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Combination of soluble coxsackievirus-adenovirus receptor and anti-coxsackievirus siRNAs exerts synergistic antiviral activity against coxsackievirus B3

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ABSTRACT

Coxsackievirus B3 (CVB-3) is a major causative agent of chronic heart muscle infections. The present study describes a cell culture system with an ongoing virus infection to evaluate two novel inhibitory strategies, either individually or combined: (1) RNA interference (RNAi) to degrade cytoplasmatic CVB-3 RNA and (2) a vector-delivered soluble variant of the coxsackievirus-adenovirus receptor fused to a human immunoglobulin (sCAR-Fc), which inhibits cellular uptake of CVB-3. Both approaches were capable of inhibiting CVB-3 in persistently infected human myocardial fibroblasts. The antiviral effect of a single treatment lasted for up to one week and could be extended by repeated applications. Each of the single treatments initially reduced the virus titer by approximately 1-log, whereas the combination of both approaches resulted in 4-log inhibition and retained substantial antiviral activity at later time points, when the effect of sCAR-Fc or siRNAs alone had already disappeared. Further analysis revealed that sCAR-Fc protects cells from virus-induced lysis but does not diminish the virus load. Reduction of the virus titer was only achieved with additional destruction of viral RNA by RNAi. Taken together, combination of RNAi and a protein-based antiviral strategy was found to result in a strong synergistic inhibition of an ongoing virus infection.

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1. Introduction

Enteroviruses such as coxsackievirus, poliovirus and echovirus are small non-enveloped viruses belonging to the picornavirus family (Racaniello, 2007). They possess a single-stranded RNA genome in positive orientation that acts directly as mRNA in infected cells. Picornaviruses are of high clinical relevance (Zeichhardt and Grunert, 2003) with coxsackievirus B3 (CVB-3) being an important member which can cause heart muscle infection. Currently, no specific clinical therapy is available for this class of viruses, although a number of antiviral agents are under development (Chen et al., 2008; De Palma et al., 2008). Among the most advanced small molecular compounds to inhibit picornaviruses are pleconaril and derivatives thereof (Schmidtke et al., 2009), but none of these substances has been approved by the FDA to date.

Acute myocarditis caused by CVB-3 can persist chronically and develop into a dilated cardiomyopathy (DCM) (Pauschinger et al., 1999), which is one of the most frequent causes of heart transplantation. In biopsies of DCM patients persistent enterovirus infections were detected, which were characterized by genomic plus and minus-strand RNA as well as VP1 protein expression but the absence of infectious virus progenies (Andréoletti et al., 2000; Pauschinger et al., 1999). The discovery of enterovirus persistence in the human heart was supported by the observation of coxsackievirus persistence in a murine model of chronic myocarditis (Reetoo et al., 2000). Virus persistence in the heart is also associated with restricted viral RNA and capsid protein synthesis. Strand-specific detection in chronic myocarditis mouse models indicated a change in the ratio of viral plus and minus-strand RNA compared to lytic coxsackievirus infections (Kandolf et al., 1999; Klingel et al., 1992). Further analysis of coxsackievirus-infected mice and cardiac cells showed a deletion in the 5′-UTR region of the virus genome,

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resulting in replication deficient particles containing minus-strand RNA (Kim et al., 2005). In addition, interaction of the virus with the host immune system also plays an essential role for initiation and maintenance of a viral persistence (Oldstone, 1989). Cultured human foetal heart cells infected with CVB-3 showed completely lysed myocytes within a few days, whereas the myocardial fibroblasts survived and multiplied (Kandolf et al., 1985). Continuous production of CVB-3 indicated a carrier state infection of human myocardial fibroblasts.

The host cell receptor for group B coxsackieviruses is the coxsackievirus-adenovirus receptor (CAR) (Bergelson et al., 1997; Freimuth et al., 2008; Tomko et al., 1997). This transmembrane protein is involved in the formation of tight junctions in the endothelium and in cell adhesion. Group B coxsackieviruses become internalized by CAR, which is located in the tight junctions (Coyne and Bergelson, 2005). According to the current model, group B coxsackieviruses initially interact with the decay accelerating factor as a co-receptor on the apical surface of the cells (Coyne and Bergelson, 2006). This interaction induces a signalling cascade, which finally permits virus movement to the tight junction, where the virus becomes internalized by CAR. Various soluble variants of CAR (sCAR) have been discovered, which originate by alternative splicing (Dörner et al., 2004). Just like other soluble virus receptors, several sCAR variants were found to inhibit CVB-3 infection in vitro (Dörner et al., 2004), and in later studies the antiviral activity was confirmed in vivo (Dörner et al., 2006). It can be assumed that sCAR acts as a decoy and saturates epitopes on the virus surface that are essential for the interaction with the cellular receptor. To enhance solubility and extend half-life, the extracellular domain of CAR was fused to the Fc domain of human immunoglobulin G1 (IgG) (Powers et al., 2001). Basically, sCAR-Fc proved to be a potent antiviral tool as it was suitable to protect cells and animals from CVB-3 infection (Lim et al., 2006; Yanagawa et al., 2004). Under therapeutic conditions, however, when animals were treated with sCAR-Fc after CVB-3 infection, the antiviral efficiency decreased substantially (Yanagawa et al., 2004).

