Research Article

Adenovirus-Mediated In Vivo Silencing of Anaphylatoxin Receptor C5aR

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C5a, one of the most potent inflammatory peptides, induces its inflammatory functions by interacting with C5a receptor (C5aR) that belongs to the rhodopsin family of seven-transmembrane G protein-coupled receptors. C5a/C5aR signaling has been implicated in the pathogenesis of many inflammatory and immunological diseases such as sepsis and acute lung injury. Widespread upregulation of C5aR has been seen at both the protein level and transcriptional level under pathological conditions. Here, we show that C5aR gene expression can be specifically suppressed by siRNA, both in vitro and in vivo. A panel of chemically siRNA oligonucleotides was first synthesized to identify the functional siRNA sequences. The short hairpin RNAs (shRNAs) were also designed, cloned, and tested for the silencing effects in C5aR transfected cells. The effective shRNA expression cassettes were then transferred to an adenovirus DNA vector. ShRNA-expressing adenoviruses were intratracheally administered into mouse lung, and a significant in vivo silencing of C5aR was obtained four days after administration. Thus, C5aR shRNA-expressing adenoviruses appear to be an alternative strategy for the treatment of complement-induced disorders.

INTRODUCTION

The complement system was initially identified as an important innate immune mechanism of host defense to eradicate microbial pathogens. Recently, complement activation has been implicated in the pathogenesis of many inflammatory and immunological diseases, including sepsis [1], acute respiratory distress syndrome [2], rheumatoid arthritis [3], glomerulonephritis [4], multiple sclerosis [5], ischemia-reperfusion injury [6], and asthma [7]. Complement activation exerts its harmful roles through the generation of complement protein split products, especially C3a and C5a (also known as anaphylatoxins). C5a induces its inflammatory functions by interacting with C5aR that belongs to the rhodopsin family of seven-transmembrane G protein-coupled receptors [8–10]. Traditionally, C5aR expression was thought to be present only on hemopoietic cells, bone marrow cells [11], neutrophils [12], monocytes [13], and eosinophils [14]. However, recent studies have demonstrated the presence of C5aR on nonmyeloid cells, including cells in human lung and liver [15–17], rodent type II alveolar epithelial cells [18], astrocytes [19], kidney tubular epithelial cells [20], mesangial cells [21], and hepatocyte-derived cell lines [22, 23]. Widespread upregulation of C5aR has been seen in organs (heart, liver, lungs, kidneys) from septic animals [24].

Due to the detrimental effects of complement activation under pathologic conditions, interventions aimed at blocking C5a/C5aR signaling represent promising targets for therapeutic treatment in the inflammatory disorders. Peptide antagonist (C5aRa) to the C5aR markedly reduced the lung permeability index (extravascular leakage of albumin) in mice after intrapulmonary deposition of IgG immune complexes [25]. C5aRa treatment substantially reduced I/R-induced pathological markers [26, 27]. In addition, mice injected at the start of CLP with a blocking antibody to C5aR showed dramatically improved survival [24].

RNA interference (RNAi) is an emerging technology that specifically inhibits target gene expression in vitro and in vivo. Tuschl and colleagues demonstrated that exogenously introduced short (19–23 nt) synthetic RNA oligonucleotides can silence genes in somatic cells without activating nonspecific suppression by dsRNA-dependent protein kinases [28]. Successful gene silencing has been achieved in vivo by intravenous injection of siRNA oligos in a large volume of saline solution [29–31] or by injecting smaller volumes of siRNAs that are packaged in cationic liposomes [32]. However, these strategies are limited by the in vivo stability of siRNA molecules and the efficiency with which they are taken up by target cells and tissues. DNA vector-based siRNA expression system would facilitate transfection experiments in

cell cultures, and allow the use of transgenic or viral delivery systems [33–36]. Several viral vectors have been used to induce RNAi silencing in cultured cells and in experimental animals, including lentivirus [37, 38], retrovirus [33], adenovirus [39, 40] and adeovirus-associated viruses (AAV) [41, 42]. Adenoviruses can infect a wide range of cells and have been shown to silence gene expression in vivo [39, 43, 44]. In this study, we demonstrated that systemic application of an adenovirus expressed siRNA can specifically inhibit C5aR gene expression in vivo.

MATERIALS AND METHODS

Cells and antibodies

Mouse alveolar macrophages (MHS cell line) were purchased from ATCC and was cultured in RPMI1640 medium (Life Technologies) supplemented with 10% fetal calf serum as well as 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml), and 0.05 mM 2-mercaptoethanol. HEK293 cell was cultured in DMEM medium (Life Technologies) supplemented with 10% fetal calf serum.

Anti-mouse C5aR polyclonal antibody was generated against a 37 aa peptide spanning the N terminus of the mouse C5aR (MDPIDNSSFEINYDHYGTMDPNIPADGIHLPKR-QPGDC) [45]. The antipeptide specific Ab was purified by affinity chromatography using the synthetic peptide coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ). HA antibody (12CA5) was obtained from BABCO (Berkeley Antibody Company).

Cecal ligation puncture-induced sepsis

C57BL/6 male mice (6 to 8 wk of age weighing 25–30 g; Jackson Laboratories, Bar Harbor, ME) were used in all experiments. Mice were anesthetized with ketamine. A 1 cm long midline incision was made to expose the cecum and adjoin the intestine. With a 4–0 silk suture, the cecum was tightly ligated below the ileocecal valve without causing bowel obstruction. The cecum was punctured through with a 21 gauge needle and gently squeezed to extrude luminal contents, ensuring patency of the two puncture holes. The abdominal incision was then closed with a 4–0 nylon suture and skin metallic clips (Ethicon, Somerville, NY). Sham-operated animals underwent the same procedure except for ligation and puncture of the cecum.

Cloning of mouse C5aR

According to the mouse C5aR sequence [46], two primers (forward primer: 5'-CGG AAT TCC GAT GGA CCC CAT AGA TAA CAG C-3'; reverse primer: 5'-GAA GAT CTT CTA CAC CGC CTG ACT CTT CCG-3') were designed to amplify mouse C5aR from mouse liver RNA using reverse transcription-polymerase chain reaction. PCR products were digested with EcoR I and Bgl II and then cloned into pCMV-HA, a mammalian expression vector that contains the hemagglutinin epitope (PYDVPDYA).

TABLE 1: Sequences and locations of siRNA oligos.

No.	Sense sequences (5' −> 3')	Locations
1	CGCCAUCUGGUUUCUGAAUd(TT)	210
2	CUACUGGUACUUUGAUGCCd(TT)	297
3	ACAUCUGCUACACCUUCCUd(TT)	656
4	CCCUAUCAUCUACGUCAUGd(TT)	888

siRNA oligos

The 21 nt sense and antisense siRNA oligomers targeting against mouse C5aR mRNA were designed and synthesized by Qiagen. Their locations and sequences are shown in Table 1 (only the sense sequences are shown). The oligos were numbered based on the nucleotide position within the coding region of mouse C5aR sequence. Sense and antisense oligos were annealed in HEPES buffer (100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) to obtain siRNA duplexes. Rhodamine labeled control (nonsilencing) siRNA was also purchased from Qiagen.

Cell transfection and western blot

For MHS cell transfection, cells were plated in 6-well plates (8 $\times 10^5$/well) and transfected with 6 μl of TransIT-TKO (Mirus) and 30 pmol of siRNA duplexes. Silencing effects were detected by semiquantitative RT-PCR two days after transfection. For HEK293 cell transfection, cells plated in 35 mm dishes (5 $\times$ 10^5 cells/dish) were transfected with HA-tagged C5aR using Lipofectamine 2000(Invitrogen). Two days after transfection, cells were placed in lysis buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 2 mM MgCl$_2$, 150 mM NaCl, 1 mM dithiothreitol, and 1 mM PMSF. Thirty microliters of the whole cell lysates were electrophoresed in 10% SDS-PAGE and then transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with TBST (40 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.1% Tween 20), containing 5% nonfat dry milk for 1 hour at room temperature. The membrane was then incubated with anti-mouse C5aR serum (1:500 dilution) overnight at 4°C. After three washes in TBST, the membrane was then incubated in a 1:10 000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia). The membrane was developed by enhance chemiluminescence according to the protocol of the manufacturer (Amersham Pharmacia).

Detection of C5aR mRNA by semiquantitative RT-PCR

Total RNA was isolated from cells or lung tissue with the Trizol reagent according to the manufacturer's instructions (Invitrogen). Digestion of any contaminating DNA was achieved by treatment of samples with RQI RNase-free DNase (Promega). RT-PCR was performed with 1 μg of total RNA using the one-step RT-PCR system (Invitrogen) according to the protocol of the manufacturer. Primers for

TABLE 2: Sequences and locations of short hairpin RNAs (note: "**G**" indicates an extra nucleotide added to the target sequence).

No.	Hairpin sequences $(5' - > 3')$	Locations
1	gatcc**G**CTACTGGTACTTTGATGC_tt_caagaga[a](AS[b])TTTTTg	300
2	gatcc**G**CCCATCTGGTGTCAGAAG_tt_caagaga[a](AS[b])TTTTTg	420
3	gatccGTGTACCGGGAGGCATAA_tt_caagaga[a](AS[b])TTTTTg	517
4	gatcc**G**ACATCTGCTACACCTTCCT_tt_caagaga[a](AS[b])TTTTTg	656
5	gatccGAGGGTGGAGAAGCTGAAC_tt_caagaga[a](AS[b])TTTTTg	831
6	gatcc**G**CCCTATCATCTACGTCATG_tt_caagaga[a](AS[b])TTTTTg	888

[a] hairpin loop sequence.

[b] antisense sequence.

C5aR were

(i) forward primer: 5′-GTTGCAGCCCTTATCATCTA-C-3′,

(ii) reverse primer: 5′-TTCCGGGTTGAGGTGTCGTCT-G-3′.

The primers were designed for a 908 bp DNA fragment amplification (nucleotides 112-1019). The primers for the "housekeeping" gene GAPDH were

(i) forward primer: 5′-ACCACCATGGAGAAGGCTGC-3′,

(ii) reverse primer: 5′-CTCAGTGTAGCCCAGGATGC-3′.

After a reverse transcription step for 30 min at 50°C, 25–35 cycles were used for amplification with a melting temperature of 94°C, an annealing temperature of 60°C, and an extending temperature of 72°C, each for 30 seconds, followed by a final extension at 72°C for 7 min. RT-PCR products were confirmed by electrophoresis of samples in 1% agarose gel. To ensure that DNA was detected at the linear part of the amplification curves, PCR was performed with different cycle numbers for C5aR and GAPDH primers. Thirty cycles were used for C5aR amplification in CLP mice, and thirty-two cycles were used in control mice. Twenty five cycles for GAPDH were found to be in the linear range of PCR amplifications.

Immunocytochemistry and confocal microscopy

HEK293 cells were plated on glass bottom 6-well plates (no. 1 thickness coverslips). Two days after transfection, cells were fixed in paraformaldehyde. Fluorescence microscopy was performed as previously described [47]. HA-tagged C5aR was visualized with the affinity purified anti-mouse C5aR antibody (1:500 dilutions) and goat anti-rabbit Alexa 568 (Molecular Probe) secondary antibody (1:1000 dilutions) in the lissamine-rhodamine channel. Cells were imaged on a LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) with a 63 × water lens.

Plasmids expressing short hairpin RNAs

Vectors that express C5aR short hairpin RNAs (shRNAs) under the control of U6 promoter were constructed by inserting pairs of annealed DNA oligonucleotides into the linearized RNAi-Ready pSIREN-DNR-DsRed-Express Vector (BD knockout adenoviral system 2) between the BamH I and EcoR I sites. Sequences and locations of shRNAs are shown in Table 2 (only the top strands are shown).

Generation of siRNA-expressing adenoviruses

U6-driven shRNA cassettes and the CMV-driven DsRed expression cassette in pSIREN-DNR-DsRed donor vector ware transferred to the adenoviral acceptor vector pLP-Adeno-X-PRLS by cre-loxP mediated recombination according to the protocol of the manufacturer. HEK293 cells were transfected with Pac I-digested adenoviral DNA using lipofectamine 2000. One week after transfection, cytopathic effect (CPE) was detected and cells were spun down and lysed in 500 μl PBS with three consecutive freeze-thaw cycles. Supernatants containing infectious adenoviruses were amplified twice by infecting larger scale of HEK293 cells. Viruses were purified by column (Puresyn, Inc) and concentrated by YM-50 centricon (Millipore). Titers of the viruses were determined by Adeno-X rapid titer kit (BD clontech).

Isolation of peritoneal macrophages and adenovirus infection

Macrophages were isolated from the peritoneal cavities of 4- to 6-week-old C57BL/6 mice 4 days after intraperitoneal injection of 0.5 ml 3% thioglycollate, yielding ≥ 95% macrophages as demonstrated by cytospin and differential stain analysis. The cells were seeded at a density of 2×10^6 cells/ml and plated into 6-well plates at 2 ml/well [48] in the same culture medium as MHS cells.

MHS cells and peritoneal macrophages plated in 6-well plates were infected with 100- to 2000-MOI of adenoviruses in a volume of 150 μl of culture medium for one-hour. During the one hour incubation, plates were shaked occasionally at a 15 min interval. Cells were changed to 2 ml fresh medium after the incubation and cultured for another two days for the examination of silencing effects.

