rats fed a high-sodium diet ($p < 0.001$, Figure 2). After 13 weeks of the gene delivery, serum IL-10 concentrations significantly increased in the IL-10-transduced rats compared to the normal untreated rats or control EGFP-transduced rats (986.6 ± 278.5 pg/ml versus <3 or 20.8 ± 18.1 pg/ml, $p < 0.001$, respectively; Figure 3). At this time point, the EGFP transduction generated a slight but significant increase of endogenous IL-10 levels compared to control ($p < 0.01$).

Anti-hypertensive effects of IL-10

SBP gradually increased in the EGFP group, resulting in levels of 184 ± 7 mmHg at 15 weeks of age (Figure 4). At 9 weeks (i.e. after 4 weeks of the vector injection), SBP in the IL-10 group (151 ± 7 mmHg) was significantly lower

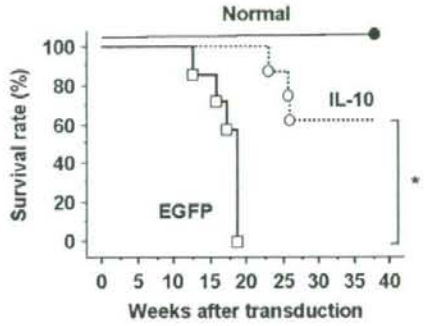

Figure 2. The pro-survival effects of IL-10 in DS rats. The 5-week-old rats were intramuscularly injected with AAV1-IL-10 or AAV1-EGFP at 1×10^{11} g.c./body. Kaplan–Meier survival analysis was performed. Closed circle, normal group; open circles, IL-10 group; open squares, EGFP group ($n = 8$ each). *$p < 0.001$ versus EGFP group

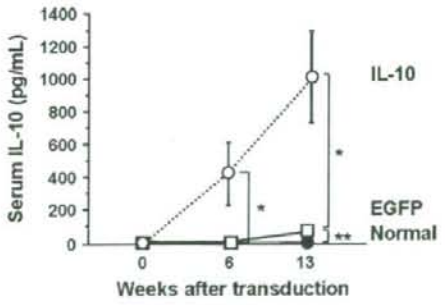

Figure 3. AAV vector-mediated systemic IL-10 expression in DS rats. AAV1-EGFP or AAV1-IL-10, at 1×10^{12} g.c./body, respectively, was injected bilaterally into the anterior tibial muscles of the 5-week-old rats. Serum IL-10 levels were determined periodically by ELISA. The normal group includes DS rats fed a low-sodium diet and not administered the vector injection. The results are presented as means ± SD ($n = 10$ each). *$p < 0.001$, **$p < 0.01$

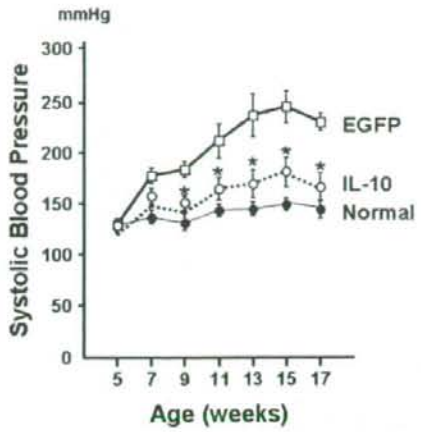

Figure 4. The anti-hypertensive effect of IL-10. Longitudinal tracing of systolic blood pressure evaluated by the tail-cuff method after injecting the AAV vectors in 5-week-old DS rats. Open squares, EGFP group; open circles, IL-10 group; closed circles, normal group ($n = 10$ each). The results are presented as means ± SD. *$p < 0.001$ versus EGFP group

Table 1. Effects of IL-10 on left ventricular hypertrophy and function

	RWT (mm)		%FS (%)	
Age (weeks)	5	11	5	18
Normal	0.46 ± 0.03	0.48 ± 0.02	58.7 ± 3.7	57.2 ± 3.9
EGFP	0.45 ± 0.03	0.63 ± 0.04*	59.8 ± 1.9	32.9 ± 4.4*
IL-10	0.45 ± 0.04	0.49 ± 0.02**	59.4 ± 2.6	59.2 ± 4.6**

M-mode echocardiograms of the LV at the papillary muscle level were traced for analysis. RWT of the LV as an index of LV hypertrophy and %FS as an index of systolic LV function were calculated as described in the Materials and methods. The results are presented as means ± SD ($n = 10$ each). *$p < 0.0001$ versus Normal group, **$p < 0.0001$ versus EGFP group at the same time-point, respectively.

than that in the EGFP group ($p < 0.0001$). The anti-hypertensive effect of IL-10 persisted until the animals were sacrificed at 18 weeks of age.

Effects of IL-10 on left ventricular hypertrophy, function and CHF

Echocardiography exhibited a 22.0% reduction in the RWT of the LV posterior wall at 11 weeks of age ($p < 0.0001$) and a 26.3% improvement in %FS of the LV wall at 18 weeks of age ($p < 0.0001$) in the IL-10 group compared to the EGFP group (Table 1). As compared to EGFP expression, IL-10 expression caused a 21.7% or 52.7% decrease in the heart or lung weight/body weight index, respectively (all $p < 0.05$; Figures 5a and 5b). Similarly, the cardiac ANP mRNA level significantly increased in the EGFP group compared to the control (46.5 ± 23.8-fold); whereas, IL-10 transduction significantly suppressed this increase (9.28 ± 5.2-fold) compared to control (Figure 5c).

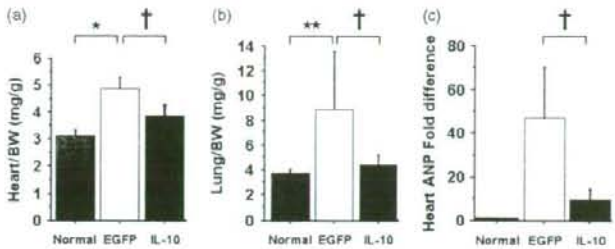

Figure 5. Effects of IL-10 on congestive heart failure. The hearts (a) and lungs (b) of DS rats were harvested and weighed at 18 weeks of age. Data were expressed after normalization using body weight. The cardiac ANP mRNA levels determined by real-time RT-PCR (c). The total RNA was extracted from the heart at 18 weeks of age. The mRNA levels were corrected by using the GAPDH mRNA level of each animal and then normalized to the mean value of the normal group. The results are presented as means ± SD ($n = 10$ each). *$p < 0.01$ versus Normal group, **$p < 0.05$ versus Normal group, †$p < 0.05$ versus EGFP

Effects of IL-10 on pathological cardiac remodelling

H&E staining demonstrated increased interstitial and perivascular cell infiltration in the failing heart of the EGFP-transduced rats (Figure 6a). Azan–Mallory staining demonstrated that interstitial and perivascular fibrosis increased in the EGFP group (Figure 6b). IL-10 transduction inhibited fibrosis and significantly decreased the cardiac TGF-β_1 levels in DS rats compared to the EGFP transduction (64.5 ± 45.3 pg/mg protein versus 197.1 ± 91.9 pg/mg protein, $p < 0.05$; Figure 6c).

Effects of IL-10 on renal function

Compared to control rats, DS rats fed a high-sodium diet exhibited a 68.0% increase in serum creatinine, a 243.0% increase in urine protein levels, and a 49.9% decrease in glomerular filtration rate (all $p < 0.05$; Figure 7). Sustained IL-10 expression reduced these changes by 88.2%, 100% and 45.8%, respectively (all $p < 0.05$).

Discussion

The present study demonstrates that systemic IL-10 expression via the AAV serotype 1 vector prevented the progression of hypertension, CHF and renal dysfunction in DS rats. A single intramuscular injection of AAV1-IL-10 achieved long-term systemic IL-10 expression, leading to the prolonged survival of the rats. The IL-10 transduction not only preserved systolic LV function, but also reduced fibrosis of the LV at the heart failure phase. The anti-hypertensive effect of IL-10 occurred prior to the

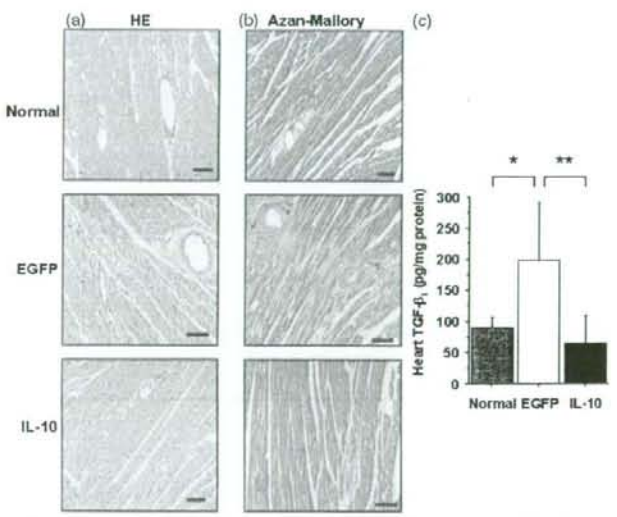

Figure 6. Histopathology and cardiac TGF-β_1 levels of the 18-week-old DS rats. (a) Representative micrographs of the H&E staining. (b) Representative micrographs of Azan–Mallory staining. Magnification, ×200; scale bar = 100 μm. (c) TGF-β_1 concentrations in the heart homogenates determined by ELISA. The results are presented as means ± SD ($n = 10$ each). *$p < 0.05$ versus Normal group, **$p < 0.05$ versus EGFP group

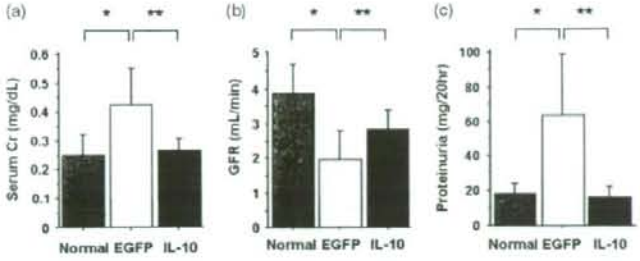

Figure 7. Effects of IL-10 on renal function in DS rats. (a) Serum creatinine (Cr), (b) glomerular filtration rate (GFR) and (c) urine protein levels were determined at 18 weeks of age. The results are presented as means ± SD ($n = 10$ each). *$p < 0.001$ versus Normal group, **$p < 0.05$ versus EGFP group

development of CHF and LVH, suggesting that this effect may largely contribute to amelioration of sodium-induced hypertensive organ damage.

Many studies have suggested the therapeutic potentials of IL-10 for CHF. Serum IL-10 levels decrease in CHF patients [19], and exogenous IL-10 administration retards progression of the disease in many cardiovascular disease models [20]. However, these studies used CHF models in which CHF was a result of acute viral or autoimmune myocarditis, and they examined the short-term IL-10 effects against initial inflammatory responses [11,12]. In the present study, we demonstrated the effects of long-term IL-10 expression against chronic CHF progression, hypertension and inflammatory changes of the cardiac tissue.

We detected a slight but significant increase of endogenous IL-10 levels in the heart failure phase in control DS rats. However, this increase was insufficient to cause beneficial effects. On the other hand, conventional IL-10 therapies based on recombinant drugs or plasmids require frequent administration for sufficient and sustained IL-10 expression. Thus, we used AAV vectors that permit long-term transgene expression *in vivo* [14]. Previously, we demonstrated that a single intramuscular injection of the AAV5-based vector caused systemic IL-10 expression for 1 year [21]. Since AAV1 is more efficient for muscle transduction than AAV2 or AAV5 [22], we used AAV1 as the vector in the present study [23].

A clinical trial using infliximab, a chimeric monoclonal antibody to TNF-α, failed to prolong the survival of CHF patients over the long term [5]. We speculate that the failure might be in part based on an insufficient regulation of the cytokine network, which may be involved in the progression of CHF and other related diseases such as hypertension and renal failure. Recent studies have shown the marked anti-hypertensive effects of an immunosuppressant mycophenolate mofetil (MMF) in DS rats [3,4]. MMF administration also ameliorates renal dysfunction via anti-inflammatory effects. Interestingly, an intramuscular injection of AAV1-IL-10 successfully ameliorated renal function in a rat model after nephrectomy [24]. We also observed that systemic IL-10 expression significantly attenuated hypertension and renal dysfunction, along with a decrease of inflammatory cell infiltration, in the kidney of stroke-prone spontaneously hypertensive rats (T. Nomoto *et al.*, unpublished data). In the present study, we demonstrate that IL-10 gene therapy successfully ameliorated heart failure and renal dysfunction along with a suppression of severe hypertension in DS rats. These observations suggest that anti-inflammatory action of IL-10 may attenuate the target organ damage related to high blood pressure. However, precise mechanism underlying the anti-hypertensive effect of IL-10 require further investigation.

The synthesis of ANP, a cardioprotective hormone predominantly produced by the ventricle, as well as its circulating levels, increases in accordance with the severity of CHF [25,26]. Administration of exogenous ANP ameliorates CHF in clinical settings via its diuretic and vasodilatory effects. In the present study, the cardiac ANP mRNA level significantly decreased in the IL-10 group. These observations suggest that IL-10 ameliorated CHF independently of direct ANP production but inhibited the adaptive increase in ANP levels.

The present study demonstrates that IL-10 expression attenuated pathological cardiac remodelling with reduced expression of TGF-β_1, a hallmark of cardiac fibrosis in DS rats [27]. Expression of monocyte chemoattractant protein (MCP)-1 in the endothelium of intramyocardial arterioles triggers perivascular macrophage accumulation [28]. Macrophage infiltration induces TGF-β_1 production, leading to fibroblast proliferation and extracellular matrix production [29]. Interestingly, a neutralizing antibody against TGF-β inhibits fibroblast activation, resulting in reduced collagen production and subsequent myocardial fibrosis [30]. Previously, we reported that systemic IL-10 expression significantly decreased serum MCP-1 levels, perivascular macrophage infiltration, and pulmonary tissue TGF-β_1 levels *in vivo* [10,21]. These observations suggest that the reduced macrophage-derived TGF-β_1 expression following MCP-1 suppression might be responsible for the anti-remodelling effects of IL-10. However, the direct effects of IL-10 on TGF-β_1 in the pathogenesis of CHF remain unclear.

Epidemiological studies have demonstrated that the increased pro-inflammatory cytokine expression is related to the incidence of pre-hypertension [31]. These results

suggest a possible link between the inflammatory response and the development of hypertension. This is the first study to demonstrate the anti-hypertensive effects of IL-10, which might be a key molecule to explain this relationship. Exploring the mechanisms underlying the effects of IL-10 would provide new molecular targets for refractory hypertension and its sequalae.

In conclusion, the sustained IL-10 expression achieved by the single AAV-IL-10 injection ameliorated CHF and prolonged survival in DS rats. IL-10 expression attenuated salt-sensitive hypertension, LV remodelling and renal dysfunction. These results suggest that our IL-10-based strategy potentially prevents the progression of refractory hypertensive organ damage in humans.

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References

1. Levine B, Kalman J, Mayer L, *et al*. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* 1990; **323**: 236–241.
2. Vasan RS, Sullivan LM, Roubenoff R, *et al*. Inflammatory markers and risk of heart failure in elderly subjects without prior myocardial infarction: the Framingham Heart Study. *Circulation* 2003; **107**: 1486–1491.
3. Mattson DL, James L, Berdan EA, *et al*. Immune suppression attenuates hypertension and renal disease in the Dahl salt-sensitive rat. *Hypertension* 2006; **48**: 149–156.
4. Tian N, Gu JW, Jordan S, *et al*. Immune suppression prevents renal damage and dysfunction and reduces arterial pressure in salt-sensitive hypertension. *Am J Physiol Heart Circ Physiol* 2007; **292**: H1018–H1025.
5. Chung ES, Packer M, Lo KH, *et al*. Randomized, double-blind, placebo-controlled, pilot trial of infliximab, a chimeric monoclonal antibody to tumor necrosis factor-alpha, in patients with moderate-to-severe heart failure: results of the anti-TNF Therapy Against Congestive Heart Failure (ATTACH) trial. *Circulation* 2003; **107**: 3133–3140.
6. Mann DL, McMurray JJ, Packer M, *et al*. Targeted anticytokine therapy in patients with chronic heart failure: results of the Randomized Etanercept Worldwide Evaluation (RENEWAL). *Circulation* 2004; **109**: 1594–1602.
7. Elenkov IJ, Chrousos GP. Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann NY Acad Sci* 2002; **966**: 290–303.
8. Cattaruzza M, Slodowski W, Stojakovic M, *et al*. Interleukin-10 induction of nitric-oxide synthase expression attenuates CD40-mediated interleukin-12 synthesis in human endothelial cells. *J Biol Chem* 2003; **278**: 37874–37880.
9. Selzman CH, McIntyre RC, Jr., Shames BD, *et al*. Interleukin-10 inhibits human vascular smooth muscle proliferation. *J Mol Cell Cardiol* 1998; **30**: 889–896.
10. Ito T, Okada T, Miyashita H, *et al*. Interleukin-10 expression mediated by an adeno-associated virus vector prevents monocrotaline-induced pulmonary arterial hypertension in rats. *Circ Res* 2007; **101**: 734–741.
11. Nishio R, Matsumori A, Shioi T, *et al*. Treatment of experimental viral myocarditis with interleukin-10. *Circulation* 1999; **100**: 1102–1108.
12. Palaniyandi SS, Watanabe K, Ma M, *et al*. Inhibition of mast cells by interleukin-10 gene transfer contributes to protection against acute myocarditis in rats. *Eur J Immunol* 2004; **34**: 3508–3515.
13. Kihara Y, Sasayama S. Transition from compensatory hypertrophy to dilated failing left ventricle in Dahl-Iwai salt-sensitive rats. *Am J Hypertens* 1997; **10**: 78S–82S.
14. Okada T, Shimazaki K, Nomoto T, *et al*. Adeno-associated viral vector-mediated gene therapy of ischemia-induced neuronal death. *Methods Enzymol* 2002; **346**: 378–393.
15. Matsushita T, Elliger S, Elliger C, *et al*. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* 1998; **5**: 938–945.
16. Okada T, Nomoto T, Yoshioka T, *et al*. Large-scale production of recombinant viruses by use of a large culture vessel with active gassing. *Hum Gene Ther* 2005; **16**: 1212–1218.
17. Nakahara T, Hashimoto K, Hirano M, *et al*. Acute and chronic effects of alcohol exposure on skeletal muscle c-myc, p53, and Bcl-2 mRNA expression. *Am J Physiol Endocrinol Metab* 2003; **285**: E1273–1281.
18. Ueno S, Ohki R, Hashimoto T, *et al*. DNA microarray analysis of in vivo progression mechanism of heart failure. *Biochem Biophys Res Commun* 2003; **307**: 771–777.
19. Stumpf C, Lehner C, Yilmaz A, *et al*. Decrease of serum levels of the anti-inflammatory cytokine interleukin-10 in patients with advanced chronic heart failure. *Clin Sci (Lond)* 2003; **105**: 45–50.
20. Ito T, Ikeda U. Inflammatory cytokines and cardiovascular disease. *Curr Drug Targets Inflamm Allergy* 2003; **2**: 257–265.
21. Yoshioka T, Okada T, Maeda Y, *et al*. Adeno-associated virus vector-mediated interleukin-10 gene transfer inhibits atherosclerosis in apolipoprotein E-deficient mice. *Gene Ther* 2004; **11**: 1772–1779.
22. Hauck B, Chen L, Xiao W. Generation and characterization of chimeric recombinant AAV vectors. *Mol Ther* 2003; **7**: 419–425.
23. Ito T, Okada T, Mimuro J, *et al*. Adenoassociated virus-mediated prostacyclin synthase expression prevents pulmonary arterial hypertension in rats. *Hypertension* 2007; **50**: 531–536.
24. Mu W, Ouyang X, Agarwal A, *et al*. IL-10 suppresses chemokines, inflammation, and fibrosis in a model of chronic renal disease. *J Am Soc Nephrol* 2005; **16**: 3651–3660.
25. Saito Y, Nakao K, Arai H, *et al*. Augmented expression of atrial natriuretic polypeptide gene in ventricle of human failing heart. *J Clin Invest* 1989; **83**: 298–305.
26. de Boer RA, Henning RH, Suurmeijer AJ, *et al*. Early expression of natriuretic peptides and SERCA in mild heart failure: association with severity of the disease. *Int J Cardiol* 2001; **78**: 5–12.
27. Ichihara S, Obata K, Yamada Y, *et al*. Attenuation of cardiac dysfunction by a PPAR-alpha agonist is associated with down-regulation of redox-regulated transcription factors. *J Mol Cell Cardiol* 2006; **41**: 318–329.
28. Kuwahara F, Kai H, Tokuda K, *et al*. Hypertensive myocardial fibrosis and diastolic dysfunction: another model of inflammation? *Hypertension* 2004; **43**: 739–745.
29. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994; **331**: 1286–1292.
30. Kuwahara F, Kai H, Tokuda K, *et al*. Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction, in pressure-overloaded rats. *Circulation* 2002; **106**: 130–135.
31. Chrysohoou C, Pitsavos C, Panagiotakos DB, *et al*. Association between prehypertension status and inflammatory markers related to atherosclerotic disease: the ATTICA Study. *Am J Hypertens* 2004; **17**: 568–573.