Another promising new strategy for the inhibition of viruses is the application of RNA interference (RNAi). This evolutionary conserved mechanism of post-transcriptional gene silencing is triggered by double-stranded RNA molecules, which induce sequence-specific degradation of a target RNA (for recent reviews, see Kim and Rossi, 2007; Kurreck, 2009; Rana, 2007). The mechanism of RNAi has originally been described for the nematode *Caenorhabditis elegans* (Fire et al., 1998). In mammalian cells, double-stranded RNA molecules shorter than 30 nucleotides, known as small interfering RNAs (siRNAs), are usually employed to trigger RNAi without inducing an unspecific interferon response (Elbashir et al., 2001). The siRNAs become incorporated into a protein complex referred to as the RNA-induced silencing complex (RISC), in which the antisense strand of the siRNA acts as a guide to the target RNA, while the sense strand is degraded. After binding of activated RISC, cleavage of the target RNA by the Argonaut 2 protein is initiated. For the design of active siRNAs, thermodynamic features of the duplex (Reynolds et al., 2004) as well as accessibility of the target region have to be taken into consideration (Overhoff et al., 2005; Schubert et al., 2005b; Westerhout and Berkhout, 2007). Comparative studies revealed a much higher efficiency of RNAi approaches as compared to traditional antisense applications (Grünweller et al., 2003).

Among other applications, RNAi has been found to efficiently inhibit viruses (Haasnoot et al., 2007), and clinical trials to treat infections with the respiratory syncytial virus, the human immunodeficiency virus and the hepatitis B virus have already been initiated (Haussecker, 2008). Successful application of RNAi for various enteroviruses was reported, including the inhibition of poliovirus (Gitlin et al., 2005; Saulnier et al., 2006), enterovirus 71 (Lu et al., 2004; Tan et al., 2007), echovirus 30 (Rothe et al., 2009) and CVB-3 in vitro (Ahn et al., 2005; Lee et al., 2007; Schubert et al., 2005a; Werk et al., 2005; Yuan et al., 2005) and in vivo (Fechner et al., 2008; Kim et al., 2007; Merl et al., 2005). Silencing was shown to be mediated via the genomic plus-strand rather than the intermediate minus-strand (Schubert et al., 2007). In addition to the general delivery problem, a big challenge for the development of RNAi-based antiviral approaches is the rapid emergence of escape mutants (Merl and Wessely, 2007; Westerhout et al., 2005), which can be counteracted by the combination of multiple siRNAs. Furthermore, just like for the sCAR-Fc approach, pretreatment with RNAi efficiently protected cells from CVB-3 infections, but the antiviral activity was substantially lower when the curative approach was carried out with an ongoing CVB-3 infection (Fechner et al., 2008).

In the current study, the curative potential of $3D^{pol}$-siRNAs and sCAR-Fc for CVB-3-infected cardiac cells was investigated. In vivo, CVB-3 pathology primarily emerges from the infection of cardiomyocytes. Still, for the present study, we chose a human myocardial fibroblast cell culture that is the only system described so far allowing persistent infection and continuous production of CVB-3 (Harms et al., 2001). Each of the approaches, individual treatment with $3D^{pol}$-siRNA or sCAR-Fc led to a significant decrease of virus proliferation, but most importantly, the combination of both strategies resulted in a considerable synergistic increase of the antiviral effect.

2. Materials and methods

2.1. Cells and virus

The human myocardial fibroblast (HMF) cell line $HMF_{1226K/I}$, HEK293 and HeLa cells (Wisconsin strain; courtesy of Dr. R. Rueckert, Madison) were propagated in monolayer culture in minimal essential medium (MEM) containing 5% heat inactivated fetal calf serum (FCS), 1% antibiotic/antimycotic, gentamicin and non-essential amino acids. Cell lines were propagated at 37 °C in a humidified atmosphere with 5% carbon dioxide. CVB-3 (Nancy, VR-30) was obtained from the American Type Culture Collection (ATCC) and propagated in Vero cells (ATCC). CVA-21 was obtained from the Institute for Clinical and Experimental Virology, Free University Berlin, as an original clinical isolate and was propagated in HeLa cells.

2.2. Coxsackievirus infection of HMF cell line and cell viability assay

For the lytic infection assays, HMF cells were first transfected with siRNAs and/or transduced with AdG12 and inoculated with CVB-3 at a multiplicity of infection (m.o.i.) of 1 plaque forming unit (pfu) per cell in medium without FCS 4 h thereafter for 30 min and maintained in cell culture medium. To generate persistently infected HMF cells, nearly confluent cells were inoculated with CVB-3 at an m.o.i. of 30. Medium was changed every other day. More than 90% of cells died within one week. Single cell clones grew up slowly and during the second week the cells were passaged. Henceforth, the infected cells were propagated by passaging twice a week in medium with a reduced FCS content of 2%. Virus titer in the supernatant was controlled regularly. After storage in liquid nitrogen and subsequent re-culturing, cells still produced high virus titer.

For the experiments, infected cells were seeded in Corning 96 Half Area Well Microplates with a cell growth area of 0.16 cm^2 and maintained for more than one week without passaging. As a measure of cytopathic effects induced by the CVB-3 in these non-subcultured HMF cells, cell viability was determined at several time

points after treatment using the Cell Proliferation Kit II (Roche, Mannheim, Germany) according to the manufacturer's instructions. Measured absorbance at 492 nm correlated directly with cell viability.

2.3. siRNAs and transfection

siRNAs with two nucleotide overhangs used in this study were purchased from MWG Biotech (Ebersberg, Germany). Both, siRNA2 (target sequence CUA AGG ACC UAA CAA AGU U) and siRNA4 (target sequence GUA CAG GGA UAA ACA UUA C), are directed against the 3D RNA dependent RNA polymerase ($3D^{pol}$) of CVB-3 (GenBank acc. no. M33854; target nucleotides 6315–6333 and 6735–6753, respectively). As a control, we used an siRNA designed by Qiagen (Hilden, Germany) with no known homology in the human and viral genome: UUC UCC GAA CGU GUC ACG UTT. Furthermore, two control siRNAs with one mismatch in the center of the target site of siRNA2, siR2mut1 (CUA AGG ACC *A*AA CAA AGU UTT), and three mismatches, siRmut3 (CUA AGG ACC *A*AA C*U*A C*G*U UTT), were used. For transfection, HMF cells were seeded in 24-well plates at a density of 1.2×10^5 cells per ml in a volume of 500 μl without antibiotics. The next day, cells were transfected with 12.5 nM siRNA 2 and 4 or 25 nM control siRNA and 2 μl Lipofectamine™ 2000 (Invitrogen, Karlsruhe, Germany) per well, following the manufacturer's instructions.

