

Gene transfer of soluble interleukin-17 receptor prolongs cardiac allograft survival in a rat model[☆]

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Received 25 November 2005; received in revised form 16 January 2006; accepted 17 January 2006; Available online 7 March 2006

Abstract

Objective: Interleukin-17 (IL-17), a potent proinflammatory cytokine, has been implicated in allograft rejection. We analyzed the efficacy of an adenoviral vector expressing an IL-17 inhibitor in delaying acute allograft rejection in a rat model of heart transplantation, and the biological mechanisms underlying the protective effect. **Methods:** We constructed an adenoviral vector expressing a soluble IL-17 receptor-immunoglobulin (IL-17R-Ig) fusion protein. IL-17R-Ig activity was assessed by inhibition of IL-17-induced IL-6 release in HeLa cells preincubated with the vector. Intracoronary vector administration was performed in F344 donor hearts that were placed as vascularized grafts into Lewis hosts. Inflammatory cells infiltrating the graft were analyzed by immunohistology. Cytokine transcripts in the graft were determined by real-time RT-PCR. **Results:** IL-17R-Ig gene transfer resulted in prolonged allograft survival (16.1 ± 3.1 days vs 10.3 ± 2.5 days with control virus and 10.1 ± 2.1 days with virus dilution buffer alone; $p < 0.001$). IL-17R-Ig gene transfer reduced inflammatory cell infiltrates, especially monocytes/macrophages and CD4⁺ T cells ($p < 0.05$). It also reduced intragraft cytokine transcripts for interferon- γ and transforming growth factor- β ($p < 0.05$) and, to a lesser extent, IL-1 β and tumor necrosis factor- α ($p = 0.083$). **Conclusions:** Local expression of soluble IL-17 receptor-immunoglobulin attenuates T helper type 1 (Th1) cytokine responses and leukocyte infiltration in rat cardiac allografts, thereby mediating prolonged graft survival. Intragraft IL-17 inhibition may be useful as an adjuvant therapy to systemic immunosuppression in heart transplantation.

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Keywords: Heart transplantation; Cytokine; IL-17; IL-17R; Gene therapy

1. Introduction

Proinflammatory cytokines mediate inflammatory and immune responses during ischemia–reperfusion injury and early rejection after heart transplantation in humans [1,2]. Interleukin-17 (IL-17) is a potent proinflammatory cytokine originally referred to as cytotoxic T lymphocyte-associated serine esterase-8 (CTLA-8) [3]. Murine IL-17 is a 21-kDa

glycoprotein, consisting of 147 amino acids, with a 63% amino acid homology with human IL-17, but no obvious homology with other cytokines (except a 57% homology with vIL-17 from *Herpesvirus saimiri*) [3–6]. Activated CD8⁺ and CD8⁺CD45RO⁺ memory T cells produce IL-17 in humans [7]. Although CD4⁺ cells can produce IL-17 in mice [8], the cytokine is preferentially produced by TCR α/β ⁺CD4⁺CD8⁻ T cells at physiologically relevant levels [9]. A high-affinity receptor for IL-17 (IL-17R) has been isolated from mouse EL4 thymoma cells [5]. This receptor shares no homology with other cytokine receptor families and shows a ubiquitous tissue distribution.

IL-17 stimulates stromal cells and macrophages to secrete a host of inflammatory mediators, such as IL-1 β , IL-6, IL-8, tumor necrosis factor- α (TNF- α), granulocyte-colony stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1), and prostaglandin E2 [9,10]. IL-17 has been shown to induce expression of intercellular adhesion molecule-1

[☆] Abstracts presented at the 24th Annual Meeting and Scientific Sessions of the International Society of Heart and Lung Transplantation, 24 April 2003, San Francisco, USA; the XXV Congress of the European Society of Cardiology, 1st September 2003, Vienna, Austria; the 12th Congress of the European Society of Organ Transplantation, 6th October 2005, Geneva, Switzerland.

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(ICAM-1) on fibroblasts [5]. ICAM-1 up-regulation on endothelial cells mediates leukocyte adhesion and contributes to ischemia–reperfusion injury. In addition, IL-17 stimulates proliferation of alloreactive T cells [11], anti-tumor natural killer (NK) cell activity [12], and maturation of dendritic cell progenitors in vitro [11]. IL-17-deficient mice exhibit abnormal contact, delayed-type, and airway hyper-sensitivity responses, along with impaired T-dependent antibody production [13]. These abnormalities suggest an important role for IL-17 in allergen-specific T cell-mediated immune responses.

Increased expression of IL-17 protein on infiltrating mononuclear cells was detectable on day 2 after experimental renal transplantation, and preceded IL-6 and IL-8 expression [14]. Elevated IL-17 levels have been associated with renal graft rejection in humans [14,15]. In a mouse model of heart transplantation, intraperitoneal administration of an IL-17 inhibitor (soluble IL-17R-Ig fusion protein) resulted in prolonged graft survival [11]. However, the impact of IL-17 inhibition on inflammatory cells infiltrates and cytokine expression in the graft was not characterized. In the present study, we used a gene transfer-based approach to produce the IL-17 inhibitor in the graft itself, as opposed to systemic administration. Gene therapy is an attractive approach to organ transplantation because it can be carried out in the isolated donor organ under well-controlled conditions immediately before transplantation, and because the therapeutic factor can be produced in the graft for extended periods of time. Localized production of the protective protein minimizes systemic effects.

We constructed an adenoviral vector that expresses a soluble IL-17R-Ig fusion protein, and we evaluated its efficacy, along with the mechanisms underlying the protective effect, in a rat model of acute cardiac allograft rejection.

2. Methods

2.1. Construction of the AdIL-17R-Ig vector

The cDNA sequence coding for the IL-17R extracellular domain was cloned using a RT-PCR protocol from C57BL/6 mouse spleen mRNA. After confirmation by bidirectional sequencing, the cDNA was subcloned in frame with an *Xba*I fragment encoding the constant and hinge region (Fc γ fragment) of mIgG1. The resulting cDNA containing the IL-17R coding sequence was placed into a CMV promoter-driven expression cassette in the pC5 plasmid. This allows for the construction of adenoviral vectors by using a novel method based on direct DNA ligation [16]. Recombinant adenovirus AdIL-17R-Ig was generated by pC5 co-transfection with *Pac*I-restricted viral DNA from AdGFP (a Δ 1-deleted recombinant adenovirus containing a unique *Pac*I site [16]) onto 293 cells. Concentrated virus stocks were prepared using two CsCl₂ ultracentrifugation gradients and stored in storage buffer (10 mmol/L Tris–HCl, pH 7.4, 1 mmol/L MgCl₂, 10% glycerol) according to standard protocols. An adenoviral vector containing no transgene (AdNull) was used as a control. The titer of AdIL-17R-Ig and AdNull stock preparations determined by plaque titration assay was 1×10^{11} plaque forming units (PFU)/mL (virus particles/PFU ratio \approx 5–10).

IL-17R-Ig protein expression was assessed by Western blot analysis of HeLa cells preincubated with AdIL-17R-Ig, using two different mAbs, each one recognizing one component of the fusion protein: goat anti-mouse IL-17R mAb (RnD; catalogue number AF 448) and goat anti-mouse IgG (Fc γ fragment-specific) mAb. IL-17R-Ig activity was determined by preincubating 3T3 cells with either AdIL-17R-Ig or control vector at a multiplicity of infection (MOI) of 200 for 24 h, followed by stimulation with recombinant IL-17 or IL-1 β for 6 h; the resulting IL-6 release was assessed by ELISA.

2.2. Gene transfer and heart transplantation

All animals received humane care in compliance with the European Convention on Animal Care and the study was approved by the local institutional ethics committee. Male Fischer rats (F344; 8–12 weeks old) were used as heart donors and male Lewis rats (LEW; same age) as recipients (both strains were from IFFA CREDO, L'Arbresle, France). Ex vivo gene transfer into the donor heart was performed by intracoronary instillation of vector containing solution immediately before transplantation. First, donor hearts were instilled slowly with 500 μ L phosphate-buffered saline PBS (without Ca⁺⁺/Mg⁺⁺; pH 7.4), followed by 200 μ L virus containing solution, while allowing efflux through the right pulmonary artery. This artery was then clamped and 400 μ L solution containing 10^{10} PFU viral vector were instilled slowly into the coronary arteries. Heterotopic cardiac grafts were placed in the abdominal position of rats anesthetized by isoflurane inhalation. Graft survival was monitored by daily abdominal palpation. Rejection, defined as total cessation of heart beating, was confirmed by direct graft examination.

