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Gene transfer of a soluble IL-1 type 2 receptor-Ig fusion protein improves cardiac allograft survival in rats☆

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Abstract

Objective: Interleukin-1 (IL-1) mediates ischemia–reperfusion injury and graft inflammation after heart transplantation. IL-1 affects target cells through two distinct types of transmembrane receptors, type-1 receptor (IL-1R1), which transduces the signal, and the non-signaling type-2 receptor (IL-1R2), which acts as a ligand sink that subtracts IL-1β from IL-1R1. We analyzed the efficacy of adenovirus (Ad)-mediated gene transfer of a soluble IL-1R2-Ig fusion protein in delaying cardiac allograft rejection and the mechanisms underlying the protective effect. **Methods:** IL-1 inhibition by IL-1R2-Ig was tested using an *in vitro* functional assay whereby endothelial cells preincubated with AdIL-1R2-Ig or control virus were stimulated with recombinant IL-1β or tumor necrosis factor-α (TNF-α), and urokinase-type plasminogen activator (u-PA) induction was measured by zymography. AdIL-1R2-Ig was delivered to F344 rat donor hearts *ex vivo*, which were placed in the abdominal position in LEW hosts. Intragraft inflammatory cell infiltrates and proinflammatory cytokine expression were analyzed by immunohistochemistry and real-time reverse transcriptase-polymerase chain reaction (RT-PCR), respectively. **Results:** IL-1R2-Ig specifically inhibited IL-1β-induced u-PA responses *in vitro*. IL-1R2-Ig gene transfer reduced intragraft monocytes/macrophages and $CD4^+$ cell infiltrates ($p < 0.05$), TNF-α and transforming growth factor-β (TGF-β) expression ($p < 0.05$), and prolonged graft survival (15.6 ± 5.7 vs 10.3 ± 2.5 days with control vector and 10.1 ± 2.1 days with buffer alone; $p < 0.01$). AdIL-1R2-Ig combined with a subtherapeutic regimen of cyclosporin A (CsA) was superior to CsA alone (19.4 ± 3.0 vs 15.9 ± 1.8 days; $p < 0.05$). **Conclusions:** Soluble IL-1 type-2 receptor gene transfer attenuates cardiac allograft rejection in a rat model. IL-1 inhibition may be useful as an adjuvant therapy in heart transplantation.

Keywords: Heart transplantation; Acute rejection; IL-1; Gene therapy

1. Introduction

Proinflammatory cytokines mediate ischemia–reperfusion injury and stimulate immune responses leading to acute graft rejection. Upregulation of a number of cytokines, including interleukin-1 (IL-1), the prototypical and one of the most potent early-acting inflammatory cytokines, occurs very early after graft reperfusion [1] including in human heart transplants [2,3]. Cytokine transcripts for IL-1α, IL-1β, and IL-2 increase with time after transplantation in cardiac transplant recipients [3]. Acting in concert with tumor necrosis factor-α (TNF-α), IL-1 upregulates adhesion molecules on endothelial cells and leukocytes, thereby promoting neutrophil adhesion [4]. E-selectin expression on arterioles is upregulated between 3 and 6 h, followed by intercellular adhesion molecule-1 (ICAM-1) that is upregulated on virtually all endothelial cells by 6–12 h after heart transplantation [5]. IL-1 promotes a destructive inflammation by activating macrophages and T cells that release proinflammatory cytokines, such as TNF-α and IL-8 [6,7]. Consequently, IL-1 is a central mediator of myocardial reperfusion injury and early alloantigen-independent inflammatory events following heart transplantation. This is manifested by the fact that IL-1 can induce early failure of donor hearts placed in syngeneic hosts [1,4]. In addition, IL-1 stimulates alloantigen

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presentation by endothelial cells and dendritic cells [8]. Therefore, IL-1 participates in both innate and allogeneic immune responses to the graft.

IL-1 affects target cells through two distinct types of transmembrane receptors that have a nearly identical extracellular domain [6]. However, IL-1 type 1 receptor (IL-1R1) has a 213-amino acid cytoplasmic domain that is essential for signal transduction, whereas the type 2 receptor (IL-1R2) has a shorter cytoplasmic domain and does not transduce the signal. IL-1R1 is found mainly on T cells, fibroblasts, keratinocytes, and hepatocytes, whereas IL-1R2 is found predominantly on B cells, macrophages, and neutrophils. IL-1 activity is tightly regulated by multiple mechanisms including the IL-1 receptor antagonist (IL-1Ra), membrane IL-1R2, and circulating 'soluble' IL-1R1 and IL-1R2 extracellular domains [6]. Membrane IL-1R2 acts as a 'decoy' molecule, especially for IL-1β, thereby subtracting free IL-1β released from inflammatory sites [9–11]. Cell surface IL-1R2 is the source of circulating 'soluble' IL-1R2 extracellular domains that are released by proteolytic cleavage by a so far unidentified metalloproteinase upon activation by secondary, IL-1-induced cytokines [12]. Soluble IL-1R2 is found in the circulation in healthy subjects as well as in inflammatory body fluids, such as synovial fluid [6]. Soluble IL-1R2 circulates in healthy humans at molar concentrations which are 10-fold greater than those of IL-1β measured in patients with sepsis and 100-fold greater than those observed after intravenous IL-1β administration in patients with cancer [13]. The rank of affinities for the two soluble receptors is markedly different for the three IL-1 molecules. The rank for the three ligands binding to soluble IL-1R1 is IL-1Ra > IL-1α > IL-1β, whereas for IL-1R2, the rank is IL-1β > IL-1α > IL-1Ra [6,7,14]. Soluble IL-1R2 binds IL-1β more avidly than IL-1R1, but loses affinity for IL-1Ra [14]. This is an attractive profile of ligand affinity for a candidate IL-1 inhibitor.

For this reason, we have constructed an adenoviral vector expressing a soluble IL-1R2-Ig fusion protein. The vector's functional efficacy was confirmed using an *in vitro* IL-1 inhibition assay. In a rat heart transplantation model, IL-1R2-Ig gene transfer into donor hearts mitigated allograft rejection, resulting in prolonged graft survival.

2. Materials and methods

2.1. Recombinant adenoviruses

The cDNA sequence coding for the extracellular portion of IL-1R2 was cloned by RT-PCR from mouse 70Z3 B cell mRNA. After confirmation by bi-directional sequencing, the cDNA was subcloned in frame with an *Xba*I fragment encoding the constant and hinge mIgG1 region (Fc-γ fragment). The resulting cDNA sequence encoding the IL-1R2-Ig fusion protein was placed under the transcriptional control of a CMV promoter into pC5 plasmid, yielding pC5-IL-1R2-Ig. Adenoviral vectors were constructed using a novel method based on direct DNA ligation [15]. Recombinant adenovirus AdIL-1R2-Ig was generated by co-transfection of pC5-IL-1R2-Ig with *Pac*I-restricted DNA from AdP-EGFP, a Δ1-deleted recombinant adenovirus that contains a unique *Pac*I site, [15] onto 293 cells. Concentrated virus stock solutions were prepared by two $CsCl_2$ ultracentrifugation gradients and stored in storage buffer (10 mmol/l Tris–HCl, pH 7.4, 1 mmol/l $MgCl_2$, 10% glycerol). Δ1-deleted adenoviral vectors containing no transgene (AdNull) or green fluorescent protein (AdGFP) or LacZ (AdLacZ) reporter genes driven by a CMV promoter were used as control or reporter vectors. Transducing titers of viral stock solutions ranged from 6×10^{10} to 2×10^{11} plaque-forming units (PFU)/ml by plaque titration assay (virus particles/PFU ratios ≈4–10).

2.2. Detection of IL-1R2-Ig expression in vitro

IL-1R2-Ig expression in HeLa cells preincubated with either AdIL-1R2-Ig or control adenovector was detected by Western blotting using both goat anti-mouse IgG (Fcγ fragment-specific) mAb (Jackson ImmunoResearch) and rat anti-mouse IL-1R2 mAb (BD PharMingen) as primary antibodies. In addition, IL-1R2-Ig expression was detected by flow cytometry using R-PE-conjugated anti-mouse IL-1R2 mAb.

2.3. Detection of IL-1R2-Ig functional activity in vitro

An *in vitro* functional assay was used to demonstrate specific IL-1 inhibition by IL-1R2-Ig. We took advantage of the fact that human endothelial cells (HUVECs) produce and release urokinase-type plasminogen activator (u-PA) upon stimulation with cytokines, including human and mouse IL-1β and TNF-α, and that they are easily transducible by adenoviral vectors. HUVECs were preincubated with either AdIL-1R2-Ig or control adenovirus for 24 h at a multiplicity of infection (MOI) of 200. Subsequently, they were stimulated with either recombinant mouse IL-1β or TNF-α at a concentration of 10 ng/ml during 6 h. Induced u-PA activity was measured by zymography in both cell extracts and supernatants. Specific inhibition of IL-1 activity was demonstrated as inhibition of the IL-1β, but not TNF-α-induced increase in u-PA activity in cell extracts and supernatants.

2.4. Gene transfer and heart transplantation

'Principles of Laboratory animal care' (NIH publication No. 86-23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) and institutional guidelines were followed for animal experimentation. Male Fischer (F344) and Lewis (LEW) rats (8–12 weeks old, purchased from IFFA CREDO, L'Arbresle, France) were used as heart donors and recipients, respectively. Gene transfer into the donor heart was performed by intracoronary instillation of vector containing solution immediately before transplantation. First, 500 μl phosphate-buffered saline (PBS without Ca^{++}/Mg^{++}; pH 7.4) was instilled slowly, in a retrograde manner, into the aortic root. The syringe was then changed and 600 μl virus (≈10^{10} PFU) containing solution was instilled slowly (2–3 min) into the coronary arteries. Heterotopic cardiac grafts were placed in the abdominal position of rats anesthetized by isoflurane inhalation. Separate groups of allograft recipients received a subtherapeutic regimen of cyclosporin A (CsA; 1.5 mg/kg/day IM; days-1 to 3), either alone or in conjunction with AdIL-1R2-Ig (≈10^{10} PFU). Graft

survival was monitored by daily abdominal palpation. Rejection, defined as total cessation of heart beating, was confirmed by direct graft examination.

2.5. Detection of IL-1R2-Ig expression in vivo

IL-1R2-Ig expression *in vivo* was determined by immunohistological staining of graft sections at 6 days, and by Western blot analysis of graft tissue extracts and sera at 3 and 15 days after transplantation ($n = 3$ each). IL-1R2-Ig protein expression was visualised by immunostaining using goat anti-mouse IgG (Fcγ fragment-specific) mAb, followed by biotin-conjugated anti-goat IgG (Jackson Immuno Research Lab.), StreptABComplex/HRP, and Nickel-DAB/H_2O_2 (Dako).

2.6. Immunohistology for inflammatory cells

Immunohistology was performed on cryostat sections of hearts ($n = 4$/group) harvested on day 6 post-transplantation. This time point was chosen for analysis because marked inflammatory cell infiltrates are present (histological rejection), while massive infiltration and tissue destruction (clinical rejection) have not occurred yet. Rats were sacrificed by lethal pentobarbital injection, perfused with ice-cold PBS, and cardiac grafts were immediately frozen in OCT-compound. Four series of 8-μm cross-sections per heart were cut at 500-μm steps perpendicular to the long axis from the cardiac apex to the basis. Immunostaining of inflammatory cells infiltrating the graft was performed with the following primary mouse mAbs: anti-ED1-like (1C7; PharMingen) that recognizes monocytes/macrophages; anti-T cell receptor (TCR)$\alpha\beta$ (R73; PharMingen) that recognizes T lymphocytes bearing T cell receptor$\alpha\beta$; anti-CD8α (OX-8; PharMingen) that recognizes CD8α^+ cells; and anti-CD4 (W3/25; Accurate Chemicals) that recognizes CD4$^+$ T lymphocytes and macrophages; irrelevant mouse Ab (MOPC-31C; PharMingen) was used 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 using a Hyper-HAD-Axioscop microscope and an Axiocam-MRcD system (Zeiss). Morphometric analysis was performed in eight representative microscopic fields (100×) per heart section using the NIH-Image-1.62 program, as described [16]. Inflammatory cell infiltrates were expressed as myocardial surface areas staining positive with the different immunohistochemical markers normalized for the corresponding total myocardial surface areas.

2.7. Quantitative RT-PCR

PBS-perfused hearts ($n = 4$/group) were excised on day 6 post-transplantation, and ventricular myocardial tissue (≈300 mg) was placed in ice-cold PBS, cut into thin slices, and submerged into RNAlater Stabilization Reagent. Total RNA was extracted using the Qiagen RNeasy midi kit. DNase-treated RNA was utilized to generate cDNA by 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. Hypoxanthine phosphoribosyltransferase (HPRT) was selected as an endogenous control to correct for potential variation in RNA loading or efficiency of the amplification reaction. Primer sequences for rat cytokines were as follows: interferon-γ (IFN-γ), 5′-tcatggccctctctggctgttac-3′ (sense) and 5′-caagaggaggctctttccttccatag-3′ (antisense); interleukin-1β (IL-1β), 5′-cttcaaatctcacagcagcatctcg-3′ (sense) and 5′-tccacgggcaagacataggtagc-3′ (antisense); tumor necrosis factor-α (TNF-α), 5′-ctgtgcctcagcctcttctcattc-3′ (sense) and 5′-ttgggaacttctcctccttgttgg-3′ (antisense); transforming growth factor-β (TGF-β), 5′-ctaatggtggaccgcaacaacg-3′ (sense) and 5′-tctggcactgcttcccgaatg-3′ (antisense);

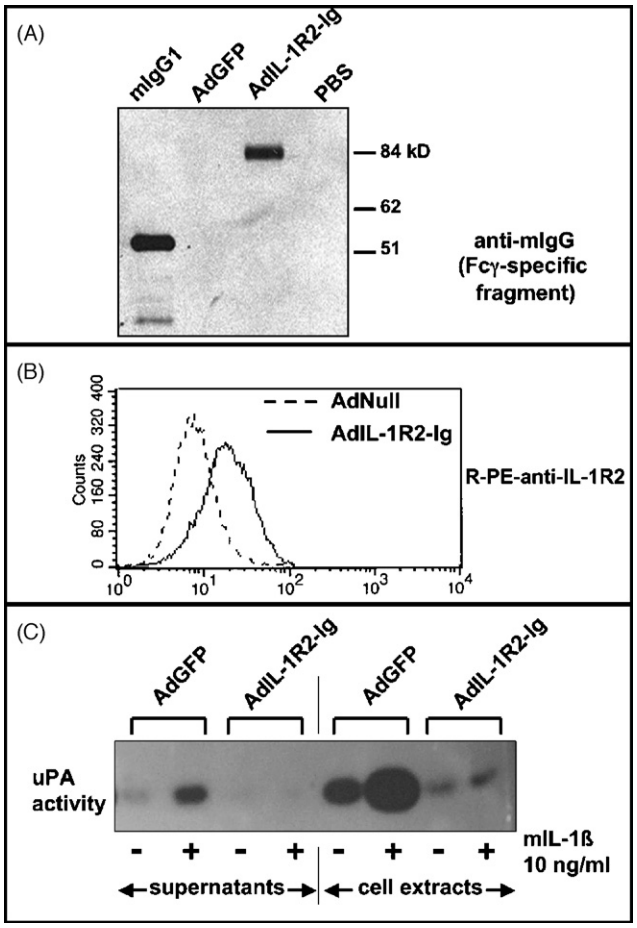

Fig. 1. IL-1R2-Ig protein expression and functional activity *in vitro*. (A) Western blot using anti-mIgG (Fcγ fragment-specific) mAb. Left to right: Purified mIgG1 (positive control); AdGFP-infected HeLa cells (MOI ≈80; adenoviral control); AdIL-1R2-Ig-infected HeLa cells (MOI ≈80); PBS (HeLa plus control solution with no virus). Predicted IL-1R2-Ig size ≈80 kDa. Comparison of Western blots under denaturing and nondenaturing conditions (data not shown) demonstrated the predicted IL-1R2-Ig dimerization. (B) Flow cytometry using R-PE-conjugated anti-IL-1R2 mAb. IL-1R2-Ig expression is evidenced as a rightward shift of the curve for AdIL-1R2-Ig (full line) versus the AdNull control (dotted line). (C) Zymography for analysis of uPA activity in supernatants and cell extracts of HUVECs pre-incubated with either AdIL-1R2-Ig or AdGFP control vector (MOI ≈200) 24 h before stimulation with 10 ng/ml mIL-1β (6 h). AdIL-1R2-Ig prevents increases in uPA activity in response to IL-1β both in supernatants and in cell extracts. uPA release also was stimulated by purified TNF-α (data not shown), but it was not inhibited by preincubation with AdIL-1R2-Ig (not shown).

