Immunosuppression Decreases Inflammation and Increases AAV6-hSERCA2a-Mediated SERCA2a Expression

Xiaodong Zhu,^{1,*} Charles F. McTiernan,^{1,*} Navin Rajagopalan,¹ Hemal Shah,¹ David Fischer,¹ Yoshiya Toyoda,² Dustin Letts,¹ Jonathan Bortinger,¹ Gregory Gibson,¹ Wenyu Xiang,¹ Kenneth McCurry,² Michael Mathier,¹ Joseph C. Glorioso,³ and Barry London¹

Abstract

The calcium pump SERCA2a (sarcoplasmic reticulum calcium ATPase 2a), which plays a central role in cardiac contraction, shows decreased expression in heart failure (HF). Increasing SERCA2a expression in HF models improves cardiac function. We used direct cardiac delivery of adeno-associated virus encoding human SERCA2a (AAV6-hSERCA2a) in HF and normal canine models to study safety, efficacy, and the effects of immunosuppression. Tachycardic-paced dogs received left ventricle (LV) wall injection of AAV6-hSERCA2a or solvent. Pacing continued postinjection for 2 or 6 weeks, until euthanasia. Tissue/serum samples were analyzed for hSERCA2a expression (Western blot) and immune responses (histology and AAV6-neutralizing antibodies). Nonpaced dogs received AAV6-hSERCA2a and were analyzed at 12 weeks; a parallel cohort received AAV-hSERCA2a and immunosuppression. AAV-mediated cardiac expression of hSERCA2a peaked at 2 weeks and then declined (to ~50%; $p < 0.03$, 6 vs. 2 weeks). LV end diastolic and end systolic diameters decreased in 6-week dogs treated with AAV6-hSERCA2a ($p < 0.05$) whereas LV diameters increased in control dogs. Dogs receiving AAV6-hSERCA2a developed neutralizing antibodies (titer $\geq 1:120$) and cardiac cellular infiltration. Immunosuppression dramatically reduced immune responses (reduced inflammation and neutralizing antibody titers $< 1:20$), and maintained hSERCA2a expression. Thus cardiac injection of AAV6-hSERCA2a promotes local hSERCA2a expression and improves cardiac function. However, the hSERCA2a protein level is reduced by host immune responses. Immunosuppression alleviates immune responses and sustains transgene expression, and may be an important adjuvant for clinical gene therapy trials.

Introduction

HEART FAILURE afflicts almost 6 million people in the United States (American Heart Association, 2009). More than 400,000 new cases of heart failure are diagnosed each year, with more than 300,000 deaths attributed to the disease. Advances in pharmacological treatments including β -blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and aldosterone antagonists have decreased hospitalizations and improved survival in patients with mild to moderate symptoms of heart failure (Hunt *et al.*, 2001). In addition, device therapy using implantable cardioverter-defibrillators and biventricular pacemakers has decreased sudden death and improved pump function (Young *et al.*, 2003), and ventricular assist devices are available as a destination therapy (Nguyen and Thourani, 2010). Heart failure is often progressive, however, and many patients eventually

develop unremitting end-stage symptoms. The only definitive therapy available for these patients is cardiac transplantation. Unfortunately, limitations in donor supply and eligibility have relegated this therapy to a minority of patients. As the population ages and the incidence of heart failure rises, the need for novel therapeutics is clear.

Gene delivery using viral vectors is one potential therapeutic option. Adeno-associated virus (AAV) has been recognized as a promising vector for gene therapy applications because it is nonpathogenic, replication defective, able to infect nondividing cells (including cardiomyocytes), and capable of long-term transgene expression (Kapli *et al.*, 1996; Xiao *et al.*, 1996). SERCA2a, the sarcoplasmic reticulum calcium ATPase 2a, loads calcium into the sarcoplasmic reticulum and has been implicated in regulating the progression of dilated cardiomyopathy (Gianni *et al.*, 2005). In cardiac tissue isolated from patients and animals with heart failure,

¹Cardiovascular Institute, University of Pittsburgh, Pittsburgh, PA 15213.

²Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15213.

³Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, PA 15213.

*These authors contributed equally to this work.

SERCA2a messenger RNA, protein, and activity levels decline (Bers *et al.*, 2003; Braz *et al.*, 2004; Del Monte *et al.*, 2004). Restoring SERCA2a levels using adenoviral vectors or adeno-associated viral (AAV) vectors has been shown to improve function, metabolism, and/or survival in small and large animal models of heart failure (Hajjar *et al.*, 1998; Del Monte *et al.*, 2001; Kawase *et al.*, 2008). A phase 1/2 clinical trial (CUPID; Celladon, La Jolla, CA), testing the safety and efficacy of catheter-based cardiac delivery of AAV1-hSERCA2a (Mydicar) in patients with advanced heart failure, has met its primary safety end points and suggested efficacy. Of note, more than 50% of potential subjects were excluded because of preexisting AAV1 neutralizing antibodies (Hajjar *et al.*, 2008; Jaski *et al.*, 2009; Jessup *et al.*, 2011).

