Prevention of Cardiac Dysfunction in Acute Coxsackievirus B3 Cardiomyopathy by Inducible Expression of a Soluble Coxsackievirus-Adenovirus Receptor

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Background—Group B coxsackieviruses (CVBs) are the prototypical agents of acute myocarditis and chronic dilated cardiomyopathy, but an effective targeted therapy is still not available. Here, we analyze the therapeutic potential of a soluble (s) virus receptor molecule against CVB3 myocarditis using a gene therapy approach.

Methods and Results—We generated an inducible adenoviral vector (AdG12) for strict drug-dependent delivery of sCAR-Fc, a fusion protein composed of the coxsackievirus-adenovirus receptor (CAR) extracellular domains and the carboxyl terminus of human IgG1-Fc. Decoy receptor expression was strictly doxycycline dependent, with no expression in the absence of an inducer. CVB3 infection of HeLa cells was efficiently blocked by supernatant from AdG12-transduced cells, but only in the presence of doxycycline. After liver-specific transfer, AdG12 (plus doxycycline) significantly improved cardiac contractility and diastolic relaxation compared with a control vector in CVB3-infected mice if sCAR-Fc was induced before infection (left ventricular pressure 59±3.8 versus 45.4±2.7 mm Hg, median 59 versus 45.8 mm Hg, P<0.01; dP/dt max 3645.1±443.6 versus 2057.9±490.2 mm Hg/s, median 3526.6 versus 2072 mm Hg/s, P<0.01; and dP/dt min −2125.5±330.5 versus −1310.2±330.3 mm Hg/s, median −2083.7 versus −1295.9 mm Hg/s, P<0.01) and improved contractility if induced concomitantly with infection (left ventricular pressure 76.4±19.2 versus 56.8±10.3 mm Hg, median 74.8 versus 54.4 mm Hg, P<0.05; dP/dt max 5214.2±1786.2 versus 3011.6±918.3 mm Hg/s, median 5182.1 versus 3106.6 mm Hg/s, P<0.05), respectively. Importantly, hemodynamics of animals treated with AdG12 (plus doxycycline) were similar to uninfected controls. Preinfection induction of sCAR-Fc completely blocked and concomitant induction strongly reduced cardiac CVB3 infection, myocardial injury, and inflammation.

Conclusion—AdG12-mediated sCAR-Fc delivery prevents cardiac dysfunction in CVB3 myocarditis under prophylactic and therapeutic conditions. (Circulation. 2009;120:2358-2366.)

Key Words: receptors ■ viruses ■ myocarditis ■ gene therapy ■ coxsackieviruses

Coxsackievirus B3 (CVB3), a member of the enterovirus group of Picornaviridae, is one of the most commonly identified infectious agents associated with acute and chronic myocarditis; it can also mediate infectious pancreatitis and meningitis. Acute enteroviral myocarditis may not lead to initial mortality, but there is strong evidence to show that it often leads to the insidious development of dilated cardiomyopathy. Currently, enteroviral myocarditis is treated nonspecifically by palliative care, because no effective antiviral therapy is available. CVB load, replication, and persistence are directly associated with cardiac injury and progression of the disease. A direct cytopathic effect of CVB in vitro and the induction of cardiac injury in immunodeficient mice in vivo support the significance of direct virus-mediated cardiac injury in disease pathogenesis. Specific targeting of CVB in viral myocarditis, therefore, will not only abrogate virus-mediated direct cardiac damage but will diminish immune response-mediated damage by blocking viral spread to uninfected tissue.
Clinical Perspective on p 2366

The coxsackievirus-adenovirus receptor (CAR) mediates cellular attachment for adenovirus subtypes A and C through F and is essential to permit infection of all 6 serotypes of CVB.5 CAR is a member of the immunoglobulin superfamily that consists of 2 extracellular immunoglobulin-like domains (D1 and D2), a transmembrane domain, and an intracellular tail of variable length.6 The N-terminal D1 domain has been shown to bind both adenovirus fiber knob protein and the canyon structure of CVB capsids.7,8 Soluble decoy viral receptors have been found to efficiently inhibit infection by rhinoviruses, measles viruses, and adenoviruses.9–11 Soluble CAR (sCAR) proteins inhibit CVB infection of susceptible target cells in vitro and in vivo.12,13 The interaction of CVB3 with sCAR leads to formation of altered particles that are characterized by loss of VP4 from the virion shell and subsequent irreversible loss of infectivity.14,15 However, severe side effects, with increased cardiac inflammation and heart injury, have been observed after treatment of CVB3-infected mice with CAR4/7, a native sCAR variant with an intact CVB3-binding D1 domain, half of the D2 domain, and a 23–aminocarboxy–C-terminal.16

Previous investigations have shown that dimeric sCAR, expressed as an immunoglobulin Fc-region fusion protein, has reduced systemic clearance and increased virus-neutralizing capacity relative to monomeric sCAR and does not induce undesirable side effects.14 Therefore, the aim of the present study was to create an in vivo delivery system that would express sCAR-Fc in the liver for systemic release under the tight control of an inducible promoter. An adenoviral vector (AdV) was constructed that only expressed sCAR-Fc in the presence of doxycycline (Dox). It was our primary hypothesis that delivery of sCAR-Fc via a recombinant adenovirus expression vector to CVB3-infected animals would inhibit myocardial CVB3 infection and improve cardiac function relative to CVB3-infected animals treated with a vector control. We used hemodynamic indices as the best measure of cardiac function and histological measurements to monitor cardiomyopathy after CVB3 infection.