RANTES, 5′-ccatatggctgcgacaccactc-3′ (sense) and 5′-gcacacttggcggttcctt-3′ (antisense); HPRT, 5′-ctcatggactgattatggacaggactg-3′ (sense) and 5′-cagcgctttaatgtaatccagcaggtc-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 expression is shown as ΔC_T (=C_T of target gene minus C_T of HPRT) [16].

2.8. Statistical analysis

Graft survival is shown as mean (±SD) survival time and Kaplan–Meier cumulative survival curves. Statistical analysis of differences in graft survival between treated and control groups was performed by product-limit (Kaplan–Meier) survival estimates and Log-Rank test (JMP program, version 5; SAS Institute). Immunohistological and RT-PCR data are shown as median values (range). Statistical analysis of differences in inflammatory cell infiltrates and cytokine mRNA expression between treated and control groups was performed by Mann–Whitney *U*-test. A p-value < 0.05 was considered to be statistically significant.

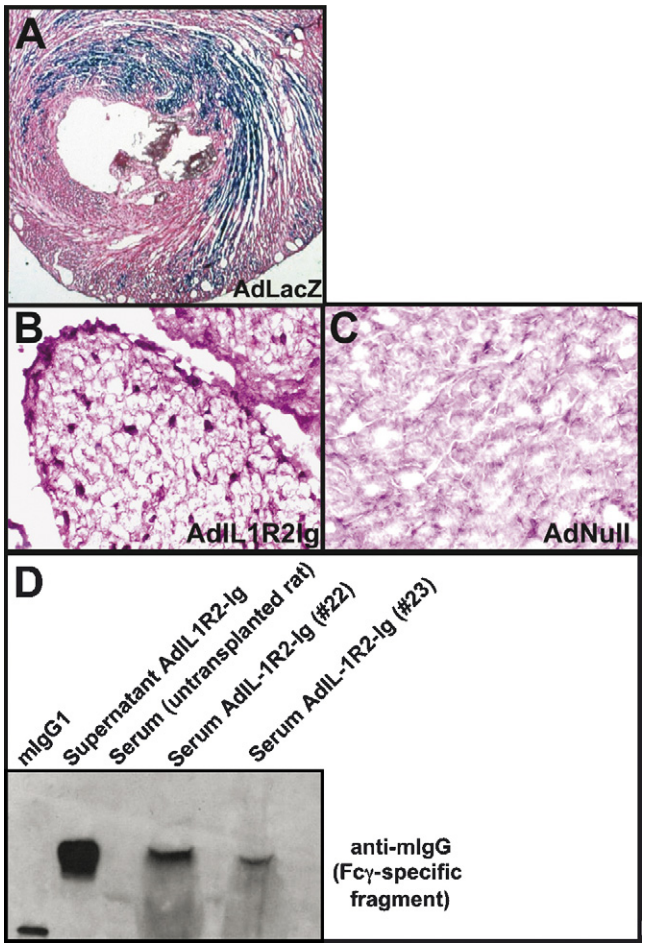

Fig. 2. IL-1R2-Ig protein expression *in vivo*. (A) X-gal histochemical staining of a transplanted heart section transduced with AdLacZ. Myocardial areas expressing the LacZ gene stain blue. (B) Immunohistochemical staining of a transplanted heart section transduced with AdIL-1R2-Ig, using anti-mIgG (Fcγ fragment-specific) mAb as the primary antibody (high magnification). (C) Immunohistochemical staining for mIgG (Fcγ fragment-specific); AdNull (control). (D) Western blot analysis of *in vivo* IL-1R2-Ig expression. Left to right: Purified mIgG1 (positive control); supernatant of AdIL-1R2-Ig-infected cells *in vitro* (positive control); rat serum, untransplanted (negative control); sera from rats transplanted with AdIL-1R2-Ig-transduced grafts (samples: #22 and #23; 3 days after transplantation). Same-size bands are present in the supernatant of AdIL-1R2-Ig-infected cells and sera of heart-transplanted rats in the AdIL-1R2-Ig group.

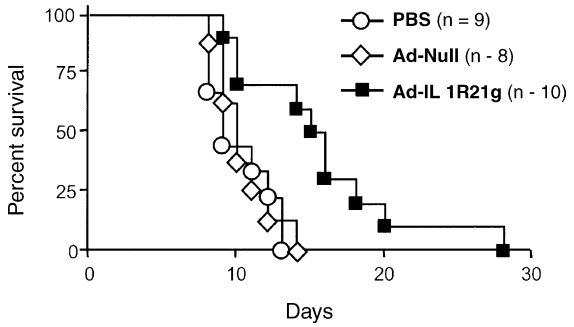

Fig. 3. Survival curves of cardiac allografts receiving AdIL-1R2-Ig, AdNull, or virus dilution solution alone (PBS; $p < 0.01$ for AdIL-1R2-Ig vs both AdNull and PBS).

3. Results

3.1. IL-1R2-Ig protein expression and functional activity in vitro

IL-1R2-Ig protein expression *in vitro* was demonstrated by both Western blot (Fig. 1A) and flow cytometry in HeLa cells preincubated with AdIL-1R2-Ig, but not in those preincubated with control adenovector (Fig. 1B). IL-1 inhibitory activity of HUVECs transduced with AdIL-1R2-Ig was evidenced by a decrease in uPA activity in the supernatant in response to IL-1β stimulation (Fig. 1C), indicating IL-1 inhibitory activity. IL-1β-stimulated uPA release in HUVECs preincubated with control adenovirus was not inhibited. TNF-α-stimulated uPA release was unaffected in HUVECs preincubated with AdIL-1R2-Ig (data not shown), indicating that IL-1R2-Ig specifically inhibited IL-1β but not TNF-α.

3.2. Gene transfer efficiency and IL-1R2-Ig protein expression in vivo

Perioperative and postoperative mortality was ≈1–2% each; significant morbidity was not observed. Preliminary studies aimed at establishing the method of gene transfer were performed in cardiac isografts (LEW/LEW) using two different doses of the AdLacZ reporter vector, 10^{10} and 10^9 PFU per heart. The higher virus dose appeared to be more efficient (Fig. 2A) and was used in subsequent experiments. Cardiomyocytes were the predominant (>90%) cell type expressing the transgene. Substantial numbers of β-gal-positive cells were still detectable 40 days after transplantation, with a ≈2.5-fold decrease as compared with day 5 [17]. IL-1R2-Ig expression in cardiac allografts was detectable by immunostaining at day 6 (Fig. 2B), and by Western blotting at day 3 (data not shown; the protein band was faint). IL-1R2-Ig was detectable in sera of rats transplanted with grafts preincubated with AdIL-1R2-Ig at day 3 (Fig. 2D). Transgene expression in grafts and sera was undetectable at day 15.

3.3. IL-1R2-Ig gene transfer and graft survival

Allograft rejection was evaluated in the F344 to LEW strain combination. Mean survival time (MST) of cardiac allografts was comparable in hearts receiving AdNull ($n = 8$) or virus dilution buffer alone ($n = 9$): 10.3 ± 2.5 days and 10.1 ± 2.1 days, respectively (Fig. 3). Survival of allografts treated with AdIL-1R2-Ig ($n = 10$) was significantly prolonged to 15.6 ± 5.7 days ($p < 0.01$ vs either control group). AdIL-1R2-Ig combined with a subtherapeutic CsA regimen ($n = 5$; one additional rat in this group died during surgery) prolonged graft survival to 19.4 ± 3.0 days, as compared with 15.9 ± 1.8 days for CyA alone ($n = 8$; $p = 0.04$). Cardiac isografts (LEW/LEW) receiving virus dilution buffer alone survived for >150 days.

3.4. Intragraft inflammatory cell infiltrates

Immunohistochemical analysis showed decreased intragraft inflammatory cell infiltrates 6 days after transplantation and IL-1R2-Ig gene transfer, as compared with the AdNull group (Fig. 4). Specifically, $CD4^+$ T lymphocytes and macrophages infiltrating the graft were reduced by 60% ($p < 0.05$) in the IL-1R2-Ig gene transfer group versus controls. In addition, there were nonsignificant trends toward decreases in the number of $CD8^+$ cytotoxic T lymphocytes and NK cell (by 40%), as well as in T lymphocytes bearing the TCRαβ receptor (by 28%) in the AdIL-1R2-Ig versus AdNull group. These results reflect attenuated recruitment of $CD4^+$ T cells and monocytes/macrophages to grafts transduced with the IL-1R2-Ig gene.

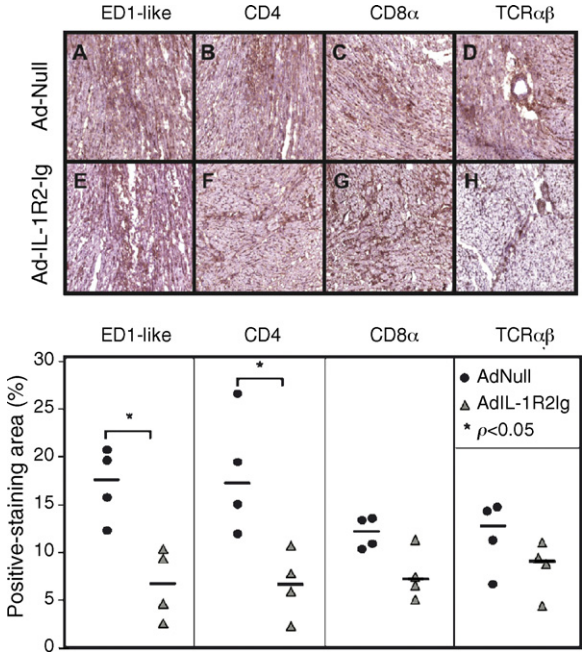

Fig. 4. Immunohistological analysis of graft infiltration by inflammatory cells. Upper panel: Photomicrographs of heart sections immunostained for the indicated inflammatory cell markers. (A—D) AdNull; (E—H) AdIL-1R2-Ig. Lower panel: Quantitative analysis of cell infiltrates staining positive for the indicated inflammatory markers. Horizontal bars are median values.

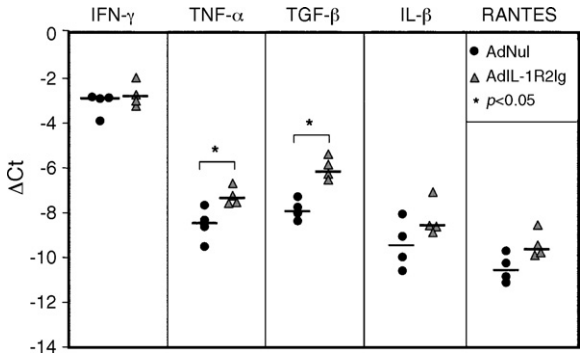

Fig. 5. Real-time RT-PCR analysis of cytokine mRNA expression in cardiac grafts. Data are shown as ΔC_T (=C_T of target gene minus C_T of HPRT). Lower values indicate higher mRNA expression. Horizontal bars are median values.

3.5. Intragraft cytokine mRNA expression

Real-time RT-PCR analysis showed decreased intragraft cytokine transcripts for TNF-α and TGF-β ($p < 0.05$). Nonsignificant trends ($p = 0.083$) toward decreased IL-1β and RANTES mRNA expression were also observed in the AdIL-1R2-Ig as compared with the AdNull group (Fig. 5). These results suggest attenuated intragraft expression of these cytokines after IL-1R2-Ig gene transfer.

4. Discussion

IL-1α and-β are upregulated early after graft reperfusion [1,4]. Here we show that expression and secretion of an IL-1R2-Ig fusion protein potently and specifically blocks IL-1β activities *in vitro* and attenuates acute cardiac allograft rejection in a rat model *in vivo*. The beneficial effect of localized IL-1R2-Ig expression *in vivo* was manifested by decreased numbers of macrophages and $CD4^+$ cells infiltrating the grafts, decreased intragraft TNF-α expression, and prolonged allograft survival. IL-1R2-Ig gene transfer combined with a subtherapeutic regimen of CsA was modestly yet significantly superior to CyA alone with respect to graft survival, indicating an additive (but no synergistic) effect of the two treatments in conjunction.

These findings are in good agreement with previous data using different IL-1 inhibitors, such as recombinant IL-1Ra, synthetic inhibitors, and neutralizing antibodies. Shiraishi et al. [18] showed that recombinant IL-1Ra enhances CsA immunosuppression and prolongs cardiac allograft survival in the DA to BN rat model (MST: 8.2 ± 0.7 days vs 5.3 ± 1.4 days in controls). Saadi et al. [19] reported that IL-1Ra reverses expression of a set of genes that are upregulated in the early steps of acute vascular rejection in a guinea pig-to-rat heart xenotransplantation model, suggesting a central role for IL-1 in acute vascular rejection. Sano et al. [20] showed that FR167653, a synthetic inhibitor of both IL-1 and TNF-α, prolongs cardiac allograft survival in the BN to LEW rat model (MST: 12.1 ± 1.5 days vs 6.8 ± 0.3 days in controls). Gerber et al. [21] reported that microspheres containing both IL-1 and TNF-α neutralizing antibodies confer protection upon mouse cardiac allografts. Finally, Suzuki et al. [22] reported attenuated ischemia—reperfusion injury after IL-1Ra gene

transfer in rat hearts; however, this approach was not tested in allografts.

