Indoleamine 2,3-dioxygenase gene transfer prolongs cardiac allograft survival

Jianping Li,1* Andrea Meinhardt,1,2* Marc-Estienne Roehrich,1,2 Dela Golshayan,3,5 Jean Dudler,4 Maria Pagnotta,4 Massimo Trucco,6 and Giuseppe Vassalli1,5

Departments of 1Cardiology, 2Microbiology, 3Nephrology, and 4Rheumatology and 5Transplantation Center, Centre Hospitalier Universitaire Vaudois and Université de Lausanne, Faculté de Biologie et Médecine, Lausanne, Switzerland; and 6Division of Immunogenetics, Department of Pediatrics, Children’s Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Submitted 25 September 2007; accepted in final form 9 October 2007

Li J, Meinhardt A, Roehrich ME, Golshayan D, Dudler J, Pagnotta M, Trucco M, Vassalli G. Indoleamine 2,3-dioxygenase gene transfer prolongs cardiac allograft survival. Am J Physiol Heart Circ Physiol 293: H3415–H3423, 2007. First published October 12, 2007; doi:10.1152/ajpheart.00532.2007.—Cells that express indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme in catabolism of tryptophan, suppress T cell responses and promote immunological tolerance. However, their role in solid organ transplantation is incompletely understood. We analyzed T cell responses to allogeneic dendritic cells (DCs) genetically modified to express the gene encoding IDO in vitro and IDO gene transfer into the donor heart in a cardiac transplant model in vivo. Bone marrow-derived DCs transduced with the gene encoding IDO produced active IDO protein. This was associated with decreased stimulation of allogeneic T cell proliferation in the mixed leukocyte reaction in vitro. In a cardiac transplant model, adenovirus-mediated IDO gene transfer into the donor heart resulted in transgene expression predominantly in cardiomyocytes. Fischer-344 rat donor hearts transduced with the gene encoding IDO survived for longer periods of time when placed in Lewis rat recipients compared with control vector or vehicle alone [median survival times of 17 (range: 12–22) days vs. 10 (range: 8–14) and 9 (range: 8–13) days, respectively, P < 0.0001]. IDO gene transfer combined with low-dose cyclosporin A (CsA) was more effective than CsA alone (P < 0.05). Numbers of monocytes/macrophages, CD4+ cells, and CD8α+ cells infiltrating the graft as well as intragraft cytokine transcript levels for IFN-γ, IL-1, TNF-α, regulated upon secretion, normal T cell expressed, and secreted/chemokine (C-C motif) ligand 5 were decreased after IDO gene transfer (P < 0.05). In conclusion, DCs genetically engineered to overexpress IDO modulate T cell alloresponses in vitro. IDO gene transfer into the donor heart attenuates acute cardiac allograft rejection. Regulation of tryptophan catabolism by means of IDO overexpression may be a useful approach in heart transplantation.

dendritic cells; transforming growth factor-β; regulated on activation, normal T-cell expressed and secreted/C-C chemokine ligand 5

INDOLEAMINE 2,3-DIOXYGENASE (IDO) is a cytosolic heme protein that catalyzes the first and rate-limiting step in the major pathway of catabolism (i.e., the kynurenine pathway) of the essential amino acid tryptophan, the least abundant of the 20 amino acids (~1% of all amino acids). IDO is expressed in many tissues (29, 32), especially in lymphoid organs and the placenta. In humans, few IDO-positive cells are detectable in normal lymphoid tissues, but focal infiltrates are present in tonsils that display features of chronic inflammation (20, 24). IDO expression and activity in peripheral blood-derived mono-ocytes/macrophages and placent al trophoblasts are induced by multiple stimuli such as IFN-γ, IL-1, CD40, lipopolysaccharides, and superoxide anions (13, 29). Specific subsets of human and mouse monocyte-derived macrophages and dendritic cells (DCs) that produce IDO in response to IFN-γ are potent suppressors of T cell proliferation (13, 15, 21). In addition, DCs can increase their production of IDO upon cross-linking of CD80/86 with antibodies directed against the T cell receptor (TCR) in tryptophan-free medium are cell cycle arrested at the mid-G1 phase and sensitized to apoptosis when exposed to anti-Fas antibody (15). In addition, tryptophan downstream metabolites induced by IDO directly inhibit CD4+ T cells, CD8+ T cells, and natural killer (NK) cells in vitro (6, 9, 30).

