Cambridge, MA, USA), for caspase-8 (1:100, NB100-116SS, Novusbio, Littleton, CO, USA), for caspase-9 (17 μg/ml, 9507S, Cell Signaling Technology, Danvers, MA, USA), for RIPK1 (1.0 mg/ml, NBP1-77077, Novusbio) and for RIPK3

(1.0 mg/ml, NB11-77299, Novusbio). The relative percentage of immunoreactive areas for caspases 3, 8, and 9, and RIPK1 and RIPK3, were measured with computer assisted imagining (Zeiss Axionvision 4.4, Carl Zeiss International). Ten photographs with 40× magnification were taken from each sample and the average area of these 10 measurements was used in statistical analyses. All analyses were performed in a blinded manner by two independent observers.

3.8. Luminence-producing enzyme activity assay and ex vivo measurement of luciferase protein in tissues

Luminence-producing enzyme activity assay was used to detect the presence and quantities of caspases 3/7 (G8090, Promega Corp., Fitchburg, WI, USA), 8 (G8200, Promega Corp.), and 9 (G8211, Promega Corp.). Samples from the apex of the allografts were homogenized with passive lysis buffer and luciferase activity determined with a luminometer (LI-COR Biotechnology, Lincoln, NE, USA). The reagent acts as a substrate for the luciferase enzyme protein, producing luminence when reacting. The sample was measured for a period of 10 s, and the data given in real light units (RLU)/10 μg of tissue protein. Luminence was detected in each sample by at least two subsequent measurement periods.

3.9. Measurement of lactic acid and lactate dehydrogenase

Rat allograft recipient serum and BEC culture growth media were collected and subsequently frozen at −20 °C until further analysis of lactic acid and LDH. The levels of lactic acid and LDH were analyzed by photometric measurement at HUSLAB, the laboratory of Helsinki University Central Hospital.

3.10. Statistics

All data are mean ± SEM and analyzed by SPSS 15.0 (SPSS Inc., Chicago, IL). For two-group comparisons, parametric variables were analyzed by Student's *t*-test. $p < 0.05$ was considered statistically significant.

4. Results

4.1. Prolonged cold ischemia in rat cardiac allografts induces mRNA of cell death factors

Donor hearts were transplanted either immediately or subjected to prolonged 4-hour cold ischemia. Warm ischemia time was standardized to 1 h. Six hours after allograft reperfusion, the mRNA expression levels of caspase-9 ($p < 0.001$, Fig. 1) and -3 ($p < 0.05$, Fig. 1), factors involved in apoptosis, and RIPK1 ($p < 0.01$, Fig. 1) and -3 ($p < 0.05$, Fig. 1), factors involved in programmed necrosis, were significantly higher in allografts subjected to clinically relevant 4-hour cold ischemia compared to those without cold ischemia. The caspase-8 mRNA levels remained constant between the study groups (Fig. 1).

Six hours after the reperfusion IHC revealed significantly increased intracardiac levels of necroptosis-related proteins, caspase-9 ($p < 0.01$, Fig. 2), and RIPK1 ($p < 0.05$, Fig. 2) in allografts subjected to 4-hour cold ischemia compared to those without cold ischemia. The levels of caspases 3 and 8, and RIPK3, remained unchanged between groups (Fig. 2). In analysis of luminence-producing enzyme activity the levels of all three caspases remained constant between the allografts with 4-hour cold ischemia and those without (Fig. 3).

4.2. Simvastatin pretreatment prevents apoptotic and necroptotic pathway activation in cardiac allograft Tx-IRI

Cardiac allograft donors and recipients were treated with a single-dose of peroral simvastatin 2 h before allograft procurement and reperfusion, respectively. The allografts were subjected to clinically relevant 4-hour cold ischemia. Simvastatin pretreatment nearly negated the effect of prolonged cold ischemia on mRNA of cell death factors. Six hours after allograft reperfusion, the intragraft mRNA levels of caspase-9 ($p < 0.001$, Fig. 1) and -3 ($p < 0.05$, Fig. 1), and RIPK1 ($p < 0.01$, Fig. 1) were lower when compared to vehicle-treated control cardiac allografts. No changes were observed in caspase-8 mRNA between simvastatin and vehicle-treated allografts (Fig. 1).

The effect on cell death was also evident when measuring immunoreactive areas in IHC and in luminence-producing enzyme activity assay. Simvastatin pretreatment inhibited the immunoreactive area of protein activity of caspase-8 ($p < 0.01$, Fig. 2), caspase-9 ($p < 0.05$, Fig. 2), and RIPK1 ($p < 0.05$, Fig. 2) 6 h after allograft reperfusion when compared to vehicle-treated control group with 4-hour cold ischemia. In enzyme activity assay simvastatin pretreatment decreased the levels of caspase-8 ($p < 0.05$, Fig. 3), and caspase-9 ($p < 0.05$, Fig. 3). No marked changes were seen in IHC or enzyme activity assay in caspase-3 levels (Figs. 2 and 3).

4.3. Simvastatin pretreatment reduces lactate and LDH levels in rat cardiac allograft recipients during Tx-IRI in vivo and human blood endothelial cell hypoxia in vitro

Next, we analyzed serum levels of lactic acid and LDH in cardiac allograft recipients 6 h after reperfusion. In the simvastatin treatment

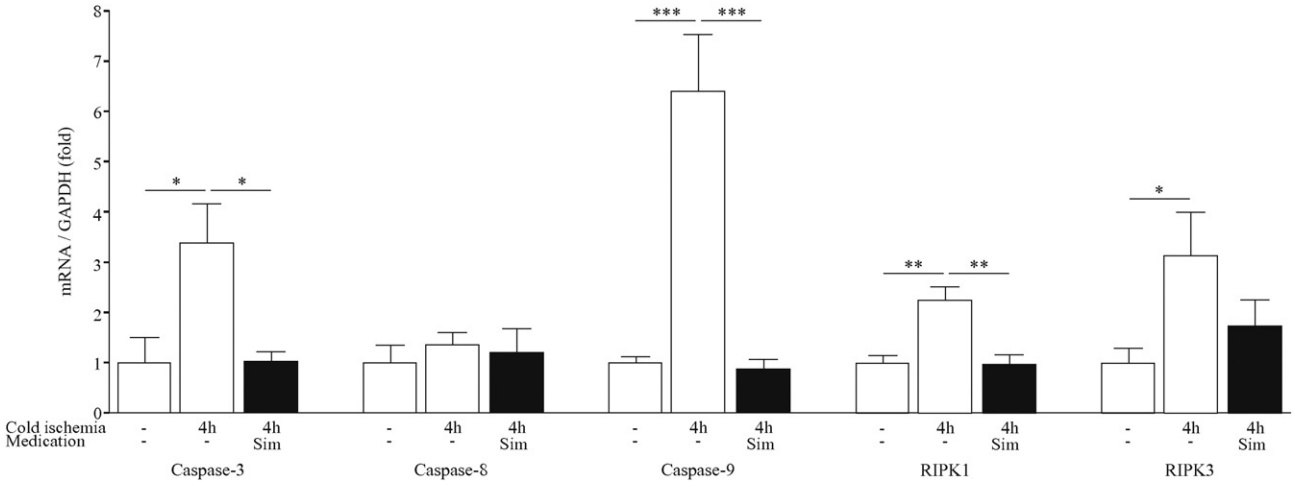


Fig. 1. Intragraft mRNA expression of cell death factors. The effect of simvastatin pretreatment on caspase-3, -8 and -9 and RIPK1 and -3 mRNA levels 6 h after ischemia-reperfusion of major MHC-mismatched rat cardiac allografts. The results are given in relation to 18S rRNA molecule numbers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