A number of studies in animal models (mostly rodents) confirmed that AAV promotes long-term target gene expression (Daya and Berns, 2008). However, studies in clinical trials and large animal models, including our studies, which delivered AAV2-TNFRII-FC into baboon hearts (McTiernan *et al.*, 2007), have revealed a range of inflammatory immune responses to AAV vectors and their transgene products, which may reduce transgene expression (Herzog *et al.*, 2001, 2002; Wang *et al.*, 2005, 2007a,b; Jiang *et al.*, 2006; Manno *et al.*, 2006; Mingozi and High, 2007) and raise the risks associated with AAV-based gene delivery procedures. Identification of an effective immunosuppressive regimen for AAV gene delivery could potentially enhance efficacy and improve safety in AAV-mediated therapies. For example, it has been reported that immunosuppression can limit development of antibody directed to AAV-encoded factor IX (Herzog *et al.*, 2001, 2002), and permit sustained AAV-mediated minidystrophin expression in dog skeletal muscle (Herzog *et al.*, 2001, 2002; Wang *et al.*, 2007b).

We are developing AAV6-hSERCA2a as a potential therapy for heart failure. In this study, a trial using the canine model of tachycardic pacing-induced heart failure was performed to test the safety and efficacy of delivering AAV6-hSERCA2a by direct injection into the hearts of large animals with heart failure. The subsequent clinical trial proposes such gene therapy delivery to occur at the time of ventricular assist device (VAD) placement in patients with end-stage heart failure. We observed that direct cardiac injection of AAV6-hSERCA2a into dog hearts promoted local robust hSERCA2a expression that was reduced by host immune responses. However, a limited immunosuppressive therapy blunted the immune responses and allowed persistent long-term transgene expression.

Materials and Methods

An expanded Methods section is available in the online supplement.

Canine heart failure and immunosuppression model

Pacemakers were implanted into the right ventricle of dogs. The dogs were paced until establishment of left ventricular (LV) dysfunction (LV ejection fraction <35%). AAV6-hSERCA2a was administered by direct injection through the epicardial surface of the left ventricle. Immunosuppression was achieved in nonpaced dogs with prednisone and cyclosporine given twice daily beginning 4 weeks after the AAV6-hSERCA2a (or solvent) injection. All

studies were reviewed and approved by the University of Pittsburgh (Pittsburgh, PA) Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

Echocardiography

Left ventricular dimensions (i.e., left ventricular end-diastolic dimension [LVEDD] and left ventricular end-systolic dimension [LVESD]) and function (percentage fractional change, FAC%) were assessed by two-dimensional and M-mode echocardiography before pacing initiation (prepacing, -4 weeks), after pacing, and before vector administration (baseline, 0 weeks), and every 2 weeks after vector (or solvent) injection. Paced heart failure dogs received AAV6-hSERCA2a or solvent and were monitored for 2 weeks ($n=5$, AAV; $n=2$, solvent) or 6 weeks ($n=6$, AAV; $n=2$, solvent) before euthanasia.

Anti-human Serca2a antiserum

Antibody specific to human SERCA2a was prepared by immunization of rabbits with a human SERCA2a peptide (PMTSGVKQKIMS, amino acid residues 535–546; NCBI Reference Sequence NP_001672.1). Antibodies cross-reactive to canine SERCA2a were removed by chromatography of the serum over an affinity gel coupled with a peptide encoding canine SERCA2a amino acid residues 535–546 (PMTPGVKQKVMS, canine SERCA2a; GenBank accession no. U94345.1).

In vitro cellular response to hSERCA2a-specific peptides

Splenocytes or peripheral blood mononuclear cells were obtained from dogs that had received saline ($n=2$) or AAV6-hSERCA2a ($n=2$). Cells were cultured with each of 2 pools containing 10 or 11 peptides encompassing the 8 amino acid residues that differ between canine and human SERCA2a (see Supplementary Fig. S3A for peptide sequences). Positive control wells contained PHA (phytohemagglutinin, M form; Gibco-Life Technologies, Grand Island, NY), and negative control wells contained medium. Each condition was assayed in triplicate. After 6 days, medium was assayed for interferon (IFN)- γ production by ELISA (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions.

Statistical analysis

Data are presented as means or as means $\pm$ SD. Significance was determined by two-tailed *t* test for paired or unpaired variables. Analysis of variance was used for continuous variables. A value of $p < 0.05$ was considered significant.

Results

Study design

The planned clinical trial will consider delivery of AAV6-encoding hSERCA2a to patients who receive a ventricular assist device for end-stage heart failure. As preclinical studies, two related investigations were undertaken. First, a canine model of tachycardia-pacing induced chronic heart failure was used to assess short-term safety and efficacy of

cardiac-injected AAV6-hSERCA2a (Davidoff and Gwathmey, 1994; Nikolaidis *et al.*, 2005). The experimental design is presented in Fig. 1. Dogs ($n=15$) to be studied at the 2- and 6-week end points first received prepacing cardiac function assessment and then underwent percutaneous pacemaker placement. After induction of heart failure, AAV6-hSERCA2a was administered on two 3×3 cm grids positioned on the beating hearts. The nine sites within each grid were injected with 0.1 ml of either AAV6-hSERCA2a ($n=11$) or solvent as control ($n=4$). In dogs injected with AAV6-hSERCA2a, one grid received the low dose (5×10^{11} viral genomes/ml) and the other received the high dose (5×10^{12} viral genomes/ml). Pacing was reinitiated 5 days after surgery to maintain heart failure.

The second arm sought to assess long-term human SERCA2a expression after delivery of AAV6-hSERCA2a, and the effects of immunosuppression (Wang *et al.*, 2007b). For the longer time point of 12 weeks, dogs ($n=15$) without pacing were used to avoid the increased morbidity and risk of mortality anticipated with extended tachycardic pacing. These dogs were placed on cardiopulmonary bypass (without circulatory arrest) and AAV6-hSERCA2a ($n=11$) or solvent ($n=4$) was delivered as described previously. Approximately half of the dogs were immunosuppressed (beginning 4 weeks after vector injection and continuing until the time of euthanasia) to simulate the clinical scenario in which a VAD-supported patient, who had received the proposed AAV-based gene delivery, proceeded to cardiac transplantation and subsequent immunosuppression.

AAV-hSERCA2a stocks were produced by a triple plasmid transfection method (Xiao *et al.*, 1997). We used vectors from two sources. Initial studies were performed with vectors produced in our laboratory, and purified via heparin chromatography (three or four dogs per group). Additional studies were performed with Good Laboratory Practice (GLP)-grade vectors prepared by the University of North Carolina (Chapel Hill, NC) Joint Vector Laboratories and purified by ultracentrifugation on CsCl gradients (two dogs

per group). Vectors purified by heparin chromatography had ~10 times more empty viral particles than vectors purified by ultracentrifugation on CsCl gradients (see the online supplement).

AAV-mediated hSERCA2a expression in dog hearts decreases with time but is preserved by immunosuppression

As SERCA2a is a highly conserved protein among mammals (Campbell *et al.*, 1992), rabbit antiserum was produced that differentiates human from canine SERCA2a protein (Fig. 2A, lane H vs. C2; see also Supplementary Fig. S1) (supplementary data are available online at www.liebertonline.com/hum). Cardiac extracts from high-dose injection and noninjected sites were subjected to Western blot analyses, which revealed increased human SERCA2a expression in dog hearts 2 weeks after receiving AAV6-hSERCA2a ($n=5$) that was not detectable in control dogs receiving solvent (Fig. 2A). hSERCA2a expression was lower in low-dose injection sites (data not shown). We observed low-level hSERCA2a expression in noninjected regions (2–4 cm from injection sites) in three dogs (data not shown). The results presented below focus on the high-dose or solvent-injected sites.

Quantitative Western blot of dog cardiac tissues (Fig. 2B) showed that AAV6-mediated human SERCA2a expression was more variable and significantly lower ($n=6$, ~50% compared with 2 weeks; $p<0.03$) 6 weeks after vector administration compared with 2 weeks. Similar reduced levels were found when analyzed 12 weeks after vector delivery in nonpaced dog hearts ($n=6$, ~50% compared with 2 weeks; $p<0.001$).

We hypothesized that immune-mediated processes led to decreased expression of hSERCA2a, and that immunosuppression would preserve hSERCA2a expression. To test this hypothesis, a group of nonpaced dogs received AAV6-hSERCA2a ($n=5$) or solvent ($n=2$), with immunosuppression commencing 4 weeks after viral delivery and continuing

A Group

Number of dogs	Tachycardic-paced	Immuno-suppression	Survival Duration	a. #of dogs injected with GLP grade vector	b. #of dogs injected with Research Grade vector	c. #of dogs injected with solvent (Control)
7	Yes	No	2 W	2	3	2
8	Yes	No	6 W	2	4	2
8	No	No	12 W	2	4	2
7	No	Yes	12 W	2	3	2

B Timeline

FIG. 1. Outline of canine toxicology study. **(A)** Table of study groups. **(B)** Schematic of the timeline of dog pacing, vector delivery, and immunosuppression. W, weeks.

FIG. 2. AAV6-mediated human SERCA2a expression in dog heart. Western blots of cardiac extracts of high-dose sites were analyzed with rabbit anti-hSERCA2a antiserum and anti-GAPDH. Human heart (H) and solvent-injected dog heart (C) extracts served as the positive and negative controls, respectively. **(A)** Western blot images from 2-week tachycardic-paced dogs receiving AAV6-hSERCA2a (lanes 1–5) or solvent (C2); 6-week tachycardic-paced dogs receiving AAV6-hSERCA2a (lanes 6–11) or solvent (C6); 12-week nonpaced dogs receiving AAV6-hSERCA2a (lanes 12–17) or solvent (C12); 12-week nonpaced dogs receiving AAV6-hSERCA2a and immunosuppression (lanes 18–22) or solvent and immunosuppression (C12+I). **(B)** Quantitative comparison of hSERCA2a expression from digitized X-ray films after normalization to a reference dog (D-70-07). Solid column, 2-week dogs plus AAV6-hSERCA2a ($n=5$); dark gray column, 6-week dogs plus AAV6-hSERCA2a ($n=6$); light gray column, 12-week normal dogs plus AAV6-hSERCA2a ($n=6$); open column, 12-week normal dogs with immunosuppression plus AAV6-hSERCA2a ($n=5$). IS, received immunosuppression; W, weeks.

for 8 more weeks until the animals were killed 12 weeks postinjection (Fig. 1B). Quantitative Western blot analyses revealed significantly ($p<0.003$) higher expression of hSERCA2a in dogs receiving immunosuppression when compared with animals that were not immunosuppressed (Fig. 2A and B). Data shown in Fig. 2 were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Normalization based on signals from Coomassie blue staining of the filter produced a similar trend (data not shown).

Cardiovascular and clinical responses to AAV6-hSERCA2a treatment

Dogs ($n=14$ total) had normal prepacing LV dimensions and function (mean $\pm$ SD: LVESD, 2.0 ± 0.3 cm; LVEDD, 3.4 ± 0.3 cm; %FAC, $42\pm7\%$) and developed increased LV systolic dimensions (LVESD, 3.2 ± 0.4 cm; $p<0.0001$), increased LV diastolic dimensions (LVEDD, 4.1 ± 0.4 cm; $p<0.001$), and decreased percentage fractional area change (%FAC, $23\pm5\%$; $p<0.001$) at baseline (after 4 weeks of pacing and before vector delivery, week 0) (Table 1). LVESD

and LVEDD significantly decreased toward baseline levels in dogs 6 weeks after AAV6-hSERCA2a delivery (6 weeks: LVESD, 2.4 ± 1.0 cm; LVEDD, 3.4 ± 0.8 cm; $p<0.04$ compared with week 0). %FAC trended to improvement at 6 weeks but did not reach statistical significance in the six dogs of this group (6 weeks, $31\pm13\%$; $p<0.08$). In contrast, solvent-treated dogs showed ongoing (relative to week 0) cardiac dilation and persistently reduced fractional shortening (6 weeks: LVESD, 3.4 cm; LVEDD, 4.3 cm; %FAC, 21%; p =not significant; all solvent treatment data represent the mean of two dogs).

Vector distribution

Vector distribution and quantification were performed on the subset of dogs receiving GLP-grade AAV6-hSERCA2a so as to assess the potential for any systemic vector delivery arising from intracardiac injection. Vector genomic DNA was detectable at between 4×10^3 and 2×10^6 copies of single-stranded DNA per microgram of genomic DNA in the high-dose and low-dose injection sites for all eight vector-treated dogs (Fig. 3). In general, low-dose regions had ~5- to 8-fold

TABLE 1. ECHOCARDIOGRAPHIC MEASUREMENT OF LEFT VENTRICULAR DIASTOLIC AND SYSTOLIC DIAMETERS OF PACED DOGS AFTER INTRACARDIAC ADMINISTRATION OF AAV6-hSERCA2a

	Prepacing				Baseline (0 weeks)				2 weeks				4 weeks				6 weeks			
	LVEDD	LVESD	FAC%		LVEDD	LVESD	FAC%		LVEDD	LVESD	FAC%		LVEDD	LVESD	FAC%		LVEDD	LVESD	FAC%	
AAV-hSERCA2a	3.4±0.3	2.0±0.3	42±7	(8)	4.1±0.4 ^b	3.2±0.4 ^b	23±5 ^b	(10)	4.2±0.4 ^b	3.2±0.5 ^b	24±8 ^b	(10)	4.0±0.4 ^b	2.9±0.4 ^b	28±8 ^b	(6)	3.4±0.8 ^d	2.4±1.0 ^d	31±13	(5)
Solvent	3.4±0.5	2.1±0.2	38±5	(4)	3.8±0.5	3.1±0.4 ^b	19±5	(4)	3.8±0.6	2.9±0.6 ^c	23±7	(4)	4.1	2.8	31	(2)	4.3	3.4	21	(2)

FAC%, percentage fractional change; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension.

No statistical analyses were performed on control groups ($n=2$). N; the number of dogs for which echocardiographic data were available. Two prepacing data point and one 6-week data point were not available for technical reasons.

^b $p \leq 0.01$ compared with prepacing.

^c $p \leq 0.05$ compared with prepacing.

^d $p \leq 0.05$ compared with week 0 (baseline).

lower vector genome copy numbers relative to high-dose regions. Among high-dose regions, no obvious differences in DNA copy number were observed between 2 and 12 weeks after injection, although regions receiving low-dose vector tended to show a reduction in AAV copy number by 6 weeks after injection. Immunosuppression had no obvious effects on AAV copy number in either low- or high-dose sites. Vector genome DNA was found at a low level (40–500 AAV genomes per microgram of genomic DNA) in noninjected areas of the heart in two vector-treated dogs. Vector DNA was also detected in kidney, liver, and spleen samples of three individual dogs. Leukocytes were positive for vector within 24 hr of injection in three of the eight dogs, and viral

FIG. 3. AAV genome copies at injection site at time of sacrifice. AAV genome copy number per microgram of dog genomic DNA was determined by GLP-level quantitative PCR. Each column represents the mean of high-dose or low-dose sites. (A) Data for high-dose injection sites. (B) Data for low-dose injection sites. IS, received immunosuppression; W, weeks. Two- and 6-week dogs received tachycardic pacing; 12-week dogs did not.

genomes were undetectable at all other time points in blood samples.

Immune responses to vector delivery; antibody production

Anti-AAV6 neutralizing antibody (NAb) titers were determined by testing the ability of serum antibody to inhibit transduction of reporter virus AAV6-LacZ into C12 cells, as described earlier (McTiernan *et al.*, 2007). In this study we used vectors purified by either chromatography or CsCl gradient density centrifugation. Chromatographically purified viral stocks typically contained ~10 times more empty capsid protein than GLP-grade stock purified by CsCl gradient. No dogs had detectable (NAb titers >1:20) AAV neutralizing antibodies before AAV injection (baseline). All dogs developed positive (>1:20) AAV NAb titers by 2 weeks after vector administration (Fig. 4A and B). Chromatographic and CsCl gradient density centrifugation purified (GLP-grade) viral stocks elicited similar NAb titers in heart failure model dogs (~1:120; Fig. 4A). However, higher NAb titers developed in nonpaced dogs that received chromatographic purified virus (>1:250) relative to those animals that received vectors prepared by CsCl gradient density centrifugation (GLP grade) (~1:100; Fig. 4B). Interestingly, immunosuppression commencing 4 weeks after viral injection and continuing for 8 weeks reduced NAb titers to baseline levels (Fig. 4B).

Sera collected from dogs 6 or 12 weeks after virus delivery were also used in Western blot analysis of human heart proteins to look for anti-human SERCA2a antibodies. Dogs expressing human SERCA2a did not have detectable

antibody reactivity to human heart proteins at the expected size of SERCA2a (see Supplementary Fig. S2).

Histopathological assessment

Histopathological assessment was performed in hematoxylin and eosin (H&E)-stained sections from paced dog hearts receiving AAV6-hSERCA2a or solvent (Fig. 5). Substantial but localized myocardial infiltration was observed in hearts that received AAV6-hSERCA2a after 2 or 6 weeks, with a tendency toward less infiltration observed in sites that received low-dose virus injection (data not shown). Infiltration was also present in 12-week dogs without heart failure that received AAV6-hSERCA2a, but was less prominent in samples from dogs that received AAV6-hSERCA2a and immunosuppression compared with those that received virus and no immunosuppression (Fig. 6A). There was no obvious difference in the severity of infiltration between dogs that received virus purified by heparin chromatography or CsCl gradient (GLP grade). Samples from dogs that received GLP-grade virus ($n=8$), and solvent controls ($n=8$), were analyzed in a semiquantitative fashion by a blinded observer. Mean data derived from the two high-dose injection sites for each of two dogs in a given treatment group showed that infiltration (chronic inflammation) was more prominent in dogs receiving AAV6-hSERCA2a, that inflammation and fibrosis were persistent from 2 weeks through 12 weeks after virus injection, and that immunosuppression reduced inflammation but not fibrosis (Fig. 6B). Inflammation and fibrosis were considerably less evident at sites that received low-dose AAV6-hSERCA2a when compared with high-dose sites and was not observed in noninjected sites (data not shown).

FIG. 4. Development of neutralizing antibodies (NAbs) against AAV6 after vector injection. **(A)** Paced dogs with survival period of 2 and 6 weeks after vector administration. **(B)** Nonpaced dogs with survival period of 12 weeks after vector administration. NAb assays were performed on blood samples collected before vector administration and at weekly or biweekly intervals after vector administration. The serum dilution value is the average of the group. CP, chromatographically purified AAV6-hSERCA2a; GLP, Good Laboratory Practice-grade preparation of AAV6-hSERCA2a purified by CsCl gradient density centrifugation; n , number of dogs per group.

FIG. 5. Hematoxylin and eosin-stained cardiac tissues from paced dogs receiving AAV6-hSERCA2a treatment for 2 or 6 weeks. **(a)** Noninjection site of a tachycardic-paced control dog 2 weeks after solvent injection. **(b)** Solvent injection site of the same dog in **(a)**. **(c)** Noninjection site of a tachycardic-paced dog 2 weeks after receiving AAV6-hSERCA2a. **(d)** High-dose injection site of the same dog in **(c)**. **(e)** Noninjection site of a tachycardic-paced control dog 6 weeks after solvent injection. **(f)** Solvent injection site of the same dog in **(e)**. **(g)** Noninjection site of tachycardic-paced dog 6 weeks after receiving AAV6-hSERCA2a. **(h)** High-dose injection site of the same dog in **(g)**. The scale bar is indicated.

Immunofluorescence microscopy of dog hearts recovered 12 weeks after injection of AAV6-hSERCA2a revealed significant CD4⁺ and CD8⁺ infiltrating cells in areas adjacent to near-normal cardiomyocytes, and interspersed with what appear to be remnants of cardiomyocytes (Fig. 6C).

There were no significant morphological changes in other organs except for (1) fibrosis of the pleura in lung samples, which was consistent with injury associated with thoracotomy; and (2) increased hepatocyte cytoplasmic clearing in the livers of immunosuppressed dogs, suggesting a mild steroid hepatopathy, which is a common phenomenon in dogs.

Cellular immune response to hSERCA2a epitopes

The cellular infiltrates concomitant with expression of human SERCA2a suggest a possible immune cell response to epitopes that differentiate human from canine SERCA2a, even though the protein sequence is >99% conserved between human and canine. To assay for cytotoxic T cell responses to cells expressing human hSERCA2a, splenocytes or peripheral blood mononuclear cells obtained from control dogs ($n=2$, saline injected) and dogs that had received AAV6-hSERCA2a (2 or 6 weeks earlier, $n=1$ each) were cultured with each of 2 pools containing 10 or 11 different peptides encompassing the 8 amino acid residues that differ between canine and human SERCA2a (Supplementary Fig. S3). Culture medium levels of IFN- γ were then determined as an assay for cytotoxic T cell activation (Rimmelzwaan *et al.*, 1991). Whereas all four cell sources produced IFN- γ in response to the positive control (1 $\times$ PHA), none of the hSERCA2a peptide pools elicited detectable levels of IFN- γ production (see Supplementary Fig. S3).

Discussion

Increasing sarcoplasmic reticulum calcium uptake and calcium transient kinetics via targeted overexpression of SERCA2a by a gene therapy-mediated approach has been

suggested as a means to improve function of the failing mammalian heart (Del Monte *et al.*, 2001; Hajjar *et al.*, 2008; Kawase *et al.*, 2008). In this report we used an AAV6-based vector encoding human SERCA2a, delivered by direct cardiac injection into nonfailing and failing dog hearts, and assessed (1) the effect of hSERCA2a expression on cardiac function in this heart failure model, (2) the persistence of hSERCA2a expression in the dog hearts, (3) the occurrence of immune responses, and (4) the effect of immunosuppression on hSERCA2a expression and immune responses. The major new observations of this study are as follows: (1) despite a limited area of injection, the AAV6-mediated overexpression of human SERCA2a was accompanied by improved measures of LV size and cardiac function in a large animal model of tachycardic-pacing induced heart failure; (2) CD4⁺ and CD8⁺ cellular infiltrates likely limit the duration and extent/amount of the hSERCA2a expression; and (3) immunosuppression 4 weeks after vector delivery limited cellular infiltration (and anti-AAV humoral immune responses), and prolonged high-level hSERCA2a expression.

This paper demonstrates AAV6-mediated expression of human SERCA2a in a large animal model of heart failure, with subsequent improvement in cardiac dimensions and systolic function. Although other AAV pseudotypes may show preferential organ tropism that varies between species (Yue *et al.*, 2008), AAV6 generates reasonably high cardiac and skeletal muscle expression in dogs (Bish *et al.*, 2008). Our approach included an antibody-based detection method to confirm overexpression of the AAV-encoded human SERCA2a as opposed to enhanced expression of endogenous canine SERCA2a. The results generally follow prior reports in which targeted overexpression of SERCA2a can improve function of the failing mammalian heart. Cardiac SERCA2a overexpression by adenoviral vector in the rat aortic banding model improves survival and cardiac metabolism (Del Monte *et al.*, 2001). In addition, long-term overexpression of SERCA2a by AAV1 vector in a volume-overload porcine heart failure model reverses cardiac dysfunction (Kawase

FIG. 6. (A) Hematoxylin and eosin (H&E)-stained cardiac tissues from nonpaced dogs 12 weeks after receiving AAV6-hSERCA2a. A1: Noninjection site of a control dog. A2: Solvent injection site of the same dog in A1. A3: Noninjection site of a dog receiving AAV6-hSERCA2a. A4: High-dose injection site of the same dog in A3. A5: Noninjection site of an immunosuppressed dog receiving AAV6-hSERCA2a. A6: High-dose site of the same dog in A5. **(B)** Severity score of fibrosis and chronic inflammation in high dose-injected heart samples. Pathological severity scores, determined by an independent pathology laboratory, are shown for H&E sections of high-dose LV sites from dogs injected with GLP-grade virus. The severity grade for fibrosis and chronic inflammation is described in Materials and Methods. The average severity score is from two high dose-injected sites within one heart and subsequently averaged between two dogs in each group (i.e., data from $n=2$ per group). Fibrosis in the 6-week control was scored zero. AAV, received AAV6-hSERCA2a; CTL, control (received solvent); IS, received 8 weeks of immunosuppression starting 4 weeks after injections; W, weeks after injections. **(C)** Immunofluorescently stained cardiac tissues from nonpaced dogs 12 weeks after receiving AAV6-hSERCA2a. C1, C2: Noninjection site of a control dog. C3, C4: AAV6-hSERCA2a-injected sites. Green, phalloidin (sarcomeres); blue, DAPI (nuclei); red, anti-CD4 (C1, C3) or anti-CD8 (C2, C4). The scale bar is indicated.

et al., 2008). The canine tachycardic-pacing model, believed to closely mimic many aspects of human nonischemic heart failure (Davidoff and Gwathmey, 1994), has not been previously used as a large animal model of cardiac gene therapy. Although the delivery method (direct cardiac visualization and injection) is more invasive than may be envisioned for clinical trials, it permits high transduction in a localized identifiable area for subsequent analyses, minimizes collateral viral uptake in other organs, and could be used at the time of cardiac surgery in a clinical setting.