A seminal report by Munn et al. (26) showed that IDO is expressed at the maternal-fetal interface during pregnancy and that specific inhibition of IDO resulted in the rejection of mouse fetuses. IDO appeared to confer immune privileges upon semiallogeneic fetuses through local tryptophan depletion and specific inhibition of antigen-activated T cells. Major roles for IDO in physiological immunoregulation have also been reported in antitumor immunity (23), chronic infection (32), autoimmune responses (8), and cell transplantation (1). However, little is known about the role of IDO in solid organ transplantation. We analyzed IDO gene transfer into DCs and the resulting effect on allogeneic T cell proliferation in the mixed leukocyte reaction (MLR) in vitro. IDO gene transfer into the donor heart was studied in a cardiac transplant model.

MATERIALS AND METHODS

Recombinant adenoviruses. The adenoviral vector Ad-IDO was constructed as previously described (1). Briefly, total RNA was isolated from macrophages and was reverse transcription-treated for cDNA synthesis. The murine IDO gene was cloned by PCR using specific primers chosen to include the native ATG transcription start signal as well as the transcription termination signal of the IDO gene and yielded a fragment size of 1.3 kb. Additional nucleotides were included in the primer sequences to create the Pmel restriction endonuclease sites necessary for ligation into the transfer vector used for adenovirus production. After PCR, the IDO product was cloned in...
a pBluescript vector (Stratagene, La Jolla, CA) using the Prime PCR Cloner Cloning System (5Prime33Prime, Boulder, CO) and inserted into the pQBI-AdCMV5-BFP transfer vector (ADENO-QUEST Kit, Q-Biogene, Carlsbad, CA), which contains the strong cytomegalovirus (CMV) 5 promoter driving the expression of the inserted sequence. In addition, this transfer vector contains the coding region for blue fluorescent protein (BFP) under the control of the weaker CMV promoter. Thus, the final transfer vector contains both the IDO gene and the fluorescent marker BFP. Adenoviral vectors containing no transgene (Ad-Null) or CMV promoter-driven β-galactosidase (Ad-LaZ) or an enhanced green fluorescent protein cassette (Ad-EGFP) were used as control and reporter vectors, respectively. Concentrated viral stocks were prepared using two CsCl ultracentrifugation gradients and stored in storage buffer [10 mmol/l Tris·HCl (pH 7.4), 1 mmol/l MgCl2, and 10% sucrose]. Titer of Ad-IDO and Ad-Null stock solutions ranged from $8 \times 10^{10}$ to $3 \times 10^{11}$ plaque-forming units (PFU)/ml. As a control infusate, virus storage buffer was diluted in PBS in the same proportions as was done for the virus stock.

**Transduction of mouse DCs in vitro**. Bone marrow (BM) cells were isolated from femurs and tibias of C57Bl/6 (B6) and C3H mice. DCs were generated in IMDM culture medium supplemented with 10% FCS (Euroclone, Pero, Italy), penicillin-streptomycin (Sigma, St. Louis, MO), 50 μM β-mercaptoethanol (AppliChem, Cheshire, CT), and granulocyte-monocyte colony-stimulating factor (GM-CSF; 1% from F558 cell culture supernatant). On day 2, 2 mM aspirin (Hänseler, Heisatsu, Switzerland) was added to the culture. On day 3, the culture medium was changed and replaced with fresh medium containing aspirin. On day 5, CD11c+ DCs were purified with immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated with adenoviral vectors [multiplicity of infection (MOI) = 100–1,000] in the presence of Polybrene (1.5 μg/ml, Chemicon, Billerica, MA) using a spinoculation technique (1,500 g at 22°C for 2 h). The maturation status of DCs was analyzed by CD80, CD86, and major histocompatibility class II (MHC-II) surface molecule expression analysis by flow cytometry using fluorochrome-conjugated monoclonal antibodies (eBioscience, San Diego, CA).

**Western blot analysis**. IDO protein expression was assessed by Western blot analysis of cell and tissue extracts using mouse anti-IDO monoclonal antibody (Upstate, Billerica, MA) or human anti-IDO antibody (Molecular Probes, Eugene, OR; 1:400) for 40 min at room temperature and mounted with Mowiol 4–88 antifade medium (Poly-3,4-diamidino-2-phenylindole (Molecular Probes). T cells were purified from spleens of naïve B6 and C3H mice. Splenocytes were washed once in PBS and resuspended in 3 ml ACK buffer (5 min, room temperature). T cells were purified with immunomagnetic microbeads (Miltenyi Biotec). T cells ($5 \times 10^4$ or $10^5$) were added to DC cultures (final volume: 200 μl/well). On day 3, [3H]thymidine (1 μCi/well, Amersham Pharmaeuca, Baie d’Urfe, QC, Canada) was added, and cell proliferation was assessed as [3H]thymidine incorporation at 20 h by liquid scintillation counting.

**Gene transfer into donor hearts and transplantation**. Animal experimentation conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (1996) and the Swiss law on animal protection and was approved by the Veterinary Office of Canton Vaud (Lausanne, Switzerland). Male Fischer-344 (F344) and Lewis (LEW) rats (8–12 wk old, Charles River, L’Arbresle, France) were used as heart donors and recipients, respectively. Ex vivo gene transfer was performed by an intracoronary infusion of vector-containing solution into donor hearts immediately before transplantation. First, hearts were instilled slowly with 500 μl PBS (without Ca2+/Mg2+), followed by 200 μl of virus-containing solution, while allowing efflux through the right pulmonary artery. This artery was then clamped, and an additional 400 μl of virus-containing solution were instilled slowly into the coronary arteries. Vascularized cardiac grafts were placed in the abdominal position. In a preliminary series of experiments, cardiac allografts (n = 4 rats/group) received varying doses of Ad-IDO (10⁸, 10¹⁰, and 3 × 10¹⁰ PFU), Ad-Null (10¹⁰ PFU), or vehicle alone. Three LEW isografts were included as controls. In a second series of experiments, donor hearts (n = 9 rats/group) received Ad-IDO or Ad-Null (10¹⁰ PFU each) or vehicle alone. In a third series experiments, recipients (n = 7 rats/group) received low-dose cyclosporin A (CSA; 1.5 mg·kg⁻¹·day⁻¹, Novartis, Basel, Switzerland) on posttransplant days 0–4 alone or in conjunction with IDO gene transfer (10¹⁰ PFU). A separate small group of donor hearts (n = 4) received an intramyocardial Ad-IDO administration (10¹⁰ PFU in 200 μl virus dilution buffer given at 5 injection sites). Graft survival was monitored by daily abdominal palpation. Rejection (defined as total cessation of heart beating) was confirmed by direct graft examination.

**Immunohistochemistry**. Gene transfer in donor hearts was visualized by immunobinding of BFP produced by Ad-IDO (1) (this approach was chosen because of suboptimal results using anti-IDO monoclonal antibodies). No BFP fluorescence was observed on graft sections by epifluorescence microscopy (in contrast to HEK-293 cells transduced with Ad-IDO in vitro; data not shown). For immunobinding, cryosections were fixed in acetone (−20°C) for 3 min, blocked with PBS, 0.3% Triton X-100, and 1% BSA for 1 h, and incubated with a mixture of goat anti-GFP antibody (which also recognizes BFP due to large sequence homologies between GFP and BFP; 1:700) and rabbit anti-rat von Willebrand factor (vWF) antibody (ab6994, Abcam, Cambridge, UK; 1:1,000) in PBS, 0.3% Triton X-100, and 1% BSA at 4°C overnight. After being washed 3 times with PBS, sections were incubated with Alexa 594-conjugated donkey anti-goat secondary antibody (Molecular Probes, Eugene, OR; 1:400) for 40 min at room temperature, followed by three washes with PBS and an incubation with Alexa 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes; 1:400) for 40 min at room temperature. After being washed three times with PBS, sections were stained with 4',6-diamidino-2-phenylindole (Molecular Probes) for 5 min at room temperature and mounted with Mowiol 4–88 antifade medium (Poly-3,4-diamidino-2-phenylindole (Molecular Probes). T cells were prepared from spleens of naïve B6 and C3H mice. Representative data from 1 of 3 experiments are shown.
Siences, Warrington, PA). Intragraft leukocyte infiltrates were analyzed at day 6 posttransplant. Three series of 8-μm sections per heart were cut at 500-μm steps parallel to the atrioventricular groove from the cardiac apex to the base and stained with the following primary monoclonal antibodies: anti-ED1-like (1C7) detecting monocyte/macrophages, anti-TCR-αβ (R73), anti-CD8α (OX-8, all from PharMingen, San Diego, CA), anti-CD4 (W3/25, Accurate Chemicals, Westbury, NY), or irrelevant mouse (MOPC-31C; PharMingen), followed by biotin-conjugated rabbit F(ab)2 anti-mouse Ig (Jackson Immunoresearch, West Grove, PA), StreptABComplex/HRP (Dako), and DAB/H2O2 (Dako). Morphometric analysis was performed in eight microscopic fields (×10) per section using the NIH Image 1.62 program, as previously described (7, 10). Positive-staining areas for each antibody were expressed as percentages of the corresponding myocardial areas of interest.