2.3. Immunohistology

Immunohistology was performed on cryostat sections of heart samples harvested 6 days after transplantation. Rats were sacrificed by lethal pentobarbital injection, perfused with ice-cold PBS, and hearts were immediately frozen in OCT-compound. Four series of 8- μ m sections per heart ($n = 4$ per group) were cut at 500- μ m steps parallel to the atrio-ventricular groove from the cardiac apex to the basis. IL-17R-Ig protein expression was visualized by immunostaining using goat anti-mouse IgG (Fc γ fragment-specific) mAb, followed by biotin-conjugated anti-goat IgG (Jackson ImmunoResearch Lab.), StreptABComplex/HRP, and Nickel-DAB/H₂O₂ (Dako). Leukocytes infiltrating the graft were immunostained with the following primary mouse mAbs: anti-ED1-like (1C7) detecting monocytes/macrophages, anti-TCR $\alpha\beta$ (R73) detecting T lymphocytes bearing TCR $\alpha\beta$, anti-CD8 α (OX-8; all from PharMingen) detecting cytotoxic T lymphocytes and NK cells), anti-CD4 (W3/25; Accurate Chemicals) detecting CD4⁺ T lymphocytes and macrophages, and irrelevant mouse (MOPC-31C; PharMingen) as a control. Detection steps were biotin-conjugated rabbit F(ab')₂ anti-mouse Ig (Jackson ImmunoResearch), followed by StreptABComplex/HRP and DAB/H₂O₂ (Dako). Images were acquired with a Hyper-HAD-Axioscope microscope and an Axiocam-MRC camera (Zeiss). Morphometric analysis was performed in eight representative microscopic fields (100 \times) per cardiac section using the NIH-Image-1.62 program. Positive-staining areas for each marker

are shown as percentages of total myocardial areas on the respective sections.

2.4. Quantitative RT-PCR

PBS-perfused hearts ($n = 4$ per group) were excised, and about 300 mg of apical myocardium was placed in ice-cold PBS and cut into thin slices, then submerged into RNAlater Stabilization Reagent. Total RNA was extracted with the Qiagen RNeasy midi kit. DNase-treated RNA was used to generate cDNA, using the reverse transcriptase Omniscript (Qiagen), random hexamer (Promega), and RNase inhibitor (Roche). cDNA equivalent to 100 ng of total RNA was used for each PCR reaction. Transcript levels for cytokines were measured by quantitative real-time PCR (Rotor-Gene 2000, Corbett Research), using the SYBR-green dye. Constitutively expressed hypoxanthine phosphoribosyltransferase (HPRT) was selected as endogenous control to correct for potential variation in RNA loading or efficiency of the amplification reaction. Primer sequences were as follows: IFN- γ , 5'-tcatggccctctctggctgttac-3' (sense) and 5'-caagaggaggctcttctcctcatag-3' (antisense); IL-1 β , 5'-cttcaatctcacagcagcatctcg-3' (sense) and 5'-tccacggcaagacataggtagc-3' (antisense); TNF- α , 5'-ctgtgcctcagcctcttctcattc-3' (sense) and 5'-ttgggaacttctcctctgttgg-3' (antisense); TGF- β , 5'-ctaagtggggaccgcaacaacg-3' (sense) and 5'-tctggcactgctcccgatg-3' (antisense); RANTES, 5'-ccatagtgctcgcacaccactc-3' (sense) and 5'-gcacacacttggcgggttcctt-3' (antisense); HPRT, 5'-ctcatggactgatttgacaggactg-3' (sense) and 5'-cagcgcttaataatccagcaggtc-3' (antisense). The threshold cycle (C_T) was defined as the fractional cycle number at which the reported fluorescence reached a defined level. Target gene normalized to HPRT was expressed as ΔC_T (C_T of target gene minus C_T of HPRT) [17].

2.5. Statistical analysis

Graft survival is shown as mean survival time (\pm SD) and by Kaplan–Meier cumulative survival curves. Statistical significance of differences in graft survival between AdIL-17R-Ig, AdNull, and PBS was analyzed using product-limit (Kaplan–Meier) survival estimates by Log Rank statistics (JMP program, version 5; SAS Institute). Immunohistological data are shown as median values (range). Statistical significance of differences in intragraft leukocyte infiltrates and cytokine transcripts between IL-17R-Ig and mock gene transfer was analyzed by Mann–Whitney U -test. A p -value < 0.05 was considered to be statistically significant.