The persistently infected cells were plated in a Corning 96 Half Area Well Microplate at a density of 10^5 cells per ml in a volume of 50 μl. These cells were transfected twice on the same day with the siRNA concentrations denoted above using 0.125 μl Lipofectamine™ 2000. The supernatant was replaced by medium 2 h after the first transfection and the second transfection mixture was left on the cells for about 20 h.

2.4. Production of adenoviral vector, transduction and induction

Generation of adenoviral vectors expressing sCAR-Fc (AdG12) is exhaustively described in Pinkert et al. (submitted for publication). The extracellular domain of human CAR (sCAR) was amplified by PCR and inserted into a plasmid, upstream of the carboxy terminus of human IgG1-Fc coding region. Subsequently, the sequence encoding the sCAR-Fc fusion protein was inserted downstream of a tetracycline response promoter Tight1 (Sipo et al., 2006) in the plasmid pZS2-CMV-rtTA (Fechner et al., 2003), which carries a cassette for expression of the reverse tetracycline-controlled transactivator rtTA(s)-M2. The promoter can be induced by tetracycline or its structural analogue doxycycline. HEK293 cells were used for amplification of recombinant adenoviral vector (AdV) and for the determination of the AdV titer as described (Fechner et al., 2000). The adenoviral construct was tested for the absence of contaminations with replication competent adenoviruses (RCA) and purified.

HMF cells were transduced with adenoviral vector at a concentration of 10 m.o.i. by the addition of the required amount to the medium. Immediately after transduction the sCAR-Fc protein production was induced by adding doxycycline (Dox: 1.5 μg/ml). Every second or third day Dox (and medium) was refreshed. When combined with siRNA double-transfections, AdG12 was transduced during the first and Dox was added after the second transfection.

2.5. Determination of CVB-3 titer

The amount of infectious CVB-3 in the supernatant of infected HMF cells was determined on HeLa cells by an agar overlaid plaque assay as described elsewhere (Werk et al., 2005). Shortly, the at least 10-fold diluted samples were incubated for 30 min on HeLa monolayers. Subsequently, cells were overlaid with agar containing Eagle's MEM. After incubation in a humidified atmosphere for two days, cells were stained with neutral red and virus titers were determined by plaque counting.

2.6. Detection of soluble CAR-Fc (sCAR-Fc)

For analysis of sCAR-Fc expression, the supernatants of induced cultures were collected at different time points and stored at −20 °C. sCAR-Fc protein levels were determined with the Human IgG Enzyme Linked Immuno Sorbent Assay (ELISA) Quantitation Kit (Bethyl Laboratories, Montgomery, TX). Following the manufacturer's instructions, MaxiSorb™ (Nunc, Langenselbod, Germany) 96-well plates were coated with a Goat anti-human IgG for 1 h. During the blocking step the collected samples were diluted 1:10 and 100 μl were transferred to the reaction plate. After an additional incubation for 1 h and intensive washing, a Goat anti-human IgG-HRP conjugate in a 1:150 000 dilution was added to each well. After addition of the tetramethyl benzidine (TMB) substrate and sulfuric acid, the oxidized product was measured in a plate reader at 450 nm. As a calibrator, human reference serum in a working range of 3.9–500 ng/ml was used in each assay in duplicate. For calculation of the results we used the calibrator as a standard curve with a four parameter logistic curve-fit.

2.7. Statistics

Statistical analysis was performed by Student's *t* test. Values are shown as mean ± SD of *n* independent experiments. Differences were considered significant at values of $p < 0.05$.

3. Results

3.1. Inhibition of coxsackievirus B3 in lytic model

In previous experiments, siRNAs against the $3D^{pol}$ of CVB-3 or sCAR-Fc released from transfected cells were shown to prevent CVB-3 infection in permissive cells in vitro. The aim of the present study was to determine the antiviral potential of siRNAs and sCAR-Fc under therapy conditions in a cardiac cell line. For a first assessment of the antiviral potential of both strategies, uninfected human myocardial fibroblasts (HMF) were initially pre-incubated with 12.5 nM of each of the siRNAs 2 and 4, both of which are directed against the viral $3D^{pol}$, and inoculated with 1 m.o.i. of CVB-3 4 h thereafter. Virus titer on subsequent days was determined by titration of culture supernatants on confluent HeLa cells. A reduction of more than 1-log was observed after 24 h and lasted for at least three days (Fig. 1). As expected, transduction with the doxycycline-(Dox-) inducible sCAR-Fc expressing adenoviral vector AdG12 did not affect the virus titer in the absence of Dox. Induction of the sCAR-Fc expression by the addition of Dox to AdG12 transduced cells resulted in a 3-log decrease of CVB-3 titer on the first day and up to 6-log lower virus titers on days two and three after infection. The combination of sCAR-Fc expressing adenoviral vector and antiviral siRNAs yielded an additive increase of the inhibitory activities resulting in an almost 7-log reduction of the virus titer. Closer statistical analysis revealed that the antiviral activity of the combination of sCAR-Fc and siRNAs was significantly higher than the inhibitory effect of the sCAR-Fc expressing vector in the presence of a control siRNA or in the absence of an siRNA on day two of the experiment.

Since siRNAs have repeatedly been shown to cause unspecific side effects like the induction of an interferon response (Jackson and Linsley, 2004) further control experiments were carried out to confirm the specificity of the RNAi approach. At first, inhibitory activity of the siRNAs on CVB-3 and the closely related coxsackievirus A21 (CVA-21), which does not contain the target sequences

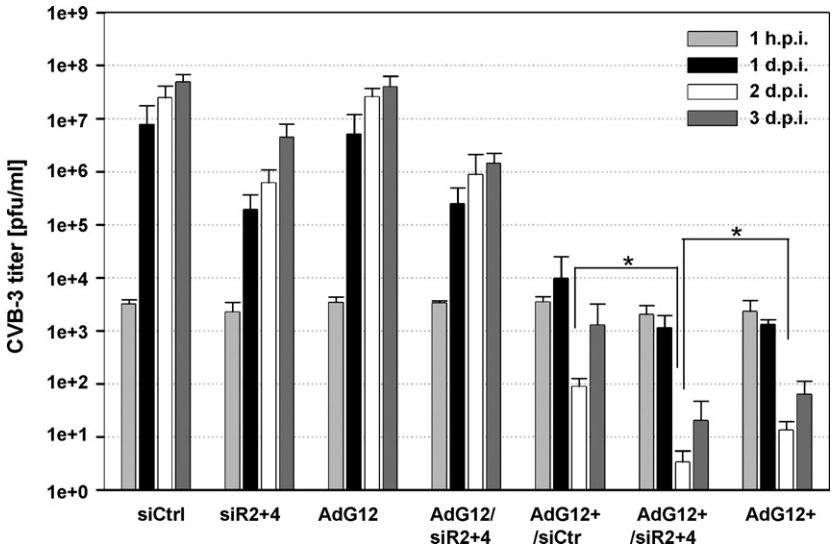

Fig. 1. Relative CVB-3 titer of infected HMF cells in the lytic phase after treatment with siRNAs or sCAR-Fc. Cells were transfected with 12.5 nM of each siRNA and/or transduced with AdG12 with (+) or without addition of Dox. Infection with CVB-3 at an m.o.i. of 1 was carried out 4 h thereafter. The supernatants were collected 1 h (light grey), one day (black), two days (white) and three days (dark grey) after infection with CVB-3 and virus titers were determined on HeLa cells. Mean values ± SD of three independent experiments each performed in duplicate are shown. siCtr: control siRNA; siR2 + 4: siRNA 2 and 4 against $3D^{pol}$ of CVB-3; AdG12: adenoviral vector expressing sCAR-Fc. $^{*}p < 0.05$.

of the siRNAs used in the present study, were compared. As can be seen in Fig. 2A, the siRNAs substantially inhibited CVB-3 to a comparable extent that was found in the experiment shown in Fig. 1. In contrast, the siRNAs did not have any effects on CVA-21. This finding clearly demonstrates that the reduction of the virus titer is a specific RNAi effect rather than an unspecific consequence of the siRNA treatment (like the induction of an interferon response).

Subsequently, additional control siRNAs were used to further investigate the specificity of the approach. Fig. 2B shows that a single point mutation in the center of the siRNA leads to a complete loss of the antiviral activity of the siRNA. The same is, of course, true for a control siRNA that contains three point mutations. These data further corroborate the high specificity of the RNAi approach. They also argue for the use of at least two siRNAs, since a persistently infected cell line can be expected to rapidly accumulate virus mutants that might escape inhibition by a single siRNA.

A further control experiment was carried out to confirm that neither the inducer doxycycline itself nor the adenoviral vector were responsible for the antiviral effect. To this end, a vector was constructed that contains a frameshift in the sCAR-sequence, which leads to a premature termination of translation at amino acid 13. In contrast to the full-length sCAR-Fc protein, this truncated version of the receptor does not enhance the antiviral effect of the siRNA after induction by doxycycline ruling out unspecific effects of either doxycycline or the vector alone (data not shown).

3.2. Set-up of persistently infected myocardial fibroblast cell line

In these experiments as well as in many of the published studies, cell cultures were treated with antiviral compounds prior to an acute infection in order to investigate their potential in vitro, although this prophylactic procedure is a restricted model for a therapeutic intervention. In a medical context, patients have to be cured of pre-existing, sometimes chronic infections. In order to further study the inhibitory activity of the tools against CVB-3, a previously described persistently infected HMF cell system was used as a more relevant model of infection (Harms et al., 2001). The CVB-3 infection in these fibroblasts proceeds through different stages. During the initial lytic infection the majority of the cells die, but some of the remaining cells survive and multiply. This culture