Methods

Coxsackievirus B3
In vitro and in vivo experiments used the genetically characterized, cardiovirulent Nancy strain of CVB3.17 Methods detailing virus propagation and titration of CVB3 in HeLa cells, as well as storage at −80°C, before infection of cells or animals have been published previously.13

Development of AdVs
sCAR-Fc was generated by fusion of the extracellular domain of human CAR with the carboxy terminus of the human IgG1 Fc coding region and inserted into an adenoviral shuttle plasmid that contained improved elements of the Tet-On gene expression system in different configurations. AdVs were generated as described previously.18 Details of cloning strategies and adenoviral generation are provided in the online-only Data Supplement.

Cell Cultures, Northern Blot, Western Blot, Virus Plaque Assays, and IgG ELISA
HeLa (human cervical carcinoma) cells and HEK293 (human embryonic kidney) cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco BRL, Karlsruhe, Germany) supplemented with 10% FCS and 1% penicillin/streptomycin. Northern and Western blot analysis and virus plaque assays were performed as described previously.19 Human IgG ELISA (Bethyl Laboratories Inc, Montgomery, Tex) for detection of the Fc tail of sCAR-Fc was performed according to the supplier’s instructions. Details are provided in the online-only Data Supplement.

Murine CVB3 Myocarditis
AdG12 was injected into the jugular vein of 6- to 8-week-old BALB/c mice. Two days after AdV injection, mice were infected with 5×10^9 plaque-forming units (pfu) of CVB3 intraperitoneally. Doxycycline (200 µg/ml) was administered orally to the mice via drinking water 2 days before CVB3 infection (prophylactic approach) or concomitantly with or 1 day after CVB3 infection (therapeutic approach). The water was replaced daily by fresh water that contained doxycycline. Seven days after CVB3 infection, the hemodynamic parameters of the mice were analyzed as described previously,19 then blood was taken and organs were harvested for histopathological analysis. CVB3 positive-strand genomic RNA in tissues was detected by in situ hybridization with single-stranded 32P-labeled RNA probes as described previously20 or standard plaque assay for CVB3 as described previously.19 All animals investigated in the present study were inbred male mice with identical genomes. For this reason, no special randomization algorithm was used. The investigation was performed in accordance with the principles of laboratory animal care and the German law on animal protection.

Statistical Analysis
Statistical analysis of the in vivo measurements was performed with SAS version 9.1 (SAS Institute Inc, Cary, NC). Nonnormally distributed parameters (left ventricular pressure [LVP], dP/dt max, dP/dt min, and virus load in the heart) are described as mean, SD, median, and 25th and 75th percentiles. Prespecified 2-group comparisons [sham (−Dox) versus sham (+Dox), AdG12 (−Dox) versus AdG12 (+Dox)] of the medians of nonnormally distributed data were performed by Mann–Whitney U test. The option “exact” for small sample sizes was selected (procedure: npar1way). Fisher’s exact test was used for analysis of differences between myocarditis scores. Differences were considered significant at P<0.05.

Results

Doxycycline-Dependent Regulation of sCAR-Fc Therapy
To achieve doxycycline-dependent sCAR-Fc expression, 2 AdVs were constructed. Each AdV contained 2 expression cassettes, 1 for constitutive expression of the second-generation reverse tetracycline transactivator rtTA-M2,21 the other for expression of sCAR-Fc from the improved second-generation tetracycline response promoter tight.22 The expression cassettes were inserted either in tandem orientation (AdR4) or in opposite orientations (AdG12) into the E1 region of an E1-deleted, E3-deleted adenovirus 5 backbone (Figures 1A and 1B). To analyze doxycycline-dependent regulation of AdV-mediated sCAR-Fc expression, HeLa cells were transduced with AdR4 or AdG12 and cultured in the presence or absence of doxycycline. Northern blot analysis found sCAR-Fc mRNA expression to be strictly doxycycline dependent, whereas rtTA-M2 expression was constitutively high (Figure 2A). However, as detected by phosphorimaging of Northern blots (Figure 2A), doxycycline-induced sCAR-Fc mRNA expression was up to 6-fold higher in AdG12- than in AdR4-transduced cells. Therefore, AdG12 was selected for further in vitro and in vivo studies.

sCAR-Fc protein was only detectable in AdG12-transduced cells and in the cell culture supernatant in the presence of
doxycycline (Figure 2B, left). As expected, Western blot analysis (under nonreducing conditions) confirmed that sCAR-Fc was expressed as a dimeric protein (Figure 2B, right). Furthermore, rapid doxycycline-dependent on/off switching of sCAR-Fc expression from AdG12 could be confirmed (Figure I in the online-only Data Supplement).

Inhibition of CVB3 Infection by AdG12 In Vitro

Next, we studied sCAR-Fc–mediated inhibition of CVB3 in vitro as a function of AdG12 dose, doxycycline concentration, and the dose of CVB3. Transduction of HeLa cells with 5 multiplicities of infection (MOI) of AdG12 and induction with doxycycline 500 ng/mL for 48 hours were sufficient to prevent CVB3 infection in sCAR-Fc–expressing cells completely. Under these transducing conditions, sCAR-Fc expression levels reached a maximum with 29.4 μg/mL in the cell culture supernatant. sCAR-Fc expressed from AdG12 efficiently blocked CVB3 doses of up to 2.5 MOI (Figures IIA through IID in the online-only Data Supplement). Therefore, sCAR-Fc expressed by AdG12-transduced cells efficiently inhibited CVB3 infection of these cells, and secreted sCAR-Fc levels were directly related to initial AdG12 MOI and doxycycline concentration.

To assess the potential of AdG12 to suppress ongoing infections, which represents the typical situation encountered in the clinical setting, HeLa cells were transduced with AdG12, and sCAR-Fc expression was induced with doxycycline at different times relative to CVB3 infection. Expression of sCAR-Fc 48 and 24 hours before CVB3 infection resulted in complete inhibition of CVB3 infection in the transduced cells. The inhibitory efficiency of sCAR-Fc was gradually reduced the later the sCAR-Fc expression was induced. However, even if sCAR-Fc expression was induced 24 hours after infection, CVB3 progeny virus number was still reduced approximately 10^6-fold compared with controls without sCAR-Fc (Figure 3), which demonstrates the high efficacy of sCAR-Fc in ongoing CVB3 infections.