3. Results

3.1. IL-17R-Ig gene transfer prolongs cardiac graft survival

To establish the method of adenovirus-mediated ex vivo gene transfer into the donor heart, we performed preliminary studies using an adenoviral vector containing the *LacZ* reporter gene (AdLacZ) in rat cardiac isografts (LEW/LEW). Gene transfer efficiency, assessed by immunohistological staining with rabbit anti- β -galactosidase Ab, was higher

using a virus dose of 10^{10} PFU/heart compared with 10^9 PFU. β -gal-positive cells decreased in number by ~ 2.5 -fold between days 5 and 40 post-transplantation. The AdIL-17R-Ig vector was tested in the allogeneic F344/LEW rat strain combination after successful functional characterization in vitro. This comprised demonstration of IL-17R-Ig protein expression in transduced HeLa cells, and of IL-17 inhibitory activity in transduced 3T3 cells; the effect of IL-1 β stimulation was unaffected (data not shown). IL-17R-Ig protein expression in cardiac allografts was visualized by immunostaining (Fig. 1A–C). Allograft survival in hearts receiving AdNull (10.3 ± 2.5 days; $n = 8$) was similar to those receiving PBS alone (10.1 ± 2.1 days; $n = 9$), whereas survival of grafts receiving AdIL-17R-Ig ($n = 8$) was significantly prolonged (16.1 ± 3.1 days; $p < 0.001$ vs AdNull and PBS; Fig. 1D). A separate group of grafts ($n = 4$) infused with the AdLacZ vector showed a similar survival (10.0 ± 2.0 days) compared with AdNull. Control isografts (LEW/LEW) survived for > 150 days.

3.2. IL-17R-Ig gene transfer reduces graft infiltration by inflammatory cells

In the AdIL-17R-Ig versus AdNull groups ($n = 4$ each), median values (range) for myocardial surface areas staining positive for ED1-like were 5.0% (3.7–8.3%) versus 17.6% (12.2–20.7%; $p < 0.05$); for CD4, 5.1% (4.2–10.0%) versus 17.2% (11.9–26.6%; $p < 0.05$); for CD8 α , 8.9% (7.1–12.2%) versus 12.5% (10.8–14.0%; $p = 0.083$), and for TCR $\alpha\beta$, 13.1% (11.7–13.4%) versus 13.0% (7.0–15.0%; NS). Thus, IL-17R-Ig gene transfer reduced graft infiltration by monocytes/macrophages and CD4 $^+$ cells by approximately 3.5-fold (Fig. 2).

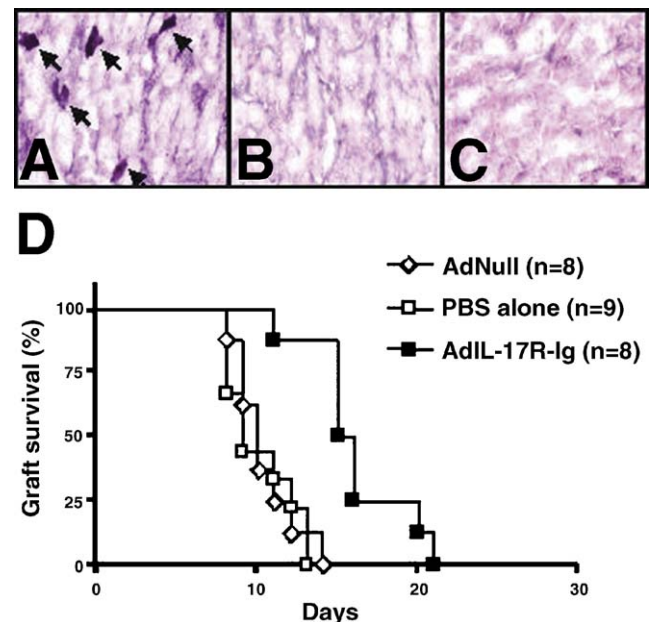


Fig. 1. (A–C) detection of transgene expression in cardiac allografts. (A) AdIL-17R-Ig. Immunostaining for mouse IgG, Fc γ fragment-specific, at day 6 post-transplant (arrows: positive-staining cells (Nickel–DAB/H $_2$ O $_2$)). (B) AdNull (control); immunostaining for mouse IgG. (C) AdIL-17R-Ig; staining with isotype-matched control antibody. (D) Kaplan–Meier survival curves of allografts receiving AdIL-17R-Ig ($p < 0.001$ vs either control), AdNull, or PBS.