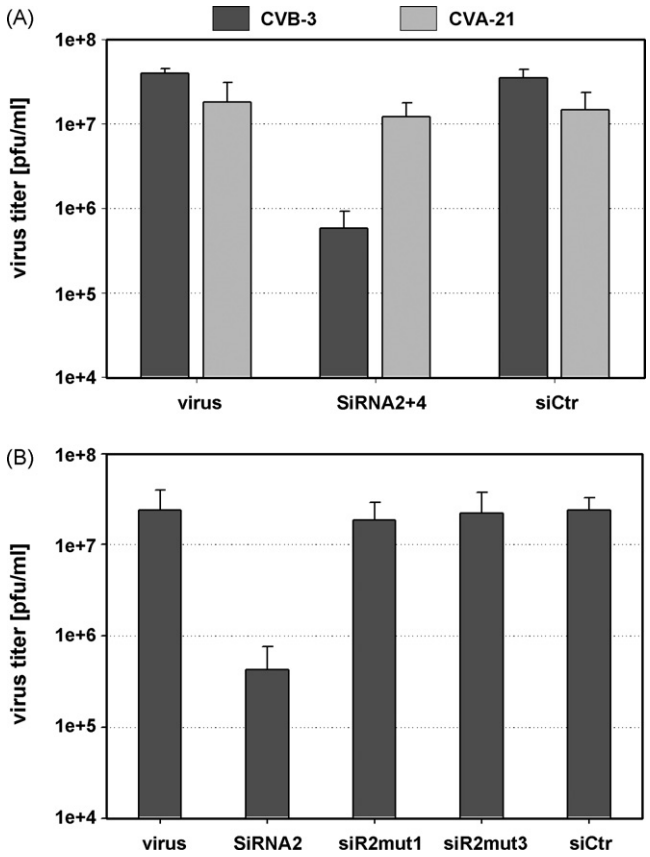

Fig. 2. Specificity of the RNAi approach. (A) HMF cells were pre-treated with siRNAs Nos. 2 and 4 and subsequently infected with CVB-3 and CVA-21, respectively, at a multiplicity of infection (m.o.i.) of 1. Two days after infection, supernatants were collected and virus titers were determined on HeLa cells. (B) Effect of additional control siRNAs on CVB-3 replication. Experiments were carried out with siRNA No. 2, a control siRNA without homology in the human or viral genome (siCtr) as well as with two control siRNAs that carry one (siR2mut1) and three (siR2mut3) mutations, respectively. All sequences are given in Section 2. Cells were treated with siRNAs 4 h prior to infection with CVB-3 and supernatants were collected two days thereafter to determine the virus titer on HeLa cells.

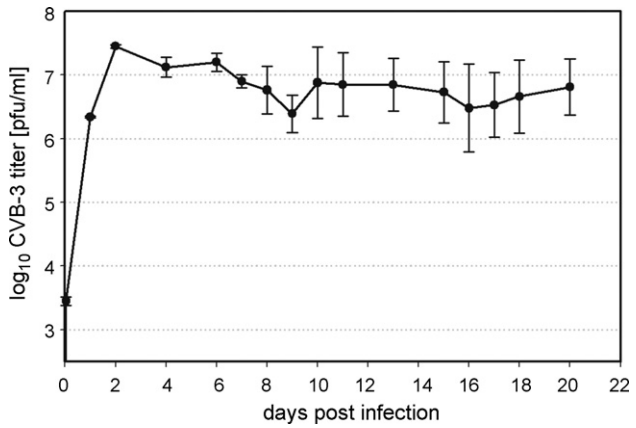

Fig. 3. Virus titer of ongoing CVB-3 infection and persistence up to 20 days post-infection. HMF cells were infected with CVB-3 at a m.o.i. of 1 and subsequently cultured with a change of the medium every second or third day without splitting the cells. At indicated time points the supernatants were harvested and stored at −20 °C. The virus titer was determined on confluent HeLa cells and calculated as plaque forming units (pfu)/ml. Shown are mean values ± SD of two independent experiments each performed in duplicate.

then proliferates and continuously produces high titers of CVB-3, indicating a persistent virus infection.

More specifically, in the time course of establishing a persistent infection, no cytopathic effects (CPE) were observed on day one after infection of the cells, but progeny virus titered on HeLa cells was clearly detectable (Fig. 3). On the next day, a strong CPE became visible and the virus titer reached an even higher level of at least 10^7 plaque forming units (pfu)/ml of culture medium. Within the next six days more than 90% of the infected fibroblasts died. Interestingly, this continuous process did not result in a substantial reduction of the virus titer. Obviously, the remaining cells and the existing virus particles were sufficient to maintain the high titer. Finally, the cells started to divide and constantly produced high CVB-3 titers of up to 10^7 pfu/ml. These persistently infected cells were either kept in culture and used for virus assays or stored at −80 °C. After thawing, CVB-3 infected HMF started to proliferate again without significant alterations in virus production.

3.3. Inhibition of CVB-3 in persistently infected cells

In the next step, we were interested in the antiviral potential of both siRNAs and AdG12 in HMF cells with an ongoing CVB-3 infection. For this purpose, we transfected the persistently infected cells simultaneously with siRNAs 2 and 4 twice. After the treatment, the virus titer decreased by 1-log (Fig. 4A). Comparable results were obtained with sCAR-Fc expressed from AdG12. In contrast to the lytic infection assays described above (Fig. 1), combination of both treatments (siRNAs plus sCAR-Fc) led not only to a slight additive increase of antiviral activity, but rather enhanced virus inhibition to give a 4-log reduction of virus proliferation in persistently infected HMF cells. As can be seen in Fig. 4A, substantial virus inhibition was obtained on day four after the treatment, but the antiviral effect was drastically diminished by the end of the week.

3.4. Time course of soluble CAR production

A possible explanation for this finding is the transient nature of siRNA-mediated silencing as well as temporally restricted production of sCAR-Fc from the adenoviral vector. To investigate the time course of sCAR-Fc expression in persistently infected HMF, the amount of protein in the supernatant was quantified by an ELISA for the detection of human IgG. For these quantifications, supernatants of cells transduced with AdG12 and induced by the addition of Dox were collected and measured. The protein level dropped drastically after day four of the experiment and could be restored by a second transduction with the AdG12 on day six (data not shown). Initially, hardly any difference was observed between cells, which were only transduced with AdG12 in the presence of Dox, and cells, which underwent additional treatment with siRNAs 2 and 4. Owing to the improved cell viability, higher sCAR-Fc levels were detected for the double-treated cells at later time points. Taken together, the time course of secreted sCAR-Fc levels in the supernatant was comparable in both types of experiments and prolonged high-level expression of sCAR-Fc can be achieved by a second transduction of the cells.