We have designed a novel IL-1 inhibitor that consists of a soluble IL-1R2-Ig fusion protein. In nature, a competition exists between the signaling IL-1 receptor, IL-1R1, and the nonsignaling receptor, IL-1R2, for the same ligand. IL-1R2 binds IL-1β tightly, thus preventing binding to IL-1R1 and receptor activation. Penton Rol et al. [23] showed that exogenous expression of physiological numbers of surface IL-1R2 molecules dampens IL-1 responses in myelomonocytic cells *in vitro*. Soluble IL-1R2 binds and blocks processing of the IL-1β precursor [14]. Physiologically, circulating soluble IL-1R2 molecules contribute to the tight regulation of IL-1 cytokine cascades in humans, thereby preventing unnecessary or exaggerated activation of this potent proinflammatory pathway. Our *in vitro* data indicate that overexpression of a soluble IL-1R2-Ig fusion protein in human endothelial cells selectively inhibits IL-1β-stimulated uPA induction.

In the transplantation model, IL-1R2-Ig expression in the donor heart reduced the number of infiltrating macrophages, $CD4^+$ T cells and, to a lesser extent, $CD8^+$ cells. Several mechanisms could potentially account for these findings, including decreases in IL-1-mediated endothelial cell activation, [6] in macrophage and Tcell stimulation, [6,7] as well as in dendritic cell maturation [8]. Previous *in vitro* data indicate that activation of endothelial cells by complement is crucially dependent on IL-1α, leading to upregulation of IL-1α and IL-1β [24]. As mentioned above, local overexpression of IL-1Ra has been associated with cardioprotection from ischemia—reperfusion injury [22]. In the present study, IL-1R2-Ig gene transfer was associated with decreased intragraft cytokine transcripts for TNF-α and TGF-β as well as, marginally, for IL-1β and RANTES. Downregulation of TNF-α and IL-1β may be explained by inhibition of self-maintaining feedback loops by which the two cytokines stimulate each other's production and secretion [6]. The RANTES chemokine has been implicated in early migration and activation of macrophages and CD45RO T lymphocytes in transplanted hearts [25].

Transgene expression was short-lived in cardiac allografts, presumably due to alloimmune responses leading to the death of transgene-expressing cells (predominantly myocytes). The intrinsically transient gene expression with adenoviral vectors may have contributed to the rapid loss of transgene expression; however, acute rejection appears to be the primary cause for this, since we have observed adenoviral LacZ expression over more than 40 days in cardiac grafts placed in syngeneic recipients.

In this proof-of-principle study, an adenoviral vector was employed to evaluate the efficacy of a novel IL-1 inhibitory molecule in a heart transplantation model. Regarding future clinical applications, gene therapy offers potential advantages, such as local expression of the protective factor by the graft itself, which reduces the risk of systemic effects. Furthermore, gene therapy could obviate the need for daily injections of a recombinant peptide, which would be required due to the short half-lives of many peptides *in vivo*, over extended periods of time. However, a number of issues still need to be solved before gene therapy can be used in clinical transplantation. Gene transfer protocols adapted to the clinical setting, flawless vectors devoid of proinflammatory effects, and reliable molecular systems that permit to regulate transgene expression levels *in vivo* ('gene dosage') are required.

Collectively, our findings indicate that IL-1R2-Ig gene transfer mitigates acute rejection of cardiac allografts, even though it ultimately fails to prevent it. Incomplete graft protection might be due to methodological limitations, such as suboptimal gene transfer efficiency and proinflammatory effects of adenoviral vectors used in the present study. Alternatively, they may be intrinsically related to the functional redundancy of molecular cascades of proinflammatory cytokines, whereby blocking a single cytokine pathway delays graft rejection, but ultimately fails to prevent it. Because IL-1 inhibition interferes with early alloantigen-independent immune responses in the context of ischemia—reperfusion injury, which are not directly targeted by current immunosuppressive agents, this approach may be potentially useful as an adjunctive therapy in heart transplantation.

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