Real-time RT-PCR. Hearts were excised at day 6 posttransplant, and ~300 mg of tissue were placed in ice-cold PBS, cut into thin pieces, and submerged into RNAlater Stabilization Reagent. Total RNA was extracted with the Qiagen RNeasy kit (Qiagen, Valencia, CA). DNase-treated RNA was used to generate cDNA with reverse transcriptase Omniscript (Qiagen), random hexamer (Promega, Madison, WI), and RNase inhibitor (Roche, Basel, Switzerland). cDNA equivalent to 100 ng total RNA was used for each PCR. Cytokine transcript levels were measured by real-time PCR (Rotor- gene multiliter real-time cycler, Corbett Research, Sydney, Australia) using SYBR green dye and hypoxanthine phosphoribosyltransferase (HPRT) as a reference gene. Primer sequences were as follows: IFN-γ, 5′-TCATGGCCCTCTCTGGGCTTAC-3′ (sense) and 5′-CAAGAGGAGGCTTCTCTTCCATAG-3′ (antisense); IL-1β, 5′-CTCCAAATCTCACGACGATCTCG-3′ (sense) and 5′-TCCACGGGCAAGACATAGTGGC-3′ (antisense); TNF-α, 5′-CTGTGCCTAGCCTTCTTCTTCTAC-3′ (sense) and 5′-TCTGGGACTAGTGGCCTCTC-3′ (antisense); transforming growth factor (TGF)-β, 5′-CTATGGCTGGACACCATC-3′ (sense) and 5′-GCA-CACACTGCGTTCTTT-3′ (antisense); and HPRT, 5′-CTCTAGACGACTTTATGGACAGCTG-3′ (sense) and 5′-CAACGGCTTTAATGTAATCCAGGTC-3′ (antisense). Results were expressed as cytokine-to-HPRT mRNA copy ratios.

Statistical analysis. Graft survival data are shown as median survival times (MST) and ranges and as Kaplan-Meier cumulative survival curves. The statistical significance of differences in graft survival between groups was evaluated using the Mantel-Cox log rank test. The statistical significance of differences in intragraft leukocyte infiltrates and cytokine mRNA expression between Ad-IDO and Ad-Null was evaluated using the Mann-Whitney U-test.

RESULTS

DCs transduced with the gene encoding IDO inhibit T cell proliferation in vitro. The purity of BM-derived DCs, as assessed by flow cytometry analysis of CD11c expression, was ~90%. The surface molecule profile of DCs before viral transduction was CD86lowCD80+MHC-IIlow (data not shown), reflecting a partially immature phenotype. Gene transfer efficiency, as assessed by flow cytometry analysis of EGFP...
IDO gene transfer prolongs cardiac allograft survival in vivo. IDO protein was detectable by Western blot analysis in graft tissue extracts at 2 and 10 days after IDO gene transfer, with an approximately sixfold decrease between the two time points (Fig. 2A). No IDO protein was detectable in nontransplanted hearts and cardiac allografts that received Ad-Null. Cardiomyocytes accounted for almost all of the BFP-positive graft cells after IDO gene transfer (Fig. 3), which were undetectable in control hearts. Virtually no BFP-positive endothelial cells were observed by double labeling with anti-vWF antibody. In preliminary experiments in a small number of animals (n = 4 rats/group), Ad-IDO at the 10^10 PFU dose prolonged allograft survival to a slightly larger extent than the 10^9 PFU dose, whereas a further increase in the virus dose to 3 x 10^10 PFU did not result in further improvement of graft survival (Table 1). Therefore, the 10^10 PFU dose was used in the second series of experiments involving a larger number of animals (n = 9 rats/group). Ad-IDO prolonged graft survival compared with Ad-Null and vehicle alone [MST of 17 (range: 12–22) days vs. 10 (range: 8–14) and 9 (range: 8–13) days, respectively, P < 0.0001; Fig. 2B]. In the third series of experiments (n = 7 rats/group), Ad-IDO (10^10 PFU) in conjunction with low-dose CsA prolonged graft survival to a larger extent than CsA alone [MST of 19 (range: 16–24) days vs. 15 (range: 13–17) days, P < 0.05; Fig. 2C]. In a separate group, an intramyocardial injection of Ad-IDO into the donor heart showed similar effects on graft survival compared with the intracoronary route of administration [MST of 17 (range: 15–18) days].