Preinfection sCAR-Fc Gene Therapy Prevents Cardiac Dysfunction and Inflammation

Encouraged by the in vitro data, we tested the ability of AdG12-transduced mice to inhibit CVB3-mediated myocar-
Mice were transduced with AdG12, and sCAR-Fc expression was induced and maintained via constant oral doxycycline administration. The AdG12 dose was reduced to 1 × 10^10 virus particles per mouse because initial experiments with 3 × 10^10 particles of AdG12 (Figure IIIA in the online-only Data Supplement) resulted in sCAR-Fc serum levels that easily exceeded therapeutic levels, previously identified to be <100 ng/mL.11 Two days after vector transduction, animals infected with 5 × 10^4 pfu of CVB3 (Figure 4A). At the point of CVB3 infection, circulating sCAR-Fc concentrations were 228.5 ± 174 ng/mL, and 7 days later, when mice were euthanized, sCAR-Fc concentrations were 99.63 ± 22.7 ng/mL. No sCAR-Fc was measured in the AdG12-transduced mice that did not receive doxycycline, which were identical to animals that did not receive AdG12. CVB3-infected mice that did not receive AdG12. CVB3-infected animals transduced with AdG12 had significantly improved cardiac contractility and diastolic relaxation compared with CVB3-infected animals transduced with AdG12 in the absence of doxycycline (LVP 59 ± 3.8 versus 45.4 ± 2.7 mm Hg, median 45.8 versus 45.5 mm Hg, P < 0.01; dP/dt_max 3645.1 versus 2057.9 ± 490.2 mm Hg/s, median 3526.6 versus 2072 mm Hg/s, P < 0.01; dP/dt_min 2125.5 ± 330.5 versus −1310.2 ± 330.3 mm Hg/s, median −2083.7 versus −1295.9 mm Hg/s, P < 0.01). Importantly, hemodynamics of CVB3-infected animals treated with AdG12 (+Dox) were similar to noninfected control animals (Figure 4B). For statistical details, see Tables IA and IB in the online-only Data Supplement. Myocardial histological grading revealed extensive areas of damage, with myocyte necrosis and infiltration of mononuclear cells in CVB-infected control mice and in CVB3-infected AdG12 (Dox) mice (myocarditis score of 3 to 4 in both groups). Cell damage and inflammation were completely absent in the AdG12 (Dox) group (myocarditis score = 0) and showed histology comparable to that of hearts of noninfected control mice (Figure 4C).

Preinfection sCAR-Fc Gene Therapy Inhibits CVB3-Mediated Cardiomyopathy and Pancreatitis

To document whether the absence of pathological changes of the heart correlated with cardiac CVB3 infection, we performed radioactive in situ hybridization experiments to visualize the presence of positive-strand CVB3 RNA at the cellular level with a high sensitivity. Animals in the AdG12 (+Dox) CVB3-infected group did not show any CVB3-infected cells in the heart and no (Figure 4D) or minimal (results not shown) levels of CVB3 RNA in the pancreas,
which is the primary site of CVB replication and the most susceptible organ for CVB infection in mice. In contrast, CVB3-infected and AdG12 (−Dox) CVB3-infected mice showed high prevalence of CVB3 RNA in the heart and pancreas. In other organs (spleen, liver, kidney, intestine, and lung), CVB3 RNA was undetectable in AdG12 (−Dox) and in the control groups by in situ hybridization (Figure 4D). Thus, sCAR-Fc efficiently protected mice from virus entry and subsequent replication in the heart and other organs.

Therapeutic sCAR-Fc Gene Therapy Improves Cardiac Contractility and Reduces Cardiomyopathy

We next analyzed the efficacy of sCAR-Fc gene therapy in a therapeutic approach. Mice were transduced with AdG12, and doxycycline-mediated induction of sCAR-Fc was delayed either to be concomitant with CVB3 infection (2 days after transduction) or to occur 1 day after CVB3 infection (day 3 after transduction; Figure 5A). By use of this experimental approach, low levels of sCAR-Fc (28.4 ng/mL) were just detectable at 16 hours after induction (ie, either 16 hours or 40 hours after CVB3 infection for the 2 groups, respectively; Figure IIIB in the online-only Data Supplement). Body weights for the concomitant-induction group were reduced by an average of 4%, whereas body weights for the 1-day delayed-induction group decreased by an average of 14% and that of untreated CVB3-infected controls decreased by an average of 18% by day 7 after infection (Figure IVB in the online-only Data Supplement). Compared with CVB3-infected animals transduced with the control vector AdG12nuac, which did not express sCAR-Fc, induction of sCAR-Fc concomitant with CVB3 infection led to significantly improved cardiac contractility (LVP 76.4 ± 19.2 versus 56.8 ± 10.3 mm Hg, median 74.8 versus 54.4 mm Hg, P < 0.05; dP/dtmin 5214.2 ± 1786.2 versus 3011.6 ± 918.3 mm Hg/s, median 5182.1 versus 3106.6 mm Hg/s, P < 0.05), with values that surprisingly were in the range of uninfeeted control animals. The diastolic relaxation in the CVB3-infected group with concomitant sCAR-Fc expression showed no significant improvement relative to control vector–transduced animals but revealed a clear trend toward improvement (dP/dtmin −3756.6 ± 1418 versus −2212 ± 745.7 mm Hg/s, median −3624.3 versus −2217.43 mm Hg/s, P = 0.073). However, animals with induced sCAR-Fc expression 1 day after CVB3 infection did not show improved hemodynamics (Figure 5B).