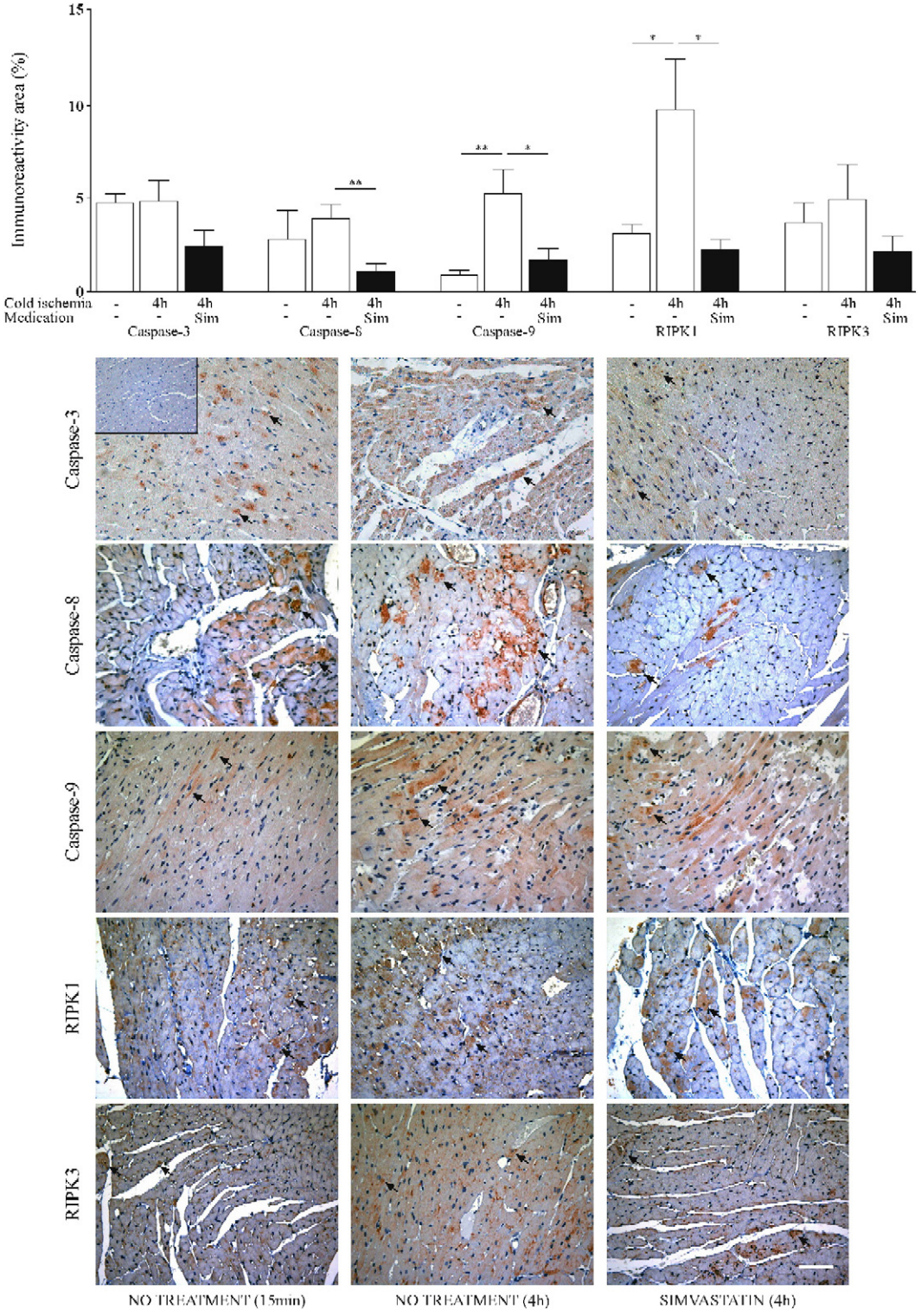


Fig. 2. Measurement of IHC stainings of immunoreactive areas for necroptosis-associated proteins in rat cardiac allograft. The effects of prolonged cold ischemia and simvastatin pretreatment on protein levels of caspase-3, -8, -9, and RIPK1 and -3, 6 h after allograft reperfusion of major MHC-mismatched rat cardiac allografts, measured by computer-assisted analysis. Representative photos of stainings under 40× magnification. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

group, serum levels of lactic acid ($p < 0.01$, Fig. 4A) and LDH ($p < 0.001$, Fig. 4A) were significantly lower compared to those in vehicle-treated rats. Furthermore, we measured the growth media levels of lactic acid and LDH in human BEC cultures subjected to 24-hour hypoxia. In the simvastatin 1 μM pretreated cell cultures, extracellular levels of lactic acid and LDH ($p < 0.05$ for both, Fig. 4B) were significantly lower than in control cultures with vehicle treatment.