Adenovirus-mediated siRNA delivery in animals

Eight- to 10-week-old C57BL/6 mice (weighing 25–30 g) were used in this study. The 50 μl viral suspensions with a
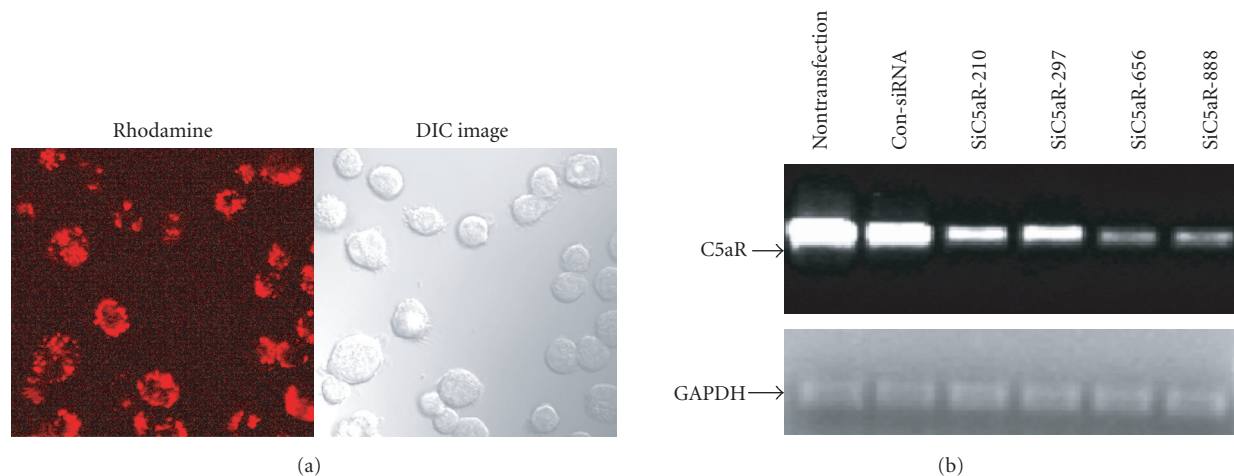
(a)

(b)

Figure 1: Screening of functional mouse C5aR siRNA oligos in MHS cells. MHS cells in 6-well plates were transfected with 30 pmol of control siRNA and four C5aR siRNA oligos using Mirus TransIT-TKO transfection reagent. (a) Eight hours after transfection, cells transfected with Rhodamine-labeled control siRNA were plated on glass-bottom plate and washed twice with phenol-red-free medium and subjected to confocal microscopy analysis (Zeiss). The transfected cells were visualized by red fluorescence (left). (b) Total RNA extracted from transfected MHS cells was quantified for C5aR mRNA expression by semiquantitative one-step RT-PCR (upper panel). GAPDH was used as endogenous control (lower panel).

dosage of 1×10^9 plaque-forming units (pfu) were injected intracheally into mouse lungs. Four days after the injection, mouse lung were extensively flushed with DPBS, and frozen in liquid nitrogen. The 2 ml Trizol reagent was added into one lung for RNA isolation procedure.

RESULTS AND DISCUSSION

siRNA duplexes efficiently inhibited endogenous C5aR in MHS cells

The 21- to 23-nucleotide siRNAs were generated by ribonuclease III through cleavage of longer dsRNAs. They have been shown to act as the mediators of post-transcriptional gene silencing in cells and animals [49, 50]. For the initial screening of the functional siRNA sequences of mouse C5aR, we used synthesized 21-nucleotide siRNA duplexes with 3′-(dTT) overhangs (Table 1) to transiently transfect MHS cell, a cell line that expresses C5aR mRNA endogenously.

Similar to other macrophages, none of the standard transfection methods (e.g., calcium phosphate, lipid, or electroporation) can efficiently transfer DNA plasmids into MHS cells. As a minimum, seventy percentage transfection efficiency is required to study the silencing effects. We used rhodamine-labeled control siRNA to determine the transfection efficiency. Different from larger DNA plasmids, short siRNA oligos could be efficiently transferred into MHS cells by a lipid-mediated method (TransIT-TKO). All of the cells showed red fluorescence eight hours after transfection (Figure 1(a)). No fluorescence was observed for the control cells without TransIT-TKO reagent (data not shown). To confirm that these oligos actually entered the cells, a Z-stack protocol of confocal microscopy was performed and the scanning results

confirmed the cytosolic localization of the fluorescence-labeled siRNA.

All four synthesized siRNA duplexes showed silencing effects on the endogenous C5aR expression as examined by semiquantitative RT-PCR (Figure 1(b)). SiC5aR-210 and siC5aR-297 had moderate inhibition effects, whereas siC5aR-656 and siC5aR-888 suppressed 90 percentage of the endogenous gene. No silencing effects were observed for the control siRNA (scrambled sequences). The specificity of these siRNA oligos was verified by BLAST search against the gene bank.

