

Inhibition of Acidic Mammalian Chitinase by RNA Interference Suppresses Ovalbumin-Sensitized Allergic Asthma

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Abstract

Asthma, a chronic helper T cell type 2-mediