Comparison of IL-10 and MCP-1-7ND gene transfer with AAV9 vectors for protection from murine autoimmune myocarditis

Ziya Kaya1*, Christoph Leib1, Stanislas Werfel1, Stefan Göser1, Renate Öttl1, Barbara Leuchs2, Gabriele Pfitzer3, Hugo A. Katus1, and Oliver J. Müller1*

1Department of Internal Medicine III, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany; 2Applied Tumorvirology, German Cancer Research Center, Heidelberg, Germany; and 3Institute of Vegetative Physiology, University of Cologne, Cologne, Germany

Received 13 December 2010; revised 9 February 2011; accepted 23 February 2011; online publish-ahead-of-print 25 February 2011

Time for primary review: 27 days

Aims
Overexpression of therapeutic genes with potential disease-limiting effects, specifically at the site of inflammation, remains a major clinical challenge. In this study, we investigate the potential of adeno-associated virus (AAV)-9-mediated cardiac expression of the anti-inflammatory mediators interleukin (IL)-10 and a dominant-negative inhibitor of monocyte chemoattractant protein-1 (MCP1-7ND) on prevention of autoimmune myocarditis.

Methods and results
Autoimmune myocarditis was induced by immunizing A/J mice with subcutaneous injection of 120 μg cardiac Troponin I (cTnI) on Days 0, 7, and 14. Two weeks prior to initial immunization, each mouse received a single systemic dose of 10^{12} AAV9 vectors carrying the coding sequence of IL-10 or MCP1-7ND transcriptionally targeted to the heart. Mice were sacrificed 28 days after initial immunization for further analysis. Only expression of IL-10 resulted in a highly significant decrease in myocardial inflammation and fibrosis, as well as an increased ejection fraction compared with controls. Further analyses of cytokine profiles of cTnI-stimulated splenocytes from IL-10 and MCP1-7ND-treated mice revealed significant alterations compared with controls. In addition, transcript levels of chemokine receptor CCR4 and T-cell activation gene were significantly reduced in hearts of IL-10-treated mice as determined by quantitative real-time PCR.

Conclusion
Our study suggests that cardiac expression of IL-10 with AAV9 vectors is a promising therapeutic approach for autoimmune myocarditis.

Keywords
Myocarditis • Gene therapy • Troponin I • Adeno-associated virus

1. Introduction
Myocarditis, either due to myocardial infection or autoimmunity, leads to active inflammatory destruction of cardiomyocytes. Cytokine imbalance plays a major role in the progression of myocarditis.1 The type II cytokine interleukin-10 (IL-10) is known to exhibit potent anti-inflammatory and anti-immune activity.2 IL-10, originally identified by Moore et al.,3 is expressed by a variety of immune competent cells, like Th1–, Th2–, Th17–, CD8+ T-cells, monocytes, and some subsets of dendritic cells.4 In vivo administration of recombinant IL-10, either by electroporation or subcutaneously, has been shown to be protective in viral myocarditis, as shown by a milder disease outcome and decreased levels of pro-inflammatory mediators tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-2.4,5 Furthermore, we showed an important role for IL-10 in inducing nasal tolerance in a mouse model of experimental autoimmune myocarditis.6 In addition to these promising results in animal models, IL-10 has been used in clinical studies in patients with Crohn’s disease7,8 with only modest clinical improvements, possibly due to the reason that IL-10 was delivered systemically. In this context, a study of Cua et al.9 revealed that delivery of IL-10 to the murine central nervous system was protective in experimental autoimmune encephalitis, whereas systemic IL-10 delivery lacked this effect.

Monocyte chemo-attractant protein-1 (MCP-1), a member of the C-C chemokine family, is critically linked to various inflammatory...
2. Methods

2.1 Generation of AAV vectors

To enable expression with the cardiac CMV-MLC promoter, the open reading frames of murine IL-10 and MCP1-7ND using intravenous injections of AAV9 vectors protect from Troponin I induced murine experimental autoimmune myocarditis.

2.2 Experimental protocol and in vivo gene transfer

Female A/J mice (5–6 weeks of age) obtained from Harlan Winkelman GmbH (33176 Borchern, Germany) were maintained in the animal facility at the University of Heidelberg and used in all experiments. All procedures involving the use and care of animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and the German animal protection code. Approval was also granted by the local ethics review board (G-178/08).

For transfer of AAV9-IL-10, AAV9-MCP, AAV9-MCP1-7ND, and AAV-luc, each mouse received a systemic single dose of 10^{12} AAV9 vectors by intravenous injection via the tail vein 2 weeks prior to initial immunization. After 2 weeks, each mouse was immunized with a subcutaneous injection of a 100 µL emulsion containing 120 µg bacteriophage lambda (λ) gene of Mycobacterium tuberculosis H37Ra (Sigma, St Louis, MO, USA) on Days 0, 7, and 14. cTnI-immunized mice which received AAV9 vectors containing the luciferase gene (n = 10) were regarded as controls for IL-10 (n = 10) and for MCP1-7ND (n = 9). Sham-immunized mice (CFA with control buffer) without previous vector injection (n = 4) were used to determine the severity of the autoimmune myocarditis and the degree of amelioration by gene therapy. Immunized mice injected with AAV9-MCP1-7ND (n = 10) were an additional control for MCP1-7ND treatment. Twenty-eight days after first immunization or sham-immunization, mice were sacrificed by cervical dislocation.

2.3 Preparation of recombinant murine cTnl

The murine cTnl was expressed in Escherichio coli and purified as previously described. In addition to purification via ion exchange chromatography, cTnl was applied to a cardiac troponin C affinity column as the second purification step. Isolated cTnl-fractions were dialyzed extensively against 1 mM HCl, and then lyophilized, and stored at −80 °C.

2.4 Determination of cTnl auto-antibody titres

Serum blood samples from each mouse were obtained retro-orbitally under general anaesthesia monitored by ocular reflexes (single intra-peritoneal injection of ketamine 120 mg/kg body weight and xylazine 16 mg/kg body weight). To measure serum anti-peptide or troponin I titres, plates were coated with 100 µL/well of cTnl (5 µg/mL) in bicarbonate buffer (pH 9.6) and incubated overnight. Anti-mouse secondary antibody diluted to 1:5000 for IgG (Sigma) was used for detection. Serum samples from test mice were diluted to 1:800 followed by 1:4 dilution steps. Normal mouse serum was used as control. Optical densities were determined at 450 nm. Antibody endpoint titres for each individual mouse were calculated as the greatest positive dilution of antibody yielding a positive signal.

2.5 Determination of serum protein levels of mIL-10 and MCP1-7ND

To determine protein levels of IL-10 and MCP1-7ND, we collected serum blood samples at Days −14, −7, 0, 7, 14, 21, and 28. Serum blood samples were collected retro-orbitally under general anaesthesia (see section 2.4). Quantikine cytokine ELISA kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) were used according to the manufacturer’s guidelines to measure IL-10 and MCP1-7ND protein levels. Serum samples were diluted 1:10 for determination of MCP1-7ND and 1:2 for IL-10 protein levels.

2.6 Echocardiography

Transthoracic echocardiography was performed as described previously using anaesthesia with ketamine (120 mg/kg body weight) and xylazine (16 mg/kg body weight). The investigator who conducted the echocardiography was blinded to the treatment status.

2.7 Histopathological evaluation

For histopathological evaluation, mice were sacrificed on Day 28 after first immunization by cervical dislocation. Hearts were removed, fixed in formalin (4%), and subsequently embedded in paraffin. We performed serial paraffin heart sections of 5 µm of thickness. Every fifth section was taken for staining with haematoxylin and eosin (to determine the level of
inflammation) and Masson’s trichrome (to determine the level of fibrosis). For evaluation of inflammation or fibrosis, we analysed five sections per heart under light microscopy and rated the whole sections by the following scoring system: Grade 1, cardiac infiltration in up to 5% of the cardiac sections; Grade 2, infiltration in 6–10%; Grade 3, infiltration in 11–30%; Grade 4, infiltration in 31–50%; and Grade 5, infiltration in >50% of cardiac sections. Histopathological evaluation was conducted by two independent examiners who were blinded to the treatment status of the respective groups.

2.8 Quantitative PCR

Total RNA was extracted from heart, liver, and skeletal muscle by using RNeasy MidiKit (Qiagen, Hilden Germany) according to the manufacturer’s protocol. Quantitative PCR (qPCR) was carried out with 1 μg of cDNA. After an initial denaturation of 95°C for 30 s, 40 cycles were performed consisting of denaturation at 95°C for 5 s and annealing at 60°C for 10 s. Primer sequences used for quantification of transcript levels are shown in Supplementary material online, Table S1. Quantification was done using the SsoFast EvaGreen Kit (Biorad, München, Germany), according to the manufacturer’s instructions.

2.9 In vitro cTnI-specific cytokine secretion profile

To measure the in vitro cytokine production of cTnI-stimulated splenocytes, spleens of sacrificed mice were removed on Day 28. Splenocytes were cultured at 5 × 10^6/well in an RPMI 1640 complete medium with 30 μg of cTnI or a medium for the control group. After 48 h of incubation at 37°C (5% CO2) supernatants were collected, aliquoted, and stored at −20°C. Cytokine levels of IL-2, IFN-γ, IL-4, IL-6, IL-13, IL-17 IL-1β, and TNF-α were measured by Quantikine cytokine ELISA kits (R&D Systems, Wiesbaden-Nordenstadt, Germany), according to the manufacturer’s instructions.

2.10 Statistical analysis

As ordinal variables, the histological scores and antibody titres were evaluated by means of the Kruskal–Wallis and the Mann–Whitney U test for statistical analysis of the distribution profiles. As for the echocardiographic data and cytokine level, Student’s t-test for independent samples was used for comparison. The level of significance was set at P < 0.05. Analysis was performed with commercially available statistic software (SPSS 16.0).

3. Results

3.1 IL-10 and MCP1-7ND serum levels after AAV9-mediated gene transfer

Serum concentrations of IL-10 and MCP1-7ND were measured from Day −14 to Day 28 in an interval of 7 days by ELISA. We detected constantly high concentrations of the respective proteins in serum 1 week after gene transfer until the end of experiment on Day 28 (Figure 1).

3.2 Tissue specificity of IL-10 expression

To evaluate the expression of IL-10 mRNA in different tissues, we performed qPCR analyses in heart, liver, and skeletal muscle 42 days after an intravenous injection of a single dose of 10^{12} AAV-MLC260 genomic particles AAV9-IL-10. Although hepatic IL-10 expression was found to be almost four times lower than cardiac levels (see Supplementary material online, Figure S1), IL-10 from hepatic origin might also contribute to high serum concentrations.

3.3 Immunization and humoral immune response

To study the effect of immunization with cTnI on humoral immune response, we measured cTnI-specific total IgG autoantibody titres in serum on Day 28. Mice having received AAV9-IL-10 showed a significantly reduced cTnI-specific autoantibody titre compared with the control group (IL-10: 4.1 × 10^{-6} ± 0.9 × 10^{-6} vs. control: 1.6 × 10^{-7} ± 6.3 × 10^{-7}; P < 0.001; Figure 2A). No significant differences in total IgG autoantibody titres were found between AAV9-MCP1-7ND-treated mice and corresponding control groups (AAV9-luc; Figure 2B).

3.4 AAV9-mediated cardiac gene transfer of IL-10 and MCP1-7ND affects cytokine production

To determine the effect of immunization on cellular immune response, we measured cTnI-specific cytokine production by splenocytes on Day 28 after initial immunization. Immunized mice, which were treated with AAV9 vector containing the coding sequence of TNF-α, IL-2, IL-4, IL-6, IL-13, IL-17, and IFN-γ compared with the control group (TNF-α, IL-2, IL-4, IL-6, IL-17: P < 0.05; IL-13, IFN-γ; P < 0.001; Figure 3). AAV9-MCP1-7ND treatment led to a significant down-regulation of TNF-α levels compared with AAV9-luc-treated mice (P < 0.05). Furthermore, there was a trend towards reduced production of IL-1β, IL-4, and IL-13 (see Supplementary material online, Figure S2).
3.5 AAV9-mediated cardiac gene transfer of IL-10 results in down-regulation of chemokine and chemokine receptor transcript levels

We performed qPCR to evaluate chemokine and chemokine receptor myocardial transcript levels at Day 28. Mice which were treated with AAV9 vectors encoding IL-10 showed significantly reduced transcript levels of the chemokine receptor CCR4 (CCR4, 2.4 ± 1.7 vs. control 15.1 ± 3.0; P < 0.05) and a trend towards decreased levels of CCR2 compared with controls (Figure 4). Furthermore, AAV9-IL-10-treated mice showed significantly reduced mRNA levels of chemokine T-cell activation factor 3 (TCA-3 = CCL1) and significantly increased levels of IL-10 (TCA-3: 1.8 ± 0.8 vs. control 8.3 ± 3.3, P < 0.05; IL-10: 27682.8 ± 4571.6 vs. control 86.3 ± 82.5, P < 0.01; Figure 4). Also transcript levels of macrophage inflammatory protein 1α and MCP-1 were reduced in the same group. However, this effect was statistically not significant (Figure 4). There was no difference in the expression of the chemokine receptors CCR1 and CCR5 (data not shown) and of RANTES, IP-10, and lymphotactin between the treatment groups.

Mice having received AAV9-MCP1-7ND showed no significant effects on cytokine and chemokine transcript levels compared with AAV9-luc controls paralleling lacking effects on IFN-γ and other cytokines except TNF-α (see Supplementary material online, Figures S2 and S3). There was no difference in the expression of the chemokine receptors CCR2 and CCR4 between the treatment groups (data not shown).

3.6 IL-10 but not MCP1-7ND potently protects against myocardial inflammation and fibrosis

We determined whether AAV9-vector-based gene expression of IL-10 in cTnI-immunized mice had a protective effect on inflammation and fibrosis in heart tissue. Immunized mice treated with AAV9-IL-10 nearly abolished progression of myocarditis: haematoxylin eosin (HE) staining showed that only 1 out of 10 cTnI-immunized mice treated with the AAV9-IL-10 vector developed mild myocarditis with a low level of cellular infiltration, whereas no infiltration was detected in the other mice of this group. In contrast, control mice developed severe myocarditis and fibrosis as reflected by an increased histoscore (AAV9-IL-10: 0.1 ± 0.1 vs. AAV9-luc: 4.4 ± 0.3, P < 0.001; Figure 5A). Masson’s trichrome (MT) staining revealed no fibrosis in any of the heart sections of AAV9-IL-10 vector-treated mice, whereas in the control group 9 out of 10 mice developed severe fibrosis and scored 3 and more (IL-10: 0.0 ± 0.0 vs. control: 4.2 ± 0.3, P < 0.001; Figure 5A). Representative HE- and MT-stained heart sections are shown (Figure 5A).
Figure 4 qPCR analysis of myocardial mRNA levels of chemokines and chemokine receptors upon AAV9-IL-10 ($n = 5$) gene therapy on Day 28 after first immunization compared with AAV9-luc- ($n = 5$) and sham-immunized controls ($n = 4$). Error bars represent SEM. *$P < 0.01$.

Figure 5 AAV9-IL-10 and AAV9-MCP1-7ND gene therapy: effects on the severity and prevalence of myocardial inflammation and fibrosis. (A) Histological scores of each mouse in the respective group is shown (AAV9-IL-10 ($n = 10$); AAV9-luc ($n = 10$); sham ($n = 4$); AAV9-MCP1-7ND ($n = 9$); AAV9-MCP1 ($n = 10$). *$p < 0.05$; **$p < 0.001$. (B–I) Representative HE- (B, C, F, and G) and MT-stained (D, E, H, and I) heart sections of AAV9-IL-10-treated mice (B, D, F, and H) and of AAV9-luc-treated mice (C, E, G, and I). Scale bar: 100 μm.
sections of AAV9-IL10 and AAV9-luc-treated mice are shown in Figure 5B–I.

MCP1-7ND-treated mice showed decreased levels of inflammatory infiltrate and fibrosis as well. However, the protective effect of MCP1-7ND gene therapy was not as potent as IL-10 gene therapy. MCP1-7ND gene therapy showed a trend towards decreased cellular infiltration, being very close to statistical significance (AAV9-MCP1-7ND: 3.2 ± 0.5 vs. AAV9-luc: 4.4 ± 0.3, P = 0.053; Figure 5A). MT staining in these groups revealed no significant decrease in fibrosis in the AAV9-MCP1-7ND group compared with AAV9-luc controls (AAV9-MCP1-7ND: 2.9 ± 0.5 vs. AAV9-luc: 4.2 ± 0.3, P = 0.077; Figure 5A).

3.7 IL-10 prevents deterioration of left ventricular function

We performed echocardiography on Day 28 to evaluate hemodynamic parameters: AAV9-IL-10 vector-based gene therapy significantly increased fractional shortening (FS) and the ejection fraction (EF) in cTnI-immunized mice compared with AAV9-luc control-treated mice (IL-10, FS: 40.5 ± 3.0%, EF: 78.1 ± 3.4% vs. control FS: 26.6 ± 3.3%, EF: 58.3 ± 4.9%; each P < 0.05; Figure 6A). In addition, we observed a trend towards decreased left ventricular end-systolic (LVES) and end-diastolic diameters (LVED) in AAV9-IL-10-treated mice compared with mice injected with AAV9-luc (Figure 6A), paralleling suppressed myocardial inflammation and fibrosis in the AAV9-IL-10 group.

Although there was a trend towards an increased EF and FS in AAV9-MCP1-7ND-injected mice compared with the AAV9-luc group, differences were not significant (MCP1-7ND, FS: 36.7 ± 3.2% vs. AAV9-luc control 24.2 ± 3.0%; P = 0.083; EF: 73.3 ± 4.0% vs. AAV9-luc control 54.6 ± 5.2%; P = 0.083; Figure 6B). Furthermore, we found significant decreases in the EF and FS, as well as increased LVES and LVED in mice treated with AAV9-MCP compared with AAV9-MCP1-7ND, reflecting the additional pro-inflammatory effect of MCP-1 overexpression (Figure 6B).

4. Discussion

This study was designed to investigate whether adeno-associated vector-based anti-inflammatory gene therapy may be a promising strategy in the therapy of myocarditis. For this purpose, we used AAV9 vectors encoding IL-10 or MCP1-7ND cDNA under the control of a cardio-specific CMV-enhanced 260 bp MLC-2v promoter. To study a potential therapeutic effect, we immunized mice with cTnI. Recently, we showed that such immunization with cTnI induces an autoimmune response leading to severe myocardial inflammation and increased expression of pro-inflammatory cytokines.29,30 Overall, we show here that a single systemic administration of AAV9 vectors carrying an IL-10 expression cassette has a potent effect by nearly abolishing cTnI-induced myocardial inflammation and fibrosis. This positive effect at the histological level was associated with a significantly increased EF and FS and an IL-10-mediated reduced production of IL-2, IL-4, IL-6, IL-13, IL-17, IFN-γ, and TNF-α. Furthermore, the anti-inflammatory effect of IL-10 treatment was accompanied by significantly decreased myocardial transcript levels of CCR4 and TCA-3.

We additionally tested a possible effect of AAV9-mediated overexpression of MCP1-7ND in cTnI-immunized mice. MCP1-7ND, a deletion mutant which lacks the N-terminal amino acids 2–8 of MCP-1, has been first described as an inhibitor of MCP-1-mediated monocyte chemotaxis.16 In this study, MCP1-7ND gene therapy was less efficient than IL-10 to protect development of murine myocarditis in our model. While there were significant differences between MCP1-7ND and MCP1 that worsened myocarditis, comparison between MCP1-7ND treatment and luciferase controls resulted...
only in moderately reduced cardiac inflammation and fibrosis, which was associated with a significantly decreased TNF-α production and a trend towards an improved EF.

Cytokines play a central role in the progression of myocarditis. Among other cytokines, the role of the anti-inflammatory cytokine IL-10 has been studied extensively in the pathogenesis of myocarditis, and the potential of this cytokine for clinical implementation is noteworthy. For example, administration of recombinant IL-10 has been found to attenuate myocardial lesions in virus-induced myocarditis, possibly by inhibition of TNF-α production in infiltrating monocytes and lymphocytes in the heart. Furthermore, electroporation of an expression plasmid carrying murine IL-10 cDNA into the tibialis anterior muscle has been shown to be cardioprotective in a myosin-induced model of autoimmune myocarditis. Although these approaches were efficient, we wondered whether a localized IL-10 expression would be beneficial due to a higher local efficiency and less systemic side effects. We found four-fold higher IL-10 expression levels in heart tissue than in liver. Nevertheless, we cannot exclude an additional effect of the hepatic expression on systemic IL-10 levels.

The diverse effects of IL-10 on cytokine production may explain the favourable clinical outcome of IL-10 overexpression in many auto-immune disorders. In our study, IL-10 overexpression significantly reduced Th1- (IL-2 and IFN-γ), Th2- (IL-4, IL-6, and IL-13), and Th17- (IL-17 and TNF-α) specific cytokine production. Additionally, the production of the pro-inflammatory cytokines IL-6 and TNF-α was reduced and the expression of the pro-inflammatory chemokine TCA-3, and the chemokine receptor CCR4 were down-regulated. Nishio et al. reported suppressed levels of TNF-α in IL-10-treated mice, which is in line with our findings. In this context, we have previously shown that blocking IL-10 results in increased production of IL-4 and TNF-α in myosin-induced autoimmune myocarditis. The potential of IL-10 to block TNF-α release may play a critical role in suppressing inflammation after cTnI immunization.

The rather moderate influence of MCP1-7ND overexpression on development of a cardiac pathology despite significantly reduced TNF-α levels indicates that TNF-α suppression alone might not be sufficient to be efficiently protective. Recently, Chang et al. revealed that IL-10 is capable of efficiently blocking IL-17 expression in myosin-induced experimental autoimmune myocarditis, thereby preventing cardiac deterioration. This finding is supported by our study in which IL-10 results in decreased IL-17 production. IL-17, which is produced by Th17 cells, is therefore supposed to be a pro-inflammatory mediator in autoimmune myocarditis. However, the precise role of IL-17 in the progression of autoimmune myocarditis is not fully understood and remains to be clarified. Eriksson et al. demonstrated an important role for IL-6 in the pathogenesis of myocarditis. Consistent with our findings, they demonstrated that the prevalence and severity of myocarditis is markedly reduced in the absence of IL-6. Taken together, our data suggest that IL-10 exerts its rescuing effect in murine autoimmune myocarditis by influencing the production of a multiple subset of cytokines. Similarly, the surprisingly low therapeutic effect of gene transfer of MCP1-7ND might be explained by a limited effect on cytokine suppression, at least in our model.

A limitation of this proof-of-principle study is that we focused on protection from myocarditis rather than treatment. Future studies are necessary to investigate the role of this approach in early or manifest myocarditis.

Successful cardiac gene therapy of autoimmune myocarditis basically depends on at least two crucial factors: one is the ability of a certain therapeutic gene to exert anti-inflammatory effects, which finally lead to myocardial rescue. In this context, we show that especially overexpression of the anti-inflammatory mediator IL-10 improves the clinical outcome after induction of autoimmune myocarditis. The second critical factor is the question how to ensure specific targeting of the heart with sufficient expression levels of a therapeutic gene. Considering the high morbidity of patients suffering from myocarditis, a minimal invasive approach is preferable. The use of a vector system allowing a transvascular route of administration would be most suitable. Furthermore, the vector itself should not be immunogenic. Thus, the use of adenoviral vectors might be limited due to an only transient gene expression, most probably due to their high immunogenicity. In our study, we used AAV9 vectors to achieve an efficient transduction of myocardium. Among other AAV serotypes, AAV9 shows the highest tropism for adult cardiomyocytes and enables an almost complete myocardial transduction after systemic injection, with gene expression being stable for up to 1 year.

Transcriptional targeting with a 0.26 kb (MLC)-2v promoter fused to a CMV enh element further allowed a predominant cardiac expression as shown for IL-10. Combining these vectors with suitable application systems may allow validation of this approach in larger animals. Overall, our study proved that cardiac expression of IL-10 by transvascular application of AAV9 vectors may be a promising tool for novel strategies against autoimmune myocarditis.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Acknowledgements**

The authors thank Ozy Kaya, Jin Li, and Martin Andrassy for critically reading the manuscript. We thank the German Cancer Research Center (DKFZ) vector core production unit for their support in generating high-titre AAV vector stocks. The excellent technical assistance of S. Zittrich, Cologne, is gratefully acknowledged.

**Conflict of interest:** none declared.

**Funding**

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (KA 1797/4-2 to Z.K., SFB612-A2 to G.P., and MU 1654/3-2 to O.J.M.) and Deutsche Stiftung für Herzforschung (to Z.K. and O.J.M.).

**References**