IDO gene transfer attenuates graft infiltration by leukocytes. IDO gene transfer was associated with decreased graft infiltration by ED1-like+ monocytes/macrophages (4.7-fold), CD4+ cells (5.1-fold), TCR-αβ+ cells (3.0-fold), and CD8α+ cells (3.0-fold) compared with control vector (P < 0.05 for each cell marker; Fig. 4).

IDO gene transfer inhibits intragraft cytokine expression. IDO gene transfer was associated with decreased intragraft transcripts levels for IFN-γ (3-fold), IL-1β (6-fold), RANTES/CCL5 (7-fold), TNF-α (8-fold), and TGF-β (14-fold) compared with control vector (P < 0.05 for each cytokine; Fig. 5).

DISCUSSION

IDO modulates various immune responses, including antiself, antifetal, and antitumor responses. We evaluated DCs genetically modified with the gene encoding IDO in vitro as well as IDO gene transfer into the donor heart in a cardiac transplant model.

Previous studies have reported that specific subsets of DCs and monocyte-derived macrophages that produce IDO in re-
IDO GENE TRANSFER IN HEART TRANSPLANTATION

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![Graph showing positive-staining area percentage for CD4, ED1-like, CD8α, and TCRαβ.](http://ajpheart.physiology.org)
response to IFN-γ are potent suppressors of T cell proliferation (13, 15, 21, 30). We analyzed the impact of transgenic IDO overexpression by BM-derived murine DCs on allo-specific T cell stimulation in vitro. DCs transduced with the gene encoding IDO produced functional IDO, which attenuated allo-specific T cell proliferation in the MLR in vitro. However, in vitro cell manipulation and adenoviral transduction resulted in DC maturation, which counterbalanced the inhibitory effect of IDO on T cell responses.

In a cardiac transplant model, IDO ex vivo gene delivery to the donor heart reduced graft infiltration by inflammatory cells as well as intragraft cytokine expression, resulting in a moderate yet significant prolongation of allograft survival. IDO gene transfer in conjunction with a short course of low-dose CsA was more effective than CsA alone. Immunohistologically, cardiomyocytes accounted for almost all of the gene-transduced cells in the graft, suggesting that this may be the cell type responsible for the protective effect. As mentioned above, initial reports focused on IFN-γ-mediated induction of IDO-positive subsets of regulatory DCs and monocyte-derived macrophages as well as on IDO-producing placental trophoblasts. However, recent evidence suggests that endogenous or transgenic IDO expression in a variety of cell types may suppress T cell responses via tryptophan catabolism. For instance, BM stromal cells have been shown to suppress allo- genetic T cell responses via IDO-mediated tryptophan degradation (17). Tumor cells transected with the gene encoding IDO inhibited T cell proliferation in the microenvironment (19). Islet cells transduced with the gene encoding IDO induced tryptophan depletion from culture medium, suppressed T cell proliferation in vitro, and survived for extended periods of time after transplantation into nonobese diabetic (NOD)/severe combined immunodeficient (SCID) recipients and adoptive transfer of NOD diabetogenic T cells (1). In a recent study, human vascular endothelial cells transsected with the gene encoding IDO were incapable of stimulating allogeneic T cell responses and induced anergy of allo-specific T cells in vitro (4). Murine corneal endothelial cells transduced with the gene encoding IDO expressed functional IDO, which inhibited allogeneic T cell proliferation and prolonged corneal graft survival (3). Finally, lung cells transsected with the gene encoding IDO inhibited inflammation-related oxidative stress and TGF-β-induced fibrosis in vitro as well as lung allograft fibrosis in a rat model (16). Conversely, specific IDO inhibition accelerated skin (27), but not corneal (3), allograft rejection in mice. Collectively, these data are consistent with transgenic IDO expression in multiple different cell types suppressing local T cell responses. Our data suggest that this may also be the case for myocytes in cardiac grafts. Two mechanisms have been evoked to explain these findings: namely, tryptophan starvation and tryptophan downstream metabolites induced by IDO. The first one is based on the observation that T cells activated via TCR ligation in tryptophan-free medium are cell cycle arrested at the mid-G1 stage and sensitized to apoptosis when exposed to anti-Fas antibody (15) and that tryptophan supplementation reverses IDO-mediated T cell suppression (22). A GCN2 stress-response kinase mediates proliferative arrest and anergy in T cells in response to IDO (22), but exactly how IDO creates the stress that activates GCN2 in T cells is not known. The second mechanism involves tryptophan downstream metabolites induced by IDO, which directly inhibit CD4⁺ cells, CD8⁺ cells, and NK cells in vitro (6, 9, 30). Both tryptophan depletion and downstream tryptophan metabolites induce TCR ζ-chain downregulation and a regulatory phenotype in naïve T cells (9).