An important observation was a cellular immune response to vector delivery ($CD4^+$ and $CD8^+$ cardiac infiltrates) that

likely limited hSERCA2a expression encoded by the AAV6 vector. This occurred in both heart failure dogs, in which AAV was injected into the beating hearts, and in nonpaced dogs, which were injected while on cardiopulmonary bypass. These observations resemble our prior studies in baboon, in which cardiac infiltrations and overt myocarditis were observed to accompany a rapid rise and fall in the expression of an AAV2-encoded secreted gene product (TNFR2-Fc-IgG) after direct cardiac delivery of the AAV2-based vector (McTiernan *et al.*, 2007). Such immune reactions could limit the effectiveness of gene delivery, and/or lead to progressively worsened cardiac function.

Both viral capsid protein, and the virally encoded human SERCA2a could provide antigens to elicit cellular immune responses. Infiltration and fibrosis occurred when using either "research-grade" heparin chromatography-purified vectors that contain substantial levels of empty capsids relative to full particles containing AAV DNA, or "clinical trial-grade" vectors prepared under GLP standards and purified by CsCl gradient ultracentrifugation, which separates empty capsids from genome-containing vector particles. Not unexpectedly, we detected increased NAb titers to AAV6 after virus injection, which was higher in those dogs receiving "research-grade" versus "clinical-grade" preparations. Although we did not specifically assess the generation of a cellular anti-AAV response, this has been observed in prior studies in which dogs have received AAV vectors (Wang *et al.*, 2007a,b, 2010; Ohshima *et al.*, 2009). However, we did not detect anti-human SERCA2a antibodies in dogs receiving AAV6-hSERCA2a, or cellular immune responses to SERCA2a peptides containing human-specific/canine divergent amino acids, suggesting that the human SERCA2a protein did not elicit the immune responses.

Not all studies that have delivered AAV vectors to large animal striated muscle (skeletal or cardiac) have detected infiltrative responses. The delivery method, titer of AAV particles, and experimental species may contribute to such differences. Direct injection of AAV into primate hearts (McTiernan *et al.*, 2007) and dog skeletal muscle (Yuasa *et al.*, 2007; Wang *et al.*, 2005, 2007a,b) induces a cellular infiltration, whereas the inflammatory response to catheter-delivered cardiac AAV infusion is not notable (Kaplitt *et al.*, 1996; Kawase *et al.*, 2008; Raake *et al.*, 2008). Comparative studies showed that direct injection of AAV induced infiltration in canine skeletal muscle whereas limb perfusion did not (Gregorevic *et al.*, 2009; Ohshima *et al.*, 2009). Studies have also observed a vector particle dose-dependent immune response (Herzog *et al.*, 2002). Dogs may also present more severe immune responses to AAV or AAV-encoded proteins than do other species (such as mice; Yuasa *et al.*, 2007), and strong cellular immune responses to skeletal muscle delivery of AAV have been noted in dogs (Wang *et al.*, 2007a,b). However, one study detected minimal cardiac fibrosis and mononuclear cell infiltration after endomyocardial injection of AAV constructs into canine hearts (Bish *et al.*, 2008).

In this light it is interesting to note that (1) several studies have observed an unexpected and prolonged (weeks to months) persistence of, and cellular responses to, viral antigens after infection with parvovirus B19 (Isa *et al.*, 2005), vesicular stomatitis virus (Turner *et al.*, 2007), or influenza virus (Kim *et al.*, 2010); (2) some studies suggest long-term persistence of AAV-antigens after viral delivery (Jiang *et al.*, 2006; Stieger *et al.*, 2009); and (3) even low levels of viral capsid antigen:MHC complexes can target cells for recognition by peptide-specific cytotoxic T cells (Pien *et al.*, 2009). Thus a large bolus of foreign antigen (i.e., AAV capsid) delivered by localized injection into striated muscle (such as the myocardium) could generate a depot for prolonged cellular immune response to AAV capsids retained at the injection site and presented for T cell recognition. This could contribute to the contrasting results in which catheter-based delivery of AAV vector encoding human SERCA2a to the myocardium does not elicit detectable cellular immune

responses (Kaplitt *et al.*, 1996; Kawase *et al.*, 2008; Raake *et al.*, 2008; Jaski *et al.*, 2009).

Heart failure itself may alter immune responses to AAV delivery. In this study, dogs in heart failure produced lower anti-AAV titers than normal dogs. This observation may parallel those studies in which humans with heart failure produced lower viral titers after influenza vaccination when compared with healthy control subjects (Vardeny *et al.*, 2009). We did not assess differences in cellular responses to direct cardiac injection of AAV as a function of heart failure presence or absence. However, we note that the pathology inflammation score for normal dogs 12 weeks after receiving virus was at least as severe as that observed in heart failure dogs 6 weeks after virus delivery.