5. Discussion

Here, we show that the beneficial effects of simvastatin pretreatment in cardiac Tx-IRI may involve prevention of apoptosis and necroptosis. Ischemia-reperfusion is a principal risk factor for microvascular dysfunction and myocardial injury after heart transplantation. During static cold preservation, a transplant is predisposed to ischemia

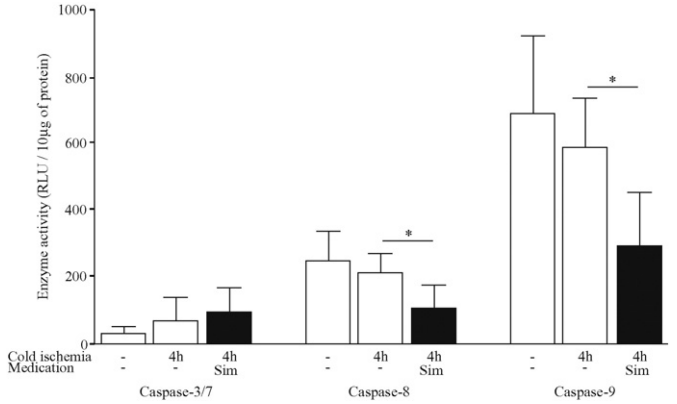
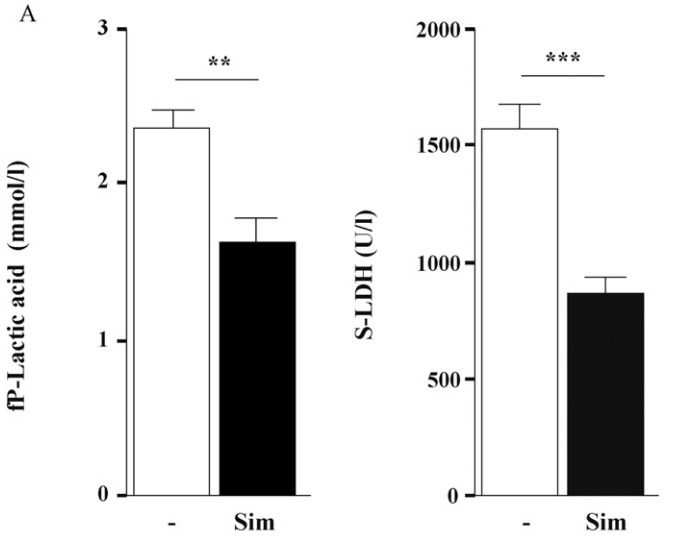


Fig. 3. Protein quantification by luciferase enzyme activity in rat cardiac allograft. The effects of cold ischemia and simvastatin pretreatment on protein levels of caspase-3/7, -8, and -9, 6 h after allograft reperfusion of fully MHC-mismatched rat cardiac allografts, as measured by luminence-producing enzyme activity (luciferase). Units given are real light units per 10 μg of tissue protein, RLU/10 μg protein. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.



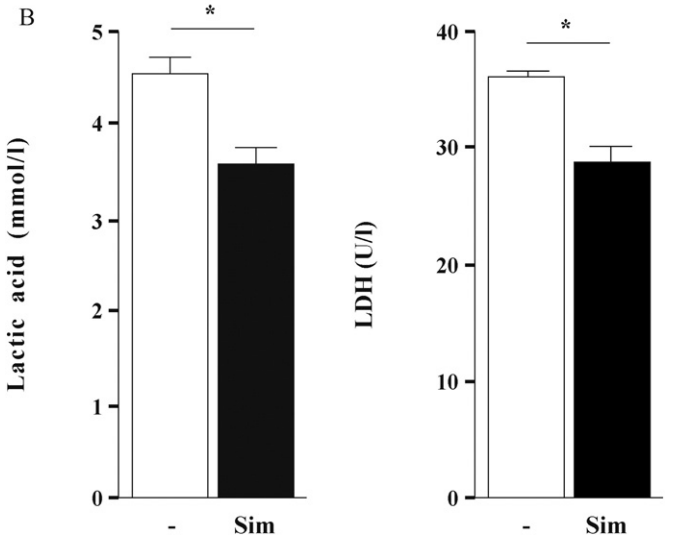


Fig. 4. Cell death in rat cardiac allograft transplantation and human blood endothelial cell culture hypoxia. Analysis of lactic acid and lactate dehydrogenase, indicators of anaerobic glycolysis and unspecific cell death in (A) serum of cardiac allograft recipients and (B) cell culture medium of hypoxic human blood endothelial cells, with and without simvastatin pretreatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and loss of nutrients. Moreover, withdrawal from blood flow excludes a transplant from physiological pulsatile vascular wall shear stress. Both ischemia and metabolic changes together with loss of shear stress disturb vascular and parenchymal homeostasis and predispose the damaged cells to release DAMPs and to activate programmed cell death in the allograft. Overall, ischemia time of the donor cardiac allograft correlates in a linear fashion for primary graft dysfunction (PGD), the progression of chronic rejection and late graft loss [22].