Cotransfection of siRNA duplexes inhibits C5aR protein expression in HEK293 cells

C5aR is a member of the seven transmembrane receptor superfamily and is ubiquitously expressed on neutrophils, macrophages, thymocytes, epithelial, and endothelial cells. However, in vitro cultured cell lines have very low or non-detectable expression of the receptor. To determine if these siRNA duplexes could also suppress C5aR protein expression, full-length mouse C5aR cDNA was cloned into a HA-tagged mammalian expression vector and transfected into HEK293 cells. Immunocytochemistry analysis showed that this C5aR construct showed a cortical pattern of expression on the membranes of HEK293 cells (Figure 2(a)). Western-blot analysis using anti-mouse C5aR antibody revealed a ∼ 45 kDa band, which is consistent to the size of the receptor expressed in tissues and primary cells (Figure 2(b)) [51].

In the cotransfection experiment, $0.8\,\mu$g C5aR plasmid was transfected with 40 pmol of control siRNA or the C5aR-siRNAs. Two days after transfection, these cell lysates were analyzed by Western blot. Compared to control group, both siRNAs (siC5aR-656 and siC5aR-888) could significantly
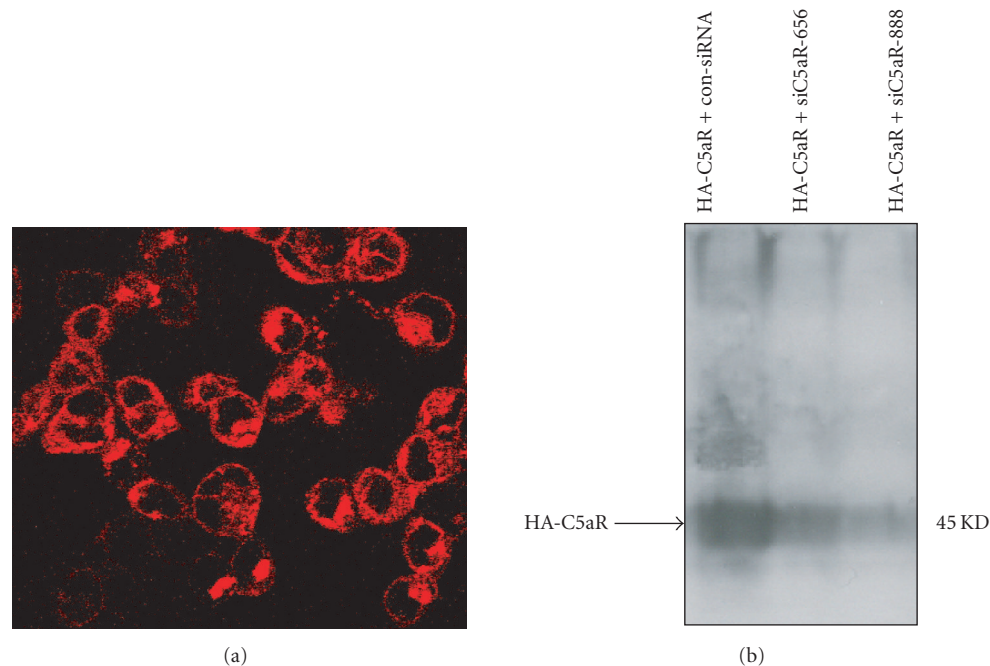
(a)

(b)

FIGURE 2: Oligo siRNA inhibition of C5aR protein expression in HEK293 cells. HEK293 cells were cotransfected with HA-tagged C5aR plasmid, control siRNA, or C5aR siRNA oligos. (a) Forty-eight hours after transfection, transfected cells were fixed in paraformaldehyde and stained with the anti-mouse C5aR primary antibody and goat anti-rabbit Alexa 568 secondary antibody. Expression and localization of C5aR were visualized by confocal microscope under lissamine-rhodamine channel (Zeiss). (b) Cell lysates from transfected cells were subjected to Western-blot analysis using anti-mouse C5aR antibody (1:500).

inhibit the protein expression of C5aR in HEK293 cells (Figure 2(b)).

Hairpin RNA constructs inhibit C5aR expression

Based on the identified C5aR siRNA oligonucleotide sequences, we designed short hairpin RNAs (shC5aR-656 and shC5aR-888) according to the design rules suggested by the manufacturer (BD PharMingen) and inserted them into a U6 promoter-driven shRNA expression donor vector, pSiren-DNR-dsRed. For the most efficient transcription initiation of RNA polymerase III, an extra "**G**" was added at the 5′ end of the sense sequence (Table 2). Sense- and antisense nucleotides were separated by a 9 nt spacer and five consecutive Ts were added at the 3′ end for the termination of short RNA transcripts.

To evaluate the silencing effects of these short hairpins, a 1:10 (HA-C5aR to hairpin RNA) ratio of plasmids was used for the cotransfection experiment in HEK293 cells. A luciferase short hairpin construct pSiren-shLuc served as negative control. Unexpectedly, neither one of the C5aR hairpin constructs (pSiren-shC5aR-888 and pSiren-shC5aR-656) efficiently inhibited C5aR expression (Figure 3).

Different from synthesized siRNA oligos, the effects of DNA vector-based hairpin RNAs are regulated by multiple components. Target sequence selection is an important component, while other factors such as the transcription efficiency, the cleavage efficiency of hairpin RNA into siRNA by

Dicer [33], and the subcellular localization of the short transcript [52], can also affect the efficacy of a hairpin RNA. To select an effective hairpin RNA structure that could be used for our in vivo adenoviral delivery, four additional plasmids, pSiren-shC5aR-300, pSiren-shC5aR-420, pSiren-shC5aR-517 and pSiren-shC5aR-831 were constructed. pSiren-shC5aR-831 (third bar) and pSiren-shC5aR-517 (fifth bar) strongly inhibited C5aR expression in HEK293 cells, while pSiren-shC5aR-420 (sixth bar) and pSiren-shC5aR-300 (seventh bar) had little inhibitory effects (Figure 3). No extra "**G**" was added to pSiren-shC5aR-420, -517 and -831 as the target sequence itself start with a "G". An extra "**G**" was added to pSiren-shC5aR-300 (Table 2).

In vitro and in vivo silencing effects of adenovirus-expressed siRNA

pSiren-DNR-DsRed is an intermediate vector of adenoviral DNA. After we identified two functional shRNAs (C5aR-517 and C5aR-831), the U6 promoter and the hairpin cassette in the donor vector were transferred to a promoterless adenoviral acceptor vector by cre-loxp mediated recombination. The adenoviral DNAs were then transfected into HEK293 cells to produce infectious viruses. Two adenoviruses (adeno-shC5aR-517 and adeno-shC5aR-831) and one control virus (adeno-shLuc) were generated for in vivo gene silencing.

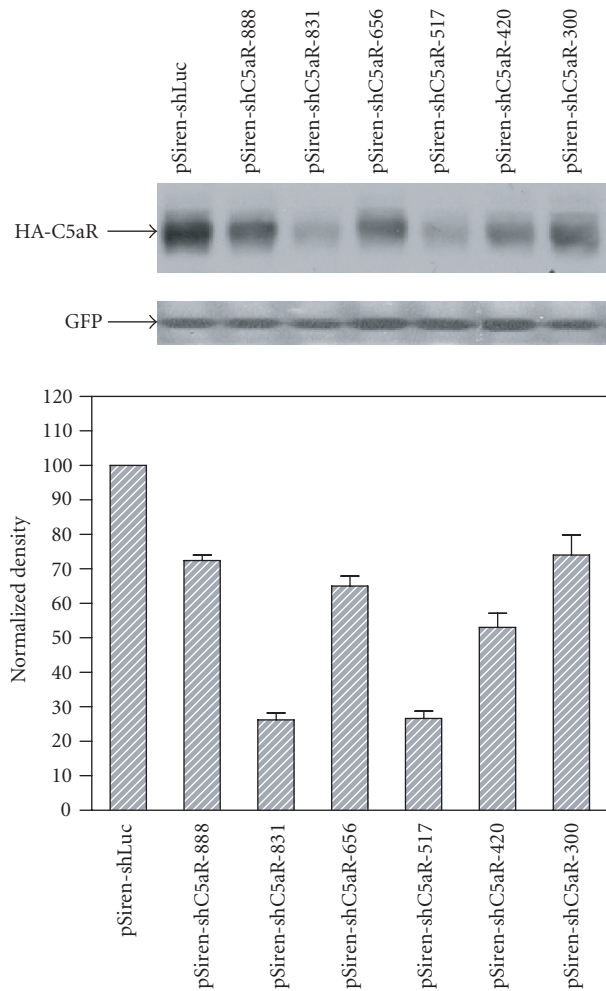Macrophages that express C5aR endogenously were used to test the silencing effects of these viruses. However, these

FIGURE 3: Silencing effects of plasmid-derived short hairpin RNAs. HEK293 cells in 6-well plates were transfected with 4 μg of a 10:1 mix of pSIREN-DNR-DsRed-shRNA to HA-tagged C5aR together with 0.3 μg gfp plasmid. Forty-eight hours after transfection, cell lysates were analyzed by Western-bloting for HA (upper panel). Expression of gfp showed equal transfection efficiency. The bottom panel showed the quantitative densitometric analysis of bands shown in the upper panel. The percentage of inhibition was normalized to control cells transfected with luciferase-shRNA. Averages are derived from 3 to 5 independent experiments (standard deviations are indicated).
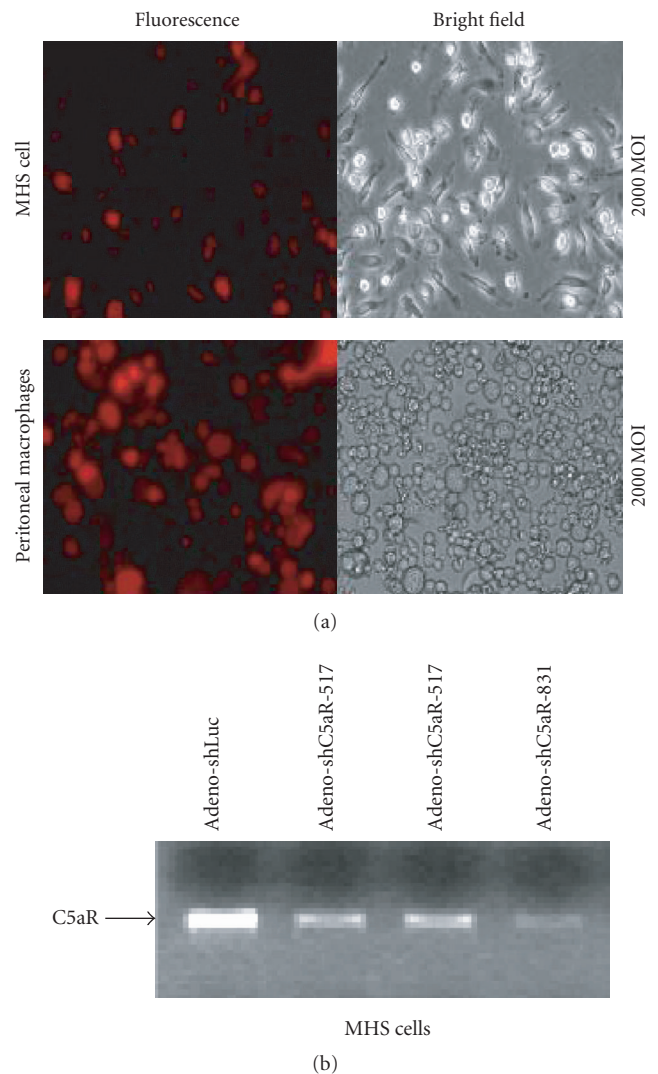

FIGURE 4: In vitro silencing by siRNA-expressing adenovirus. Both MHS cell and peritoneal macrophages were infected with 2000 MOI of adenovirus. (a) Sixty hours after infection, expression of dsRed protein in the cells was visualized by fluorescence microscope. Virus infection efficiency was examined by comparing the fluorescence images (left) and the bright field images (right). (b) Silencing effects of adenovirus-expressed siRNAs were examined by one-step RT-PCR as described in Material and Method. Adeno-luciferase siRNA was used as control.

cells do not express coxsackie receptor [53, 54] and they internalize adenovirus about 100- to 1000-fold less than receptor-expressing cells, such as epithelial cells [55]. To identify an optimal infection condition, we tested a range of infectious units (100, 500, 1000, and 2000 MOI) and found that ∼ 80% of the cells could be infected (as shown by the DsRed marker in the adenoviral DNA) at 2000 MOI (Figure 4), whereas less than half the cells were infected at 1000 MOI (data not shown). In addition to the high infectious units, we also used a low volume of medium during the incubation to enhance other virion uptake pathways (endocytosis or phagocytosis). C5aR mRNA expression in infected

MHS cells were examined by semiquantitative RT-PCR. Both adeno-shC5aR-517 and adeno-shC5aR-831 effectively inhibited endogenously expressed C5aR and the inhibition effect of adeno-shC5aR-831 appeared to be stronger (Figure 4(b)).

To evaluate the ability of virally expressed siRNAs to diminish target gene in vivo, mice were injected intratracheally with 1×10^9 plaque-forming unit (pfu) infectious units of recombinant adenovirus expressing shC5aR-831 or the control virus adeno-shLuc. Four days after infection, RNAs were isolated from mouse lungs and subjected to RT-PCR analysis. As shown in Figure 5(b), the luciferase control virus infection did not change C5aR expression in the lung tissue. However,
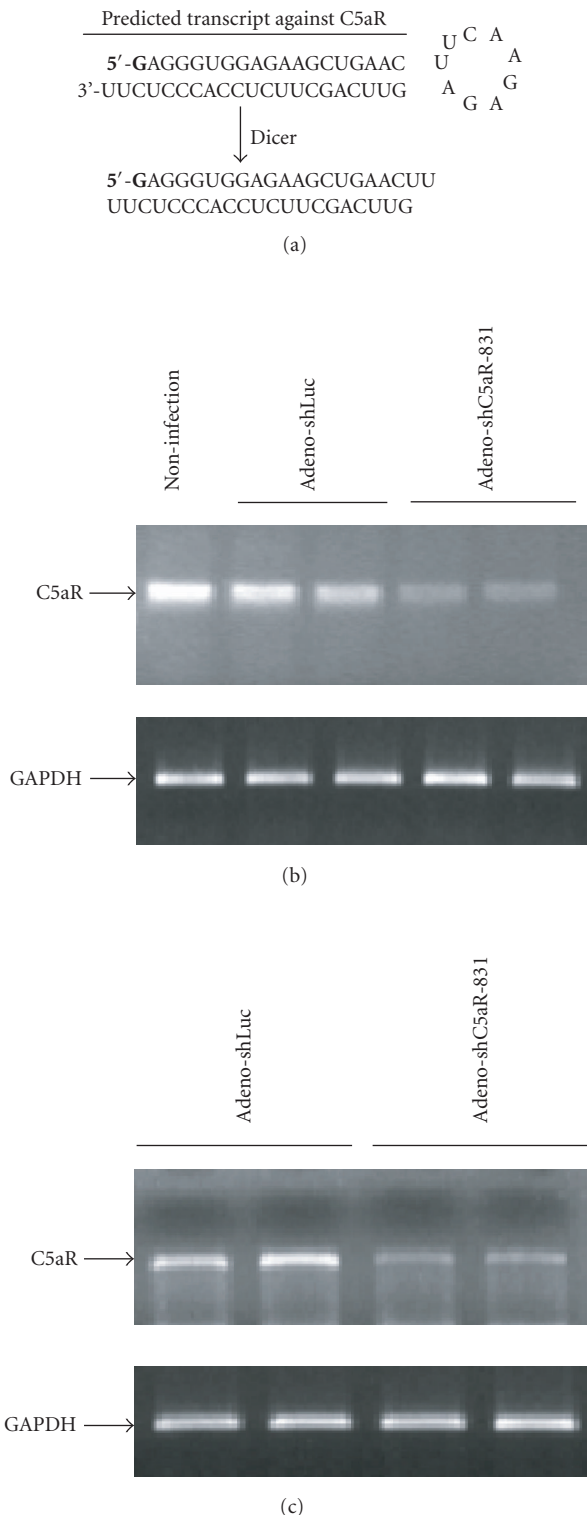
Predicted transcript against C5aR

5′-**G**AGGGUGGAGAAGCUGAAC
3'-UUCUCCCACCUCUUCGACUUG

```
      U   C   A
   U           A
      A       G
         G  A
```

↓ Dicer

5′-**G**AGGGUGGAGAAGCUGAACUU
UUCUCCCACCUCUUCGACUUG

(a)

Non-infection
Adeno-shLuc
Adeno-shC5aR-831

C5aR →

GAPDH →

(b)

Adeno-shLuc
Adeno-shC5aR-831

C5aR →

GAPDH →

(c)

FIGURE 5: In vivo silencing by adenovirus delivey of siRNA. (a) The predicted shRNA transcript from the adenovirus vector and the expected Dicer processing products in vivo. Semiquantitative RT-PCR analysis with whole lung RNAs from control (b) and septic mice (c) showed the decreased C5aR expression in mice infected with C5aR-siRNA adenovirus. Luciferase-siRNA-adenovirus infected lungs were used as controls.

adeno-shC5aR-831effectively inhibited C5aR expression in the lung tissue. To test the effect of adeno-shC5aR-831 in disease condition, sepsis was induced by CLP in mice that had received adenovirus for four days. Twenty four hours after CLP, RNAs from lungs were analyzed for C5aR and GAPDH expression. As shown in Figure 5(c), the inhibitory effect of adeno-shC5aR-831 is still effective under sepsis condition. These data indicate that complement receptor C5aR could be suppressed in vivo by an adenovirus-mediated siRNA knockdown strategy under both normal and disease conditions.

RNA interference is a powerful tool to silence gene expression post-transcriptionally. Different from gene knock out, the inhibition efficiency of siRNAs could vary dramatically by employing a different delivery method and sequence design strategy of siRNA oligonucleotides or short hairpins. In this study, it is noted that the vector-based siRNA sequences could not be simply derived from chemically synthesized oligo sequences. It may be due to the fact that the functionality of shRNAs depends on more complicated intracellular mechanisms. In fact, none of the current design rules guarantee an effective siRNA and a functional siRNA can only be identified experimentally. Another important factor that affects the application of siRNA is the efficiency and the effectiveness of delivery routes. Here we demonstrated the intratracheal administration of siRNA-expressing adenovirus that could efficiently knock down C5aR expression. Thus, C5aR siRNA-expressing adenovirus shall not only serve as a useful tool for studying the mechanisms of complement activation in inflammation, but may also have important therapeutic applications.

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