For statistical details, see Tables IIA and IIB in the online-only
Data Supplement. Myocardial pathological grading revealed reduced myocarditis scores in both the concomitant and postinfection sCAR-Fc treatment groups (Figure 5C) and significantly decreased infectious CVB3 titers in the heart compared with CVB3-infected control animals (Figure 5D). For statistical details, see Tables IIC and IID in the online-only Data Supplement. However, all animals in these groups with sCAR-Fc expression after CVB3 infection showed high pathological scoring and inflammatory damage to the pancreas (pancreatitis score 3 to 4; not shown), which indicates that only prophylaxis was capable of saving the pancreas from destruction.

Discussion

In the present study, we report inhibition of CVB3 infection by a sCAR-Fc fusion protein expressed from the newly developed doxycycline-regulated adenovector AdG12. sCAR-Fc expressed from AdG12 inhibited CVB3 infection of susceptible target cells in vitro and inhibited CVB3...
infection of the myocardium in vivo. As a consequence, sCAR-Fc prevented heart injury and cardiac inflammation and prevented the development of systolic and diastolic cardiac dysfunction in CVB3-infected animals.

Previous reports demonstrated inhibition of CVB3 myocarditis through application of purified recombinant sCAR-Fc or through delivery of sCAR-Fc from plasmids. However, plasmid-based transgene expression in vivo is inefficient owing to low-level and transient protein expression, which severely limits its use in acute CVB3 infections with high virus load. Furthermore, methods that are used to enhance plasmid uptake, eg, electroporation, appear to be inapplicable for therapeutic use in humans. Generation of purified recombinant proteins is time consuming and expensive, and ex vivo production of the proteins in bacterial or mammalian cells runs the risk of copurifying proinflammatory substances (ie, lipopolysaccharide) or altering the glycosylation of proteins that accelerate their systemic clearance. Moreover, repeated readministration frequently is necessary to maintain therapeutic protein levels for both the plasmid-based and the recombinant protein approach.

Therefore, we focused on the development of a gene therapy approach that used an AdV as a carrier to deliver sCAR-Fc. This viral vector type enables fast, strong, persistent expression of therapeutic genes in vivo. Intravenous AdG12 application resulted in a typical expression profile of sCAR-Fc after its doxycycline-mediated induction with the highest sCAR-Fc serum levels at days 4 and 5 after induction. Most importantly, sCAR-Fc was already detectable in the serum 16 hours after induction with doxycycline, and serum levels rapidly increased to >100 ng/mL within 1 day after transduction. These levels exceeded serum sCAR-Fc levels measured after plasmid-based gene transfer, and moreover, they exceeded the sCAR-Fc serum concentration that has been shown to be required to protect animals from CVB3-induced myocarditis. In addition, if the AdG12 dose of 1×10^{10} particles used for mice is adapted to human weight, it would be within the range of AdV doses previously used in clinical trials, which suggests that therapeutically relevant sCAR-Fc serum levels could be produced in humans after AdG12 treatment.

CVB3 infection is known to evoke severe left ventricular systolic and diastolic failure in humans during acute infection and in the present well-established experimental model. Here, we confirm cardiac dysfunction in CVB3-infected mice as reduced left ventricular contractility and relaxation.

The present study demonstrates for the first time that CVB3-induced cardiac failure can be prevented by sCAR-Fc, because the hemodynamic parameters (LVP, dP/dt_{max}, and dP/dt_{min}) of sCAR-Fc–expressing AdG12 (+Dox)–treated mice were significantly better than those of untreated CVB3-infected mice, almost reaching the values measured in uninfected control animals. The effect was visible when sCAR-Fc was expressed prophylactically, but much more importantly, LVP and dP/dt_{min} were also improved if sCAR-Fc was induced concomitantly with CVB3 infection, which demonstrates a strong potential for therapeutic efficacy of the sCAR-Fc gene therapy approach after CVB3 infection. Although sCAR-Fc induction corresponding to approximately 40 hours after CVB3 infection did not improve heart function or pancreatitis, histological cardiomyopathy grading and myocardial virus load were decreased significantly, which likely would lessen the risk of development of dilated cardiomyopathy. In agreement with previous reports, prolymphatic sCAR-Fc delivery abrogated pancreatic and cardiac inflammation and necrosis and resulted in negligible virus load as assessed by in situ visualization of CVB3 genome presence and parenchymal virus titer. The present study improves on previous methods of delivering sCAR-Fc by showing higher and more prolonged circulating levels of decoy receptor, without the variability of plasmid delivery or expense and potential copurification of contaminants associated with the production of purified recombinant proteins.

The decreased efficacy of sCAR-Fc gene therapy when administered after CVB3 infection likely reflects the inability of sCAR-Fc to penetrate sufficiently into the parenchyma of the heart and pancreas once initial virus infection has been established and the virus predominantly spreads between adjacent cells or perhaps is unable to fully neutralize the initially high levels of CVB3 in the serum that are seen early in infection. The present results were similar to those previously reported that examined administration of purified recombinant sCAR-Fc to CVB3-infected mice, but the present results advance these studies by showing a significant decrease in cardiac inflammation, virus load, and cardiac function if treatment begins within the first 24 hours of CVB infection.

The present in vitro data demonstrate that sCAR-Fc could neutralize up to 10^4 CVB3 particles (results not shown) and reduce CVB3 replication by up to 11 orders of magnitude. In addition to virus-neutralization capacity, we cannot rule out other antiinflammatory effects of sCAR-Fc. Other decoy receptor Fc fusion proteins (eg, CD55-Fc) and purified pooled immunoglobulins have been shown to decrease myocardial lesion area, inflammation, viral replication, and viral progeny synthesis in CVB3 infection in vivo; however, these were not as effective as sCAR-Fc. CAR also has been reported to bind human IgG and IgM, which suggests a further role for CAR in immune system interactions. Future studies examining the ability of sCAR-Fc to modulate cardiomyopathy in mouse models by other nonenteroviral pathogens will be needed to address this possibility.

Extension of the efficacy of sCAR-Fc beyond 24 hours after CVB infection may be achieved by combining viral neutralization with potent antiinflammatory therapies (for example, targeting proinflammatory cytokines, which have also been shown to be potent in inhibiting CVB3-induced myocarditis). Another potential approach would be to combine sCAR-Fc with RNA interference that specifically targets intracellular CVB3 during replication. In this context, we have shown that small hairpin RNAs directed against CVB3 RNA-dependent RNA polymerase were capable of increasing systolic and diastolic function in CVB3-infected animals.

The safety of viral vectors plays an important role in gene therapy applications. AdG12 treatment did not induce side effects in CVB3-infected or uninfected animals. Furthermore, because AdG12 is a first-generation AdV, its presence in the
body is self-limited to several weeks. Moreover, the doxycycline-dependent control of sCAR-Fc expression in AdG12 enables on/off switching and confers the ability to shut off transgene expression at any point after delivery, thereby further increasing its safety. It is a hallmark of AdG12 that there is no leaky sCAR-Fc expression in the absence of the inducer drug, doxycycline. This was achieved through the use of improved key components of the Tet-On gene expression system, the second-generation transactivator (rtTA-M2) and a new response promoter (tight1). Each was shown to be able to reduce background activity of the Tet-On system, in the context of AdVs.32,33 The resistance of tight1 to nonspecific transactivation may be responsible for the lack of leakiness of AdG12. Moreover, the use of improved components of the Tet-On system enables side-by-side insertion of cytomegalovirus-promoter–driven rtTA-M2 and tight1 promoter–driven sCAR-Fc expression cassettes into the adenoviral E1 region, thereby simplifying the construction of doxycycline-regulated AdVs.

Host immune responses often prevent readministration of an AdV of the same serotype.34 This may limit the potential clinical utility of our proof-of-concept approach in cases in which repeated AdV administration would be necessary, eg, to extend the treatment period. However, administration of AdVs derived from different serotypes,35 or the use of significantly less immunogenic vectors that are derived from adeno-associated virus, is an available tactic to overcome this limitation.

In conclusion, here we show therapeutic efficacy in treating CVB3 myocarditis with sCAR-Fc delivered from a pharmacologically regulated AdV. The treatment was highly efficient, without clinically observable side effects, and led to improved hemodynamics and heart function in CVB3-infected animals. For the purpose of the present study, the chosen sample sizes were appropriate to document statistical significance; however, if the prognostic relevance of this treatment, including survival changes, is to be investigated in the future, considerably larger study groups must be used. The combination of the sCAR-Fc approach with gene therapy methods represents a promising new approach for treatment of cardiac CVB3 infections.

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Disclosures

Dr Fechner and S. Pinkert have a patent pending on AdG12 and AdR4, their doxycycline-regulated expression cassettes, and their use for expression of soluble receptor proteins for antiviral therapy. The remaining authors report no conflicts.

References

CLINICAL PERSPECTIVE

Group B coxsackieviruses (CVBs) are the prototypical agents of acute myocarditis in humans, but an effective targeted therapy is not yet available. Acute enteroviral myocarditis may not lead to initial mortality but often results in the insidious development of chronic dilated cardiomyopathy and terminal heart failure. Here, we analyze the therapeutic potential of a soluble (s) virus receptor molecule against CVB3 myocarditis, delivered by use of a gene therapy approach. We generated an inducible adenoviral vector (AdG12) for strictly drug (doxycycline)-dependent delivery of sCAR-Fc, a fusion protein composed of the coxsackievirus-adenovirus receptor (CAR) extracellular domain and the carboxyl terminus of human IgG1-Fc. After intravenous injection, AdG12 significantly improved systolic and diastolic function in CVB3-infected mice if sCAR-Fc expression was pharmacologically induced in a prophylactic or therapeutic setting. Importantly, the hemodynamics of animals treated with AdG12 were similar to those of healthy control animals. With respect to the underlying pathomechanism, sCAR-Fc completely blocked (prophylactic) or strongly reduced (therapeutic) cardiac CVB3 infection and the resultant myocardial injury and inflammation. The strength of the cardioprotective effect and the additional safety feature mediated by the strict drug dependency of the sCAR-Fc expression make this strategy a promising approach for treatment of this life-threatening cardiac disease. It may also serve as a paradigm for analogous treatments of other severe viral infections by systemic delivery of “virus-trap” proteins, generated either by vectors as described here or by direct intravenous infusion of the encoded recombinant protein.
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Supplemental Methods

Construction of Adenoviral Shuttle Plasmids

For construction of sCAR-Fc fusion protein, total cellular RNA was extracted from HeLa cells using TRIzol® Reagent (Invitrogen GmbH, Karlsruhe, Germany) following the manufacturer’s instructions. RNA was reverse transcribed by the use of Superscript RTase (Invitrogen, Carlsbad, USA, CA). The extracellular domain of human CAR was then amplified by PCR using *Nhe*I linked hCARs (5' - cta *agt* gtc gca gcc agc atg gcg – 3') and *Not*I linked human CAR specific primer B618 (5' - ggc *ggc cgc ttt att tgaagg g g – 3'). The resulting fragment was inserted via *NheI/NotI* into the plasmid pTorsten⁰, upstream and in frame with the carboxy terminus of human IgG1 Fc coding region, leading to generation of sCAR-Fc fusion protein containing plasmid pTorsten-sCAR-Fc.