Although the phase 1/2 CUPID trial (Jaski *et al.*, 2009; Jessup *et al.*, 2011) has not reported significant inflammatory or infiltrative responses to AAV delivery, it is also clear that large animal and human clinical trials using AAV can display therapy-limiting immune responses. A clinical trial using hepatic delivery (portal vein infusion) of AAV2 expressing factor IX (AAV2-hF.IX) observed a transient elevation and subsequent decrease in serum factor IX, accompanied by an increase (and eventual fall) in serum liver enzymes, suggesting cell-mediated destruction of liver cells loaded with AAV antigens (Manno *et al.*, 2006). Subsequent studies demonstrated the presence of CD8⁺ T cells reactive to AAV capsid epitopes (Mingozzi and High, 2007; Mingozzi *et al.*, 2007) and indicated that human hepatocytes containing even extremely low levels of MHC class I molecules loaded with AAV capsid-derived peptides are targets for cytotoxic T cell destruction (Pien *et al.*, 2009), suggesting that AAV capsid epitope presentation by MHC class I molecules is an important pathway for AAV-induced cytotoxic T cell responses. Anti-AAV T cell responses and antibody production, and progressively diminishing vector-encoded gene expression, occurred after intramuscular injection of patients with AAV1 encoding lipoprotein lipase-1 or α_1 -antitrypsin (AAT), although persistent low-level AAT expression was observed for months in some patients (Brantly *et al.*, 2009). The severity of immune responses to AAV encoding factor IX in hemophiliac dogs, and the duration of factor IX expression, depended at least in part on the route of AAV delivery and the development of anti-factor IX antibodies (Niemeyer *et al.*, 2009). Thus the immune response to AAV-based vectors remains a significant issue so as to maximize safety and efficacy of AAV-based gene delivery (Mingozzi and High, 2007).

In recognition of this issue, prior studies have demonstrated the usefulness of transient and/or long-term immunosuppression in achieving longer duration, and higher level expression of the AAV-encoded transgene (Herzog *et al.*, 2001, 2002; Yuasa *et al.*, 2007). In a canine muscular dystrophy model, transient immunosuppression (using antithymocyte globulin, cyclosporine, and mycophenolate mofetil) started before AAV delivery allowed prolonged AAV-mediated microdystrophin expression 3 months after withdrawal of all immunosuppression (Wang *et al.*, 2007b). Without immunosuppression, AAV-encoded transgene expression was diminished soon after intramuscular injection because of the T cell responses (Wang *et al.*, 2007a,b). In our current study, limited immunosuppression, using prednisone and cyclosporine starting 4 weeks after the vector

administration, reduced inflammation (and AAV NAb titer) and preserved hSERCA2a expression. Interestingly, there was no substantial change in AAV genome copy number regardless of the level of hSERCA2a expression or presence of immunosuppression (Fig. 3). This may reflect, at least partially, the previously observed “shutdown” of viral promoters, such as the cytomegalovirus (CMV) promoter, in virus-mediated gene delivery constructs, and may suggest a role for the inflammatory response in the process (Löser *et al.*, 1998).

We acknowledge several limitations to this study. Because of the difficulties in maintaining tachycardic-paced dogs for more than 6 weeks, cardiac functional and immune responses in heart failure animals were limited to that period, and thus we do not know how long improved cardiac function might be maintained. We assessed human SERCA2a expression, immune responses, and the effect of immunosuppression at longer intervals after virus delivery to normal dogs. Whether immunosuppression of AAV6-hSERCA2a-treated heart failure dogs would enhance cardiac function due to enhanced hSERCA2a expression remains to be determined. It is also not clear whether the immunological changes associated with heart failure may impact the immune response to AAV vectors. We did not directly confirm the cellular response to AAV capsid as a mechanism of cardiac infiltration and decay of hSERCA2a expression. However, (1) prior studies have documented activation of cytotoxic T cells in large animals (including canines) by AAV capsid-derived antigens, and (2) the high degree of conservation between human and canine SERCA2a (>99% amino acid residues) lessens the likelihood of canine immune responses to human SERCA2a. Indeed, no humoral response to hSERCA2a was detected, and the assayed human-specific hSERCA2a peptides did not elicit a detectable cellular response as indicated by interferon- γ production. However, we cannot rule out reactivity below our level of detection, or a cellular response not revealed by IFN- γ production.

In summary, we observed that long-term expression of hSERCA2a from an AAV6-based vector can improve cardiac function in a large animal model of heart failure, but is accompanied by cellular infiltrates, humoral responses to AAV, and an eventual decrease in hSERCA2a expression. This may limit efficacy and raise safety issues, and may have implications for human SERCA2a trials. The humoral and cellular infiltrative immune responses elicited by the virus can be blunted by immunosuppressive therapy, and was associated with prolonged hSERCA2a expression. Such immunosuppressive therapies could prove to be a beneficial adjuvant to AAV-based interventions for human heart failure.

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Author Disclosure Statement

The authors have no conflicts of interest to declare.

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Address correspondence to:

Dr. Barry London
UPMC Cardiovascular Institute
University of Pittsburgh
Scaife S-572, 200 Lothrop Street
Pittsburgh, PA 15213

E-mail: londonb@upmc.edu

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