Current immunosuppressive therapies target recipient T cell mediated adaptive immunity. However, donor modalities that target Tx-IRI could restrain graft immunogenicity and the consequent adaptive immune responses. Recently, simvastatin pretreatment in combination with aspirin was shown to reduce vascular endothelial cell damage in a murine heart transplantation model. These changes were accompanied with prolonged allograft survival and were linked with increased number of regulatory T cells (Tregs) [23]. Moreover, i.v. administration of activated simvastatin prior to reperfusion reduced post-infarct myocardial injury in association with down-regulation of high mobility group box (HMGB)-1 - an innate immune ligand [24]. Here, we show that a clinically applicable pretreatment of allograft donors and recipients with simvastatin negated prolonged cold ischemia induced activation of caspase-9/-3 and RIPK1/3 pathways and reduced cell death, evidenced by reduced recipient serum LDH levels.

The elevation in RIPK1 and RIPK3 expression levels induced by prolonged cold ischemia of cardiac allografts was consistent with the previous studies concerning IRI-based damage on different organs [16–18]. Activation of necroptosis in the transplantation setting can have its basis on cellular disturbances in Ca^{2+} homeostasis, depletion of intracellular ATP and increased formation of reactive oxygen species [25]. The fact that caspase-8 expression levels remained constant in our experiment is somewhat puzzling, perhaps expressing that in Tx-IRI death receptor-mediated molecular pathways steer toward necroptosis and inflammation. But then, our results suggest that the caspase-9 expression levels were elevated after cellular stress induced by prolonged cold ischemia of cardiac allografts. The equilibrium between apoptotic and necroptotic molecular pathways in Tx-IRI remains beyond the scope of this study and needs to be further addressed. More importantly, however, necroptosis was recently described to regulate cardiac allograft rejection, and its inhibition with a selective RIPK1-inhibitor, necrostatin-1, improved cardiac allograft survival [19]. Based on these aspects, inhibition of RIPK1/3 activity by simvastatin pretreatment suggests sustained cardioprotective effects. Our data, taken together with the recent findings from other groups [23,24], may suggest a cytoprotective role for simvastatin treatment in preventing the activation of adaptive immune system. This beneficial effect may be mediated through decreased initial cell death and resulting damage from an ischemic insult, which would then lead to milder activation of innate immune system. This in turn, might tip the balance toward Treg-mediated resolution of inflammation instead of prolonged inflammatory state and unnecessary activation of cytotoxic adaptive immunity.

Pre- and early postoperative use of statins is associated with improved cardiac function after major elective surgery [26]. Moreover, findings on the cardioprotective effects of statins are supported by acute preoperative statin administration before elective surgery in clinical trials in patients undergoing percutaneous coronary intervention (PCI) [27] and coronary artery bypass grafting (CABG) [28]. Based on clinical experience, it is reasonable to administrate statins with immunosuppressive medication to reduce allograft vasculopathy and cardiovascular mortality in heart transplant recipients [29,30]. Organ donors do not have major cardiovascular disease nor tend to have previous medical track record of statin use. Donor treatment is a fascinating

approach to prophylactically target Tx-IRI induced cell death and induction of allograft immunogenicity.

Preclinical studies have shown that rapid direct vasculoprotective and anti-inflammatory effects of statins could be exploited in transplantation settings most effectively by combined donor and recipient pretreatment [31,32]. Many of those studies, however, unfortunately lack data on clinically relevant major injuries caused by brain death, cold and warm ischemia, or alloimmune response, or do not fit with the clinically appropriate pretreatment time windows. Thus, conclusions drawn from these studies are difficult to equal with the human transplantation setting. To establish the clinical relevance of oral donor simvastatin treatment in the terms of adequate HMG-CoA reductase inhibition in the target tissue pharmacokinetic analyses of the blood levels in brain-dead human organ donors indicate that simvastatin is absorbed and metabolized to the active beta-hydroxyl acid form within 2 h from drug administration [5]. Taking into account the excellent safety profile, minimal costs of statins, and the possible protective effects on multiple organs, we have initiated a randomized and double-blinded clinical trial to investigate the early and late protective effects of simvastatin pretreatment. In the future, randomized clinical trials will hopefully answer whether anti-apoptotic and anti-necroptotic properties of simvastatin medication could be exploited in extended criteria donors in expansion of the donor pool and improvement of the quality of transplants.

Disclosures

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.trim.2016.05.001.

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