3.5. Extension of virus inhibition by repeated treatment

Thus, to compensate for the loss of antiviral impact we transfected and/or transduced the HMF cells again on day six of the experiment. As can be seen in Fig. 4B, a second treatment with siRNAs directed against the virus did not restore a substantial antiviral effect. In contrast, the additional transduction of the cells with AdG12 inhibited virus replication again and led to a 1-log reduction of CVB-3 titer on day 11 of the experiment. For the double-treatment approach with both siRNAs and sCAR-Fc, the titer initially decreased

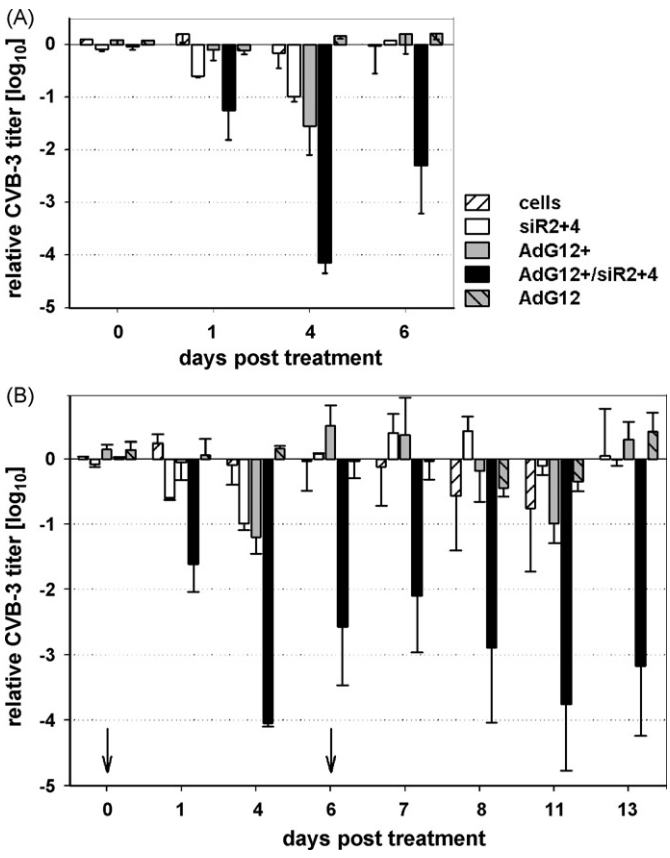

Fig. 4. Change of CVB-3 titer in persistently infected HMF cells treated with siRNAs and/or sCAR-Fc. Cultures were transfected with 12.5 nM siRNAs 2 and 4 twice (white bar) or transduced with the sCAR-Fc expressing vector in the absence (hatched grey bar) or presence of Dox (grey bar). Additional cultures were treated with both, siRNAs 2 and 4 plus the adenoviral vector (black bar). Virus titer of the collected supernatants was determined on HeLa cells. (A) Change of the virus titer after a single treatment. Shown are mean values ± SD of six independent experiments, each performed in duplicate. (B) Change of the virus titer of persistently CVB-3 infected HMF cells after repeated treatment with siRNAs and/or sCAR-Fc. Cultures were transfected and/or transduced on days zero and six of the experiment (arrows). Mean values and standard deviations of three independent experiments, each performed in duplicate, are shown.

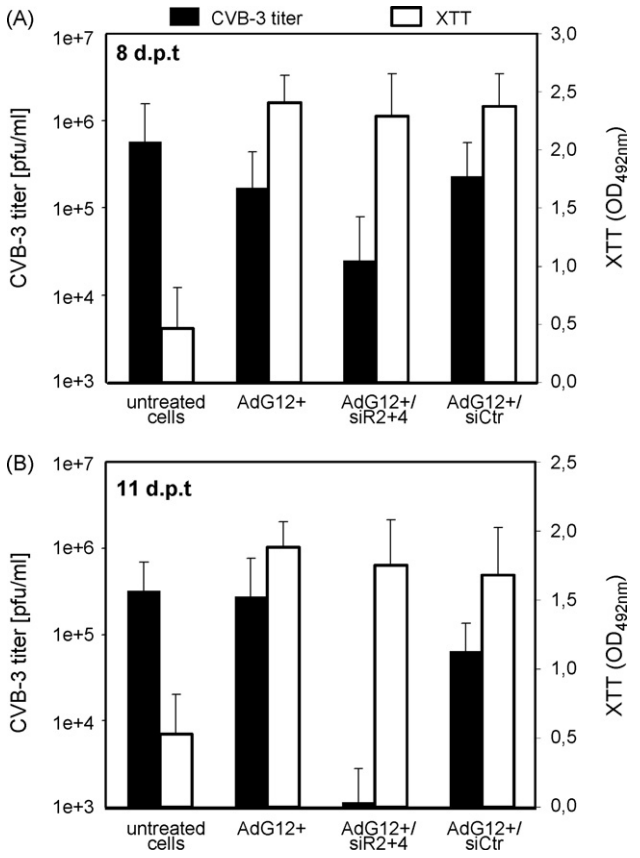

Fig. 5. Virus titer and cell viability of persistently infected HMF cells after two rounds of treatment. Initially, cells were transduced with AdG12 and transfected with siRNAs 2 and 4. Six days after the first treatment cells were transduced with AdG12 again, either without siRNA transfection or in combination with siRNAs (2 and 4) and control siRNA, respectively as indicated. Both virus titer in the supernatants and cells were analysed at day eight (A) and day eleven (B) after the first treatment. Virus titer (black bars) of the supernatant was determined on HeLa cells. XTT absorbance measured at 492 nm (white bars) correlates directly with cell viability. Untreated cells were neither treated during the first nor the second round. Shown are mean values and standard deviations of five independent experiments each performed in duplicate. siCtrl: control siRNA; siR2 + 4: siRNA 2 and 4 against $3D^{pol}$ of CVB-3; AdG12: adenoviral vector expressing sCAR-Fc; the '+' symbol denotes addition of doxycycline.

by approximately 4-logs and then rose again. After the second round of treatment, the titer was reduced by approximately 4-logs again.

3.6. Synergistic effect of soluble CAR and siRNAs

According to these results the combination strategy with siRNAs and AdG12 is considerably more efficient in inhibiting CVB-3 in persistently infected HMF cells than either of the single approaches. Furthermore, repeated treatments are required to maintain inhibition. We wondered, however, whether it will be necessary to repeat the double treatment or if it might be sufficient only to use the adenoviral vector AdG12 for the second administration. To address this question, persistently infected HMF cells were initially treated with both siRNAs and AdG12. As can be seen in Fig. 5, cultures that did not undergo a second round of treatment lost viability (white bar) concomitantly with a high virus titer (black bar) on day eight of the experiment. When cells were transduced with AdG12, cell viability was high as measured by XTT assays. However, despite the protective effect of sCAR-Fc against cell lysis, the virus titer remained comparatively high after the second application of the virus vector. In contrast, combination treatment with AdG12 and siRNAs not only maintained high cell viability, but also substantially reduced the virus titer. A reduction of the virus titer by approximately 1-log was observed on day eight (Fig. 5A), and the effect increased to an approximately 2.5-log inhibition at day eleven of the experiment (Fig. 5B), indicating the beneficial outcome of the double treatment.

4. Discussion

Despite the steadily growing medical importance of virus infections, the prospects for treatment still remain limited. We therefore explored the antiviral potency of new biological strategies to inhibit the heart-pathogenic CVB-3. Soluble variants of CAR bind to CVB-3 proteins required for the interaction with the cellular CAR and will thus prevent virus entry into the cells. In contrast, siRNAs are effective intracellularly and induce cleavage of the viral RNA in the cytoplasm. As a first comparative test, we used an sCAR-Fc fusion protein expressed from an adenoviral vector and siRNAs directed against the $3D^{pol}$ of the virus in the acute phase of HMF infection with CVB-3, i.e. the cells were treated with the antiviral agent prior to infection with CVB-3 which leads to cell lysis in untreated cells. In line with our previous findings obtained with HeLa cells (Werk et al., 2005), siRNAs reduced the virus titer by approximately 1-log. Soluble variants of CAR have been reported to inhibit CVB-3 with great efficiency (Dörner et al., 2004, 2006; Lim et al., 2006; Yanagawa et al., 2004). In agreement with these data, we found a strong, more than 6-log virus inhibition, when expressing sCAR-Fc in HMF cells from an adenoviral vector. The combination of sCAR-Fc and siRNAs led to a slight increase of the inhibitory activity by no more than 1-log, suggesting an additive effect of both strategies in the lytic phase of the infection.

These data confirm previously published findings describing the antiviral activity of the two strategies. It should be noted that these studies also concede that sCAR-Fc as well as vector-mediated RNAi lost their efficacy when applied in a therapeutic set-up, i.e. after virus infection (Fechner et al., 2008; Yanagawa et al., 2004). The latter scenario, however, reflects the situation in patients more adequately, in which the infection occurs first and treatment follows thereafter. This is particularly true for CVB-3 infections of the heart, which can persist for many years and lead to the development of dilated cardiomyopathy, one of the most frequent causes of heart transplantations. It was therefore the aim of the present study to test the antiviral strategies against an ongoing infection. To this end, a previously established in vitro model for chronic infections with persistently infected HMF cells was used (Harms et al., 2001). As described before, most of the cells died within one week after the infection, but some cells survived and developed into a cell culture, which continuously produces high CVB-3 titers in the range of 10^6 to 10^7 pfu/ml. It should be mentioned that CVB-3 changed its plaque morphology during the course of persistent infection. The plaques on HeLa cells from supernatants of persistently infected HMF cells showed a reduced plaque diameter in comparison to those obtained with the initially used native Nancy strain. This finding might indicate a mutated or restricted virus genome (Klingel et al., 1992; Reagan et al., 1984), but, importantly, the virus from persistently infected cells was still susceptible to sCAR-Fc (data not shown), i.e. the CVB-3 capsid structure does not seem to be affected.

Thus, the infected HMF cells can be considered as a model for a persistent CVB-3 infection, which is characterized by up to 10% of the cells being infected with virus or reproducing virus particles (Harms et al., 2001; Heim et al., 1995; Kandolf et al., 1985). During this period, however, cells can also carry viral RNA in the absence of active replication (Andréoletti et al., 2000; Feuer et al., 2002). Several factors, e.g. the cell cycle status, can affect coxsackievirus replication and may lead to reactivation of virus replication resulting in the production of progeny particles (Feuer et al., 2002). Our cell culture system is characterized by continuous virus production and re-infection as can be concluded from the high virus titer mea-

sured from the supernatant. It will be valuable to confirm the results obtained here with cardiomyocytes, but so far human myocardial fibroblasts are the only known cardiac cells that can be persistently infected with CVB-3 and continuously produce high virus titers.

When we transfected persistently infected HMF cells with siRNAs against CVB-3, we again observed an approximately 1-log reduction of the virus titer after four days. Surprisingly, a similar reduction of the virus level was observed with sCAR-Fc. This limited activity (compared to the acutely infected cells) can be explained by the fact that sCAR-Fc can only trap viruses by interacting with capsid proteins. A soluble variant of CAR obtained by recombinant expression was shown to inhibit CVB-3 (Dörner et al., 2006). This treatment can be expected to occur extracellularly. The *in situ* expression strategy of sCAR-Fc followed in the present study might in addition lead to the intracellular neutralization of viruses prior to their release. Regardless of the exact mechanism of action of sCAR-Fc, the persistently infected culture can be expected to contain large amounts of virus genomes inside the cells, which are hidden from sCAR-Fc and can continuously produce high amounts of virus progeny. After cell lysis, the local viral load might be too high to be completely trapped by sCAR-Fc and, as a consequence, virions may infect neighboring cells. This assumption may also explain the synergistic effect that was observed, when AdG12 and siRNA were combined. In this case, a 4-log reduction of the virus was achieved, since sCAR-Fc neutralizes virions, while the siRNAs induce cleavage of virus genomes in the cells.

The RNAi-mediated antiviral activity observed in this experiment declines drastically after approximately one week. Chemically pre-synthesised siRNAs are well-known to inhibit their target transiently and the silencing effect has been observed to vanish after several days in cell culture (Watanabe et al., 2004), most likely due to degradation of the siRNA and dilution of the molecules as a consequence of cell division. It will therefore be necessary to treat the cells repeatedly in order to maintain a high level of inhibitory activity. To this end, we transfected siRNAs twice on day one of the experiment and carried out a second double-transfection six days thereafter (Fig. 4B). We initially observed a good antiviral activity, which declined after four days and was less pronounced after the second transfection. This finding might be explained by the high number of dead, lysed cells that were observed in this experiment at the time of the second transfection (data not shown), which set free large amounts of virus particles. The antiviral effect may therefore be compensated by the increased viral load. In contrast to our experiments, it was reported that two out of three HEp-2 cultures persistently infected with a mutated poliovirus variant could completely be cured after two sets of double treatment with siRNAs (Saulnier et al., 2006). In this study, a mixture of two siRNAs was used to prevent the emergence of escape mutants. Since CVB-3 was also shown to become resistant against a single siRNA within a few days only (Merl and Wessely, 2007) and the control experiments in Fig. 2 show that a single point mutation can lead to a complete loss of the antiviral activity, the strategy to use a mixture of two siRNAs to prevent viral escape was adopted in the present study. The two siRNAs (siR2 and 4) were previously found to possess high antiviral activity (Werk et al., 2005). Interestingly, a closer analysis now revealed that despite comparable initial antiviral potencies, the inhibitory activity of siRNA 4 decreased much faster than that of siRNA 2 (data not shown). We still decided to use a mixture of the two siRNAs since persistently infected cells can be expected to host a mixed virus population and the experiments required up to two weeks under the selection pressure of siRNAs, so that it was advisable to take measures against viral escape.

Just alike the RNAi effect, the antiviral efficacy of sCAR-Fc expression declines four to six days after transduction due to a decrease of protein expression. This observation is in contrast to previous experiments, in which adenoviral vectors expressed high levels of a transgene for up to two weeks in cell culture (Fechner et al., 2007). This discrepancy may be explained by the fact that many AdV transduced cells will be co-infected with CVB-3. Lysis of these cells by CVB-3 will also terminate transgene expression from the adenoviral vector. High levels of sCAR-Fc could still be maintained with a second transduction of AdG12 as determined by anti-IgG ELISA. As a consequence, the inhibitory effect of sCAR-Fc of approximately 1-log was restored at day eleven of the experiment.

While both of the individual treatments, application of siRNAs and expression of sCAR-Fc, only led to a moderate decrease of the viral load, the combination of both approaches resulted in a strong synergistic antiviral effect. Initially, a 4-log reduction of the virus titer was achieved, and even at the end of the experiment (day thirteen) the viral load was diminished by 3-logs relative to the initial virus titer, whereas the single treatments had completely lost their inhibitory activity. Further experiments helped to elucidate the cooperative mechanism of sCAR-Fc and the siRNAs in more detail. After the initial treatment with both approaches, cells were either treated with AdG12 or with a combination of the virus vector and the siRNAs again on day six of the experiment. Soluble CAR maintained high cell viability, but did not substantially reduce the virus titer. This result is somewhat unexpected, since sCAR-Fc was reported to initiate irreversible capsid alteration after binding, leading to A-particle formation (Goodfellow et al., 2005). Further mechanistic studies will be necessary to clarify this point in more detail.

Only the combination of treatment with AdG12 and siRNAs against CVB-3 is suitable to achieve both, an increase of cell viability and a substantial reduction of the virus titer. Both antiviral agents act in a synergistic manner. While sCAR-Fc traps virus particles, siRNAs induce degradation of virus genomes that are present in the cells either at the beginning of the experiment or by entering the cells after circumventing the extracellular shield.

It might be a question whether the use of sCAR-Fc in a therapeutical setting is safe. The sCAR domain in the fusion protein may potentially interact with CAR localized on the cell surface. In preliminary immunochemical analysis with the cardiac HL-1 cell line, however, we did not observe a colocalization of sCAR-Fc and the endogenously expressed, membranous CAR (data not shown), which indicates that this interaction might not be a major problem. Furthermore, no side effects were observed in a number of in vivo studies, in which sCAR-Fc was either applied as recombinant protein (Yanagawa et al., 2004) or expressed from the liver (Pinkert et al., submitted for publication) or the skeletal muscle (Lim et al., 2006) following in vivo gene transfer. Although these studies indicate the safety of the approach, further experiments will be necessary to exclude unwanted side effects.

In recent years, RNAi has been established as a new promising strategy to treat diseases caused by overexpression of deleterious genes and a number of clinical trials based on this technology have already been initiated [summarized in Kurreck, 2009]. In preclinical studies, RNAi has been used to inhibit virtually any type of virus with medical relevance (Haasnoot et al., 2007). Likewise, the potential of soluble variants of cellular receptor has been demonstrated to exert antiviral activity not only for coxsackievirus, but for other types of viruses as well. For example, soluble CD46 was shown to prevent infections with measles virus (Seya et al., 1995) and human herpes virus 6 (Santoro et al., 1999) and a soluble form of ICAM-1 protects cells from rhinovirus infections (Greve et al., 1991). It will thus be worthwhile to assess the combination of soluble variants of virus receptors and siRNAs against the viral genomes for other types of viruses as well.

In summary, our results clearly showed a high antiviral potential of sCAR-Fc and siRNAs against CVB-3 in persistently infected HMF cells. To the best of our knowledge, this is the first study to demonstrate that the combination of a soluble receptor variant and RNAi

results in a synergistic increase in the inhibitory activity. We will now test the double treatment strategies in mouse models with an ongoing virus infection to assess whether this strategy might be a suitable approach to develop a new therapy for patients suffering from virus-induced myocarditis.

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