For generation of a Dox-regulated sCAR-Fc expressing AdV, an expression cassette (TRE-tight1-MCS-SV40pA) containing an improved Dox responsive tight1 promoter ², a multiple cloning site (MCS) and a SV40 polyA signal was amplified with the primer pair P1-*AatIII*-SpeIs (5' - aac tga *gac gtc* taa *ctg tct ctc ggt tta ctc atc agt aga ga – 3') and P2-*XhoI*-XbaIs (5' - ccg *ctc gag* gca *gtc tag agc* gag gaa gct aga gcc agt g – 3') from pTRE-tight (Clontech, Palo Alto, CA, USA) and then cloned into pCR®4Blunt-TOPO (Invitrogen). The expression cassette was cut out with SpeI and XhoI and subcloned into the adenoviral shuttle plasmid pZS2-CMV-rtTA ³, in two opposite directions, via a singular XbaI site located downstream of the second-generation reverse tetracycline (tet)-controlled transactivator rtTA-M2 ⁴ The plasmids generated were termed pAdTREtight-od-M2-G (opposite direction to that of the rtTA-M2 expression cassette) and pAdTREtight-sd-M2-R (same direction). sCAR-
Fc was then amplified using the primer pair sCARFc-\textit{Kpn}I (5’- cgg ggt acc cag cat ggc gct cct gct g – 3’) and sCARFc-\textit{Mlu}Ias (5’ - cga cgc gtc gcc att tag gtg acc act aat – 3’) in a 30-cycle PCR with \textit{Pwo} polymerase (Roche, Mannheim, Germany) from pTorsten-sCAR-Fc and cloned into pAdTREtight-od-M2-G and pAdTREtight-sd-M2-R via \textit{Kpn}/\textit{Mlu}I downstream to the TRE-tight1 promoter, resulting in the plasmids pAdG12-sCAR-Fc and pAdR4-sCAR-Fc. pAdG12-sCAR-Fc\textit{trunc} was constructed as pAdG12-sCAR-Fc. Different to pAdG12-sCAR-Fc in pAdG12-sCAR-Fc\textit{trunc} a frameshift mutation at nucleotide position 9 (deletion of a cytosine) was inserted in sCAR-Fc leading to premature translation stop at amino acid 13. Therefore, the vector do not expresses sCAR-Fc.

The correctness of all plasmids was confirmed by sequencing on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

\textbf{Development of Adenoviral Vectors}

The adenoviral shuttle plasmids pAdG12-sCAR-Fc, pAdR4-sCAR-Fc and pAdG12-sCAR-Fc\textit{trunc} were linearized with \textit{Xba}I and ligated to the 5' long arm of \textit{Xba}I-digested E1-E3- adenovirus 5 mutant RR5. Transfection into HEK293 cells and propagation were done as described \textsuperscript{5} generating the adenoviral vectors termed AdG12, AdR4 and AdG12\textit{trunc}. All adenoviral constructs were tested for RCA contamination by PCR as described before \textsuperscript{6} and the viral titres were determined using standard plaque assays on HEK293 cells.

\textbf{Northern Blot}

RNA was isolated using TRiZol\textsuperscript{®} Reagent (Invitrogen GmbH) following the manufacturer’s instructions. 10 µg of total RNA were separated on a 1 % formaldehyde agarose gel and transferred to a Hybond N nylon membrane (Amersham Biosiences, Buckinghamshire, UK). After prehybridization, the membranes were hybridized with a \textsuperscript{[32P]}dCTP-labeled single stranded antisense probe in ExpressHyb Solution (Clontech) following the manufacturer’s instructions. The probe was labelled in a PCR-like reaction as described previously \textsuperscript{7} by use of primer B618 and a DNA fragment comprising the extra
cellular domain of human CAR as template. Hybridized filters were exposed to Kodak Biomax MS film (Integra Biosciences, Fernwald, Germany). The hybridization signal intensity was determined by phosphoimaging in a Fuji Film BAS-1500 imager (Fuji Photo Film, Dusseldorf, Germany).

**Western Blot**

For detection of sCAR-Fc protein, cells were transduced with viral vectors expressing sCAR-Fc or truncated sCAR-Fc and cultured with or without Dox. Supernatants were removed and collected, while cells were lysed with lysis buffer [20 mM Tris, pH 8, 10 mM NaCl, 0.5% (v/v) Triton X 100, 5 mM EDTA, 3 mM MgCl₂]. After heating the cell-lysate and supernatants at 95°C for 5 min, protein was separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen) under denaturating and reducing conditions and transferred onto a PVDF membrane (Bio-RAD Lab. Hercules, CA, USA). For detection of sCAR-Fc, the immunoreactivity membrane was incubated with anti-CAR monoclonal antibody RmcB (Upstate Biotechnology, Lake Placid, NY, USA) in a dilution of 1:200 or anti-GAPDH polyclonal antibody 1:7.500 (Chemicon International, Hofheim, Germany) for 1 h in 5 % dry milk/TBST. After washing, membranes were incubated with secondary goat-anti-mouse (primary antibody: RmcB) antibodies or swine-anti-rabbit antibodies (primary antibody: anti-GAPDH) conjugated with peroxidase (DAKO GmbH, Hamburg, Germany) at a dilution of 1:10.000. To detect the IgG1-Fc domain of sCAR-Fc, the membrane was stripped with 1.5 % glycine, 1 % SDS, 0.001 volume Tween 20 [pH 2] for 1 h at 55°C and reincubated with horseradish peroxidase conjugated sheep anti-human IgG antibody 1:500 (Amersham Bioscience) in TBST/5 % dry milk for 1 h. Detection by chemiluminescence was achieved using the ECL system (Amersham Bioscience).
Detection of sCAR-Fc Expression *In Vivo* by Use of the IgG specific Enzyme-linked Immunosorbent Assay

Blood samples were taken from vector transduced animals by puncture of the *vena facialis*. The serum was separated by centrifugation at 4,000 rpm for 8 min and stored at -80°C. The serum sCAR-Fc protein levels were determined by a human IgG ELISA kit (*Bethyl Laboratories Inc.*, Montgomery, TX, USA).

**Virus Plaque Assay**

The amount of infectious CVB3 in HeLa cells was determined on monolayers of HeLa cells by agar overlay plaque assay, as described elsewhere. For *in vivo* analysis midventricular portions of the heart from CVB3 infected mice were homogenized in serum-free DMEM, and subsequently analyzed by plaque assay. Results to be shown were gained by two independent experiments, each performed in duplicate.

**Histopathological Analysis**

The apical parts of the hearts were fixed in 4% formalin, embedded in paraffin, and 5-µm sections were cut and stained with hematoxylin and eosin (H&E) to assess myocardial injury and inflammation. To quantify myocardial damage comprising cardiac cell necrosis, inflammation, and scarring, we applied a myocarditis score of 0 to 4 as described (score: 0, no inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes; 2, larger foci >100 inflammatory cells; 3, ≤10% of the cross-section involved; 4, 10 to 30% of the cross-section involved).

**Haemodynamic Measurements**

Left ventricular (LV) function was analyzed using pressure volume loops. As described recently, the animals were anesthetized, intubated, and artificially ventilated. A 1.2 F pressure-volume catheter (*Scisense Inc.*, Ontario, Canada) was positioned in the LV for registration of LV pressure–volume (PV) loops in an open-chest model. Systolic function
was quantified by LV end-systolic pressure (LVP) (mmHg) and dP/dt max (mmHg/s). Diastolic function was quantified by analyzing dP/dt min (mmHg/s).

**In situ Hybridization**

CVB3 positive-strand RNA in tissues was detected using single-stranded $^{35}$S-labeled RNA probes which were synthesized from the dual-promoter plasmid pCVB3-R1. Control RNA probes were obtained from the vector pSPT18. Pre-treatment, hybridization, and washing procedures of dewaxed 5-μm paraffin tissue sections were performed as described previously. Slide preparations were subjected to autoradiography, exposed for 3 weeks at 4°C, and finally counterstained with HE.
Supplemental Tables

Supplementary Table I

A. Prophylactical approach – p-values for haemodynamic parameter comparisons

<table>
<thead>
<tr>
<th>Outcome parameters</th>
<th>LVP</th>
<th>dP/dtmax</th>
<th>dP/dtmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdG12 (-Dox) vs. AdG12 (+Dox)</td>
<td>p=0.0043</td>
<td>p=0.0043</td>
<td>p=0.0087</td>
</tr>
<tr>
<td>sham (+Dox) vs. sham (-Dox)</td>
<td>p=0.0043</td>
<td>p=0.0022</td>
<td>p=0.0649</td>
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</table>

B. Statistical data of haemodynamics of the prophylactical approach

<table>
<thead>
<tr>
<th>group</th>
<th>LVP [mmHg]</th>
<th>dP/dtmax [mmHg/s]</th>
<th>dP/dtmin [mmHg/s]</th>
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<tbody>
<tr>
<td></td>
<td>Q1 median</td>
<td>Q3 median</td>
<td>Q1 median</td>
</tr>
<tr>
<td>AdG12 (-Dox)</td>
<td>6</td>
<td>43.68</td>
<td>45.77</td>
</tr>
<tr>
<td>AdG12 (+Dox)</td>
<td>5</td>
<td>58.24</td>
<td>59.03</td>
</tr>
<tr>
<td>sham (-Dox)</td>
<td>6</td>
<td>57.19</td>
<td>67.49</td>
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<tr>
<td>sham (+Dox)</td>
<td>6</td>
<td>43.15</td>
<td>45.84</td>
</tr>
</tbody>
</table>

N=number of animals; Q1: 25th percentile; Q3: 75th percentile; STD= standard deviation
### Supplementary Table II

#### A. Therapeutical approach – p-values for haemodynamic parameter comparisons

<table>
<thead>
<tr>
<th>Outcome parameters</th>
<th>LVP dP/dtmax dP/dtmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdG12 (+Dox, 0d) vs. AdG12trunc (-Dox)</td>
<td>p=0.048 p=0.0303 p=0.0732</td>
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<tr>
<td>AdG12 (+Dox, 1d) vs. AdG12trunc (-Dox)</td>
<td>p=0.1649 p=0.1282 p=0.1649</td>
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<tr>
<td>sham (+Dox) vs. sham (-Dox)</td>
<td>p=0.0424 p=0.0121 p=0.0242</td>
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</tbody>
</table>

#### B. Statistical data of haemodynamics of the therapeutical approach

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Q1</th>
<th>Median</th>
<th>Q3</th>
<th>Mean</th>
<th>STD</th>
<th>Q1</th>
<th>Median</th>
<th>Q3</th>
<th>Mean</th>
<th>STD</th>
<th>Q1</th>
<th>Median</th>
<th>Q3</th>
<th>Mean</th>
<th>STD</th>
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</thead>
<tbody>
<tr>
<td>AdG12 (+Dox, 0d)</td>
<td>5</td>
<td>61.29</td>
<td>74.83</td>
<td>93.03</td>
<td>76.38</td>
<td>19.18</td>
<td>3722.60</td>
<td>5182.13</td>
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<td>AdG12 (+Dox, 1d)</td>
<td>7</td>
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<td>AdG12trunc (-Dox)</td>
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<td>sham (-Dox)</td>
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<td>471.88</td>
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</table>

N=number of animals; Q1: 25th percentile; Q3: 75th percentile; STD= standard deviation
C. Therapeutical approach – p-values for CVB3 load in the heart comparisons

<table>
<thead>
<tr>
<th>CVB3 titer</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdG12 (+Dox, 0d) vs. AdG12\text{trunc} (-Dox)</td>
<td>=0.0025</td>
</tr>
<tr>
<td>AdG12 (+Dox, 1d) vs. AdG12\text{trunc} (-Dox)</td>
<td>=0.0006</td>
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</table>

D. Statistical data of plaque assays of the therapeutical approach

<table>
<thead>
<tr>
<th>group</th>
<th>N</th>
<th>Q1</th>
<th>median</th>
<th>Q3</th>
<th>mean</th>
<th>STD</th>
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</thead>
<tbody>
<tr>
<td>AdG12 (+Dox, 0d)</td>
<td>5</td>
<td>5.70</td>
<td>294.00</td>
<td>9090.00</td>
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<td>AdG12 (+Dox, 1d)</td>
<td>7</td>
<td>5020.33</td>
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<td>AdG12\text{trunc} (-Dox)</td>
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<td>180380.50</td>
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</tbody>
</table>

N=number of animals; Q1: 25th percentile; Q3: 75th percentile; STD= standard deviation
Supplementary Figure I

+ Dox
0 1 2 3 4

- Dox
1 2 3 4
day

cells
Anti-IgG-Fc
Anti-GAPDH

super-natant
Anti-IgG-Fc
Supplementary Figure II

A

CVB3 in pfu/ml vs. AdG12 in MOI

B

CVB3 in pfu/ml vs. Dox in ng/ml

C

sCAR-Fc in ng/ml vs. Dox in ng/ml

D

CVB3 in pfu/ml vs. CVB3 in MOI

- Dox

+Dox
Supplementary Figure III

A

Serum level of human IgG (ng/ml)

- AdG12 -Dox
- AdG12 +Dox

2d  5d  8d  11d  14d

B

Serum level of human IgG (ng/ml)

- AdG12 +Dox

16h  1d  2d  3d  4d  5d  6d  7d
Supplementary Figure IV

A

- sham (-Dox)
- AdG12 (+Dox)
- AdG12 (-Dox) + CVB3
- sham (+Dox)

mouse weight in %

0d  5d p.i.  7d p.i.

B

- sham (-Dox)
- 0d AdG12 (+Dox)
- 1d AdG12_trunc (-Dox)
- sham (+Dox) + CVB3

mouse weight in %

0d  2d p.i.  4d p.i.  5d p.i.  7d p.i.
Supplementary Figure I. On/off switching of sCAR-Fc expression.
HeLa cells were transduced with AdG12 at a MOI of 2 and incubated with Dox (1 µg/ml). After 24 h (day 0) medium was replaced by fresh medium and cells were cultured for an additional 4 days with Dox (left panel) or without Dox (right panel). During this time, medium was replaced daily with fresh medium. sCAR-Fc was detected in both cells and medium by Western analysis using an anti-IgG-Fc antibody. GAPDH immunoreactivity was used as loading control. sCAR-Fc complete disappeared in the cells and near complete disappeared in the supernatant on day four after withdrawal of Dox in comparison to the samples taken before.

Supplementary Figure II. Inhibition of CVB3 replication in permissive HeLa cells through sCAR-Fc.
(A) Inhibition of CVB3 replication as a function of AdG12 dose. HeLa cells were transduced with AdG12 (MOI 0 to 20) and cultured with Dox (1 µg/ml). Forty-eight hours later cell culture supernatant was harvested, pre-incubated with an MOI of 0.1 of CVB3 for 30 min at 4°C, deposited to the AdG12 transduced cells for 30 min and then replaced with fresh medium. CVB3 replication was analysed by plaque assays after 24 h of culture.
(B) Influence of Dox dose on sCAR-Fc mediated inhibition of CVB3 replication. HeLa cells were transduced with AdG12 at a MOI of 5 and then cultured with Dox in different concentrations (0 - 1 µg/ml) for 48 h. CVB3 neutralization assays were performed as described under (A) above.
(C) Measurement of Dox-dependent expression of sCAR-Fc in cell culture supernatant. HeLa cells were transduced and sCAR-Fc expression induced as described under (B) above. sCAR-Fc expression levels were measured in the cell culture supernatant by IgG ELISA.
(D) Inhibition of CVB3 replication by AdG12 as a function of CVB3 doses escalation. HeLa cells were transduced with AdG12 at a MOI of 5 and incubated in the absence or presence of Dox (1 µg/ml) for 48 h. CVB3 neutralization assays were carried out as described under (A) above.
**Supplementary Figure III. Systemic sCAR-Fc Gene Transfer Supports Inducible sCAR-Fc Delivery in vivo**

(A) Kinetics of individual sCAR-Fc serum level after transduction of mice with AdG12. Balb/c mice (n=7) were injected intravenously with $3 \times 10^{10}$ particles of AdG12. sCAR-Fc expression was induced and maintained in four animals by addition of 200 µg/ml Dox to the drinking water. The other three AdG12-transduced animals did not contain the drug. sCAR-Fc serum concentrations in AdG12 (+Dox) transduced animals roughly doubled from day 2 (254±29 ng/ml) to day 5 (464±159 ng/ml), then decreased at day 8 (147±60 ng/ml), but expression did not decrease further when measured at day 14 (141±51 ng/ml). In the absence of Dox, sCAR-Fc serum levels were indistinguishable from levels in untransduced control mice. Histopathological examination of liver and heart samples did not show any signs of tissue damage and inflammation at various time points (not shown).

(B) Kinetics of sCAR-Fc expression after delayed induction of sCAR-Fc. Balb/c mice (n=6) were injected intravenously with $10^{10}$ particles of AdG12. Measurement of sCAR-Fc expression in the serum revealed absence of sCAR-Fc in the serum two days after AdG12 transduction. Dox (200 µg/ml) was than added to the drinking water to induce sCAR-Fc. sCAR-Fc serum levels reached 28.4 ng/ml 16 h after Dox treatment, increased to 552.7 ng/ml at day 4 after induction and drop to 28.8 ng/ml at day 7 after induction.

**Supplementary Figure IV. sCAR-Fc prevents body weight loss in CVB3 infected animals.** (A) Animals were treated as described in Figure 4A. (B) Animals were treated as described in Figure 5A. The values shown in A and B are mean values. No mortality was observed in any of the groups.
Reference List