In our model, there is a remote possibility that rare DCs in the donor heart were transduced with the gene encoding IDO, left the graft, migrated into the host spleen (14), and inhibited allo-specific T cell responses. However, this mechanism seems unlikely for several reasons. Our in vitro data indicate that efficient gene transduction into DCs requires high viral MOIs that exceed by far those achievable in the donor heart. In addition, we have shown that adenovirus-induced DC maturation counterbalances the inhibitory effect of IDO overexpression in the MLR in vitro. It is therefore unlikely that gene-transduced DCs contributed to the beneficial effect of IDO gene transfer in vitro.

In our model, IDO gene transfer conferred only moderate protection upon cardiac allografts. This may be due, in part, to methodological aspects potentially amenable to a solution in the future. These aspects include vector tropism, gene transfer efficiency, vector toxicity, and delayed onset, and short duration of IDO transgene expression. Consistent with the low tropism of adenovirus vectors for the endothelium, we did not observe significant gene transfer into graft endothelial cells in our model. In this regard, whereas the vascular endothelium expresses little endogenous IDO, transgenic IDO expression by endothelial cells locally suppresses T cell responses (4). Furthermore, adenovirus vectors mediate tissue inflammation, which may offset the beneficial effects of IDO overexpression. The kinetics of transgene expression and tryptophan degradation by IDO also need to be considered. The production of IDO
protein in gene-transduced cells starts approximately hours after gene delivery and presumably reaches peak levels one to several days thereafter. In addition, several hours to a few days are needed for IDO-mediated tryptophan degradation (Fig. 1C). The immunomodulatory effect presumably depends on the degree of tryptophan depletion and/or tryptophan metabolite production but cannot be achieved in the first few hours after transplantation. Therefore, a potential role for IDO gene therapy as an adjunctive treatment is conceivable, but the use of immunosuppressive agents would remain mandatory, at least in the early posttransplant period. Finally, adenoviral transgene expression is short lived (Fig. 3A). Other vectors, such as adeno-associated virus (AAV) vectors, have a potential for sustained transgene expression [we have observed AAV-mediated EGFP expression during 1 yr in mouse hearts (31)].

Clearly, several methodological aspects of IDO gene therapy need to be refined to optimize the therapeutic effect. The present proof of principle study suggests that the regulation of tryptophan metabolism by IDO gene transfer may be a useful approach in heart transplantation.

ACKNOWLEDGMENTS
We thank Dr. Eleonora Simeoni, Dr. Sylvain Fleury, and Dr. Emma Fiorini for scientific help and Kaethy Mujynya-Ludunge for technical help. Present address of J. Li: Transplant Research Laboratories, Novartis, Basel, Switzerland.

GRANTS
Financial support was obtained from the Swiss National Science Foundation, the Teo Rossi di Montefera Foundation, the Swiss Cardiology Foundation, the Novartis Research Foundation, and the Lausanne Transplant Foundation.

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