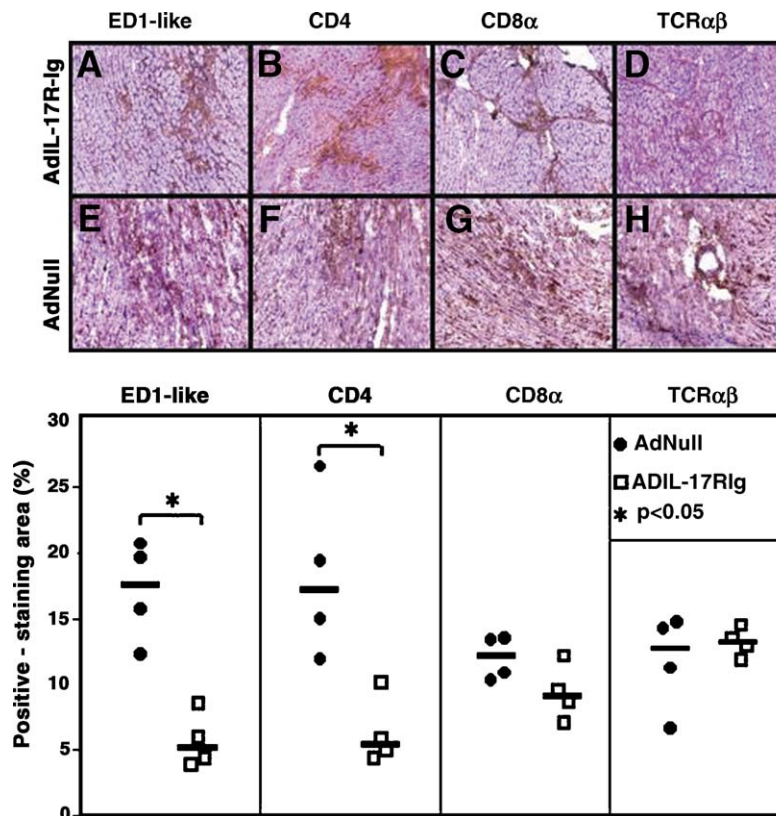


Fig. 2. Immunohistological analysis of inflammatory cells infiltrating the graft. Upper panels: photomicrographs of heart sections in the AdIL-17R-Ig and AdNull groups ($n = 4$ each) immunostained with the indicated mAbs. Lower panel: quantitative analysis of percent myocardial surface area staining positive for each mAb (AdIL-17R-Ig, opened squares; AdNull, closed circles). Horizontal bars are median values.

3.3. IL-17R-Ig gene transfer reduces cytokine transcripts in the graft

Compared with mock gene transfer, IL-17R-Ig gene transfer reduced cytokine transcripts for IFN- γ ($p < 0.05$), TGF- β ($p < 0.05$), IL-1 β ($p = 0.083$), and TNF ($p = 0.083$), but not RANTES (Fig. 3). Of note, a change in ΔC_T values by 1 unit corresponds to a change in cytokine transcripts by several folds; hence, differences in cytokine transcripts between the two groups were substantial.

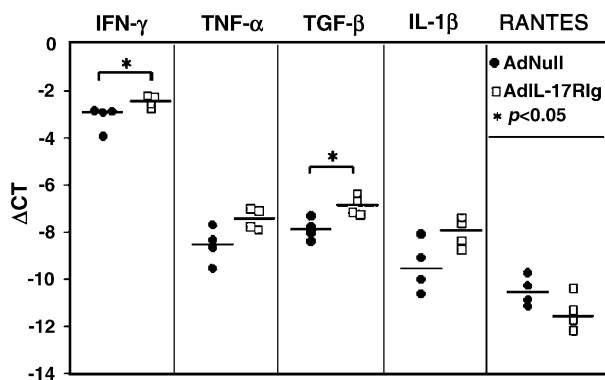


Fig. 3. Real-time RT-PCR analysis of cytokine transcripts in allografts after AdIL-17R-Ig (opened squares) or mock gene transfer (closed circles; $n = 4$ each). Data are shown as ΔC_T (more negative values indicate higher cytokine transcript levels) [17]. Horizontal bars are median values.

4. Discussion

Blocking of a cytokine's activity (e.g., using mAbs or soluble receptors) is a rational approach to the immunosuppressive therapy of allograft rejection. Here we show that localized expression of a soluble IL-17R-Ig fusion protein in donor hearts mitigates tissue inflammation and delays acute allograft rejection in a rat model. The protective effect was associated with decreased numbers of macrophages, CD4⁺ T cells, and marginally, CD8⁺ T cells infiltrating the graft. Moreover, IL-17R-Ig gene transfer reduced cytokine transcripts for IFN- γ , TGF- β , IL-1 β , and TNF- α . These results suggest that IL-17R-Ig may act by down-regulating early-phase cytokines induced by ischemia–reperfusion injury, such as TNF- α and IL-1 β [18], together with T helper (Th) type 1 cytokines, such as IFN- γ . Th1 cytokine responses have been associated with acute allograft rejection. Our results are consistent with in vitro data showing that IL-17 up-regulates IFN- γ and IL-1 β in macrophages [10]. In turn, IFN- γ stimulates macrophages to produce IL-1 β , TNF- α , IL-12, and other proinflammatory cytokines. Thus, down-regulation of IFN- γ may be an important mechanism by which IL-17 inhibition protects the graft. Additional mechanisms supported by previous in vitro data include inhibition of IL-17-induced proliferation of alloreactive T cells, and of IL-17-induced maturation of dendritic cell progenitors [11].

Our results are in good agreement and extend previous data by Antonysamy et al. [11] showing prolonged mouse

cardiac allograft survival (from 10.5 to 19 days) as a result of intraperitoneal injections of purified IL-17R-Ig protein (on post-transplantation days 0–6). However, leukocyte infiltrates and cytokine expression in the graft were not characterized in this study. Using gene transfer technology, we have investigated the effect of localized IL-17R-Ig production in the graft itself, as opposed to the systemic approach utilized in the previous study [11]. Intra-graft gene transfer may be more or less effective than the systemic approach, depending on several factors including the therapeutic molecule itself. For instance, intra-graft gene transfer of CTLA-4-Ig, an immunomodulatory protein, was more effective and caused less systemic immunosuppression than systemic CTLA-4-Ig protein treatment in a rat heart transplantation model [19]. However, this study lacked a direct comparison between intra-graft and systemic CTLA-4-Ig gene transfer, because the systemic approach consisted of recombinant CTLA-4-Ig protein administration. This approach suffers from several limitations including inter-batch variability of CTLA-4-Ig activity. In another study, gene transfer of heme oxygenase-1, a cytoprotective enzyme, achieved long-term cardiac allograft survival in a majority of mice after intravenous vector injection, but not after direct vector administration to the graft [20]. Because heme oxygenase-1 overexpression in the spleen was detected after intravenous vector injection only, it was postulated that modification of alloimmune responses in the spleen might be central to the protective effect of heme oxygenase-1. These results were consistent with data in HO-1 transgenic mice showing prolonged allograft survival of normal donor hearts placed in HO-1 transgenic hosts, whereas HO-1 transgenic donor hearts were promptly rejected by normal hosts [21]. These observations illustrate the fact that intra-graft and systemic overexpression of a therapeutic gene may induce different biological effects; hence, the interest of studying intra-graft IL-17R-Ig gene transfer.

Although IL-17R-Ig gene transfer delayed acute rejection, it ultimately failed to prevent it. This result is not surprising considering the molecular redundancy in cytokine activation cascades, whereby multiple cytokines can activate downstream effector pathways of tissue inflammation. The present proof-of-concept study does not permit to draw any conclusion regarding possible clinical applications. Data in rodent models of transplantation are of limited usefulness in humans. Nevertheless, our results suggest that further investigations on IL-17 inhibition as a potential adjuvant therapy in transplantation are warranted. Future studies will need to explore possible synergistic effects of IL-17 inhibition and currently used immunosuppressive agents in clinically more relevant transplantation models in nonhuman primates.

Acknowledgments

The authors wish to thank Kaethy Mujynya Ludunge, Maria Pagnotta, Robert Driscoll, and Roberto Salvi, PhD, for expert technical and scientific assistance.

Financial support: Swiss National Science Foundation (grant 632-058215), *Fondation Lausannoise de Transplantation Cardiaque*, *Fondation Vaudoise de Cardiologie*, Teo Rossi di Montelera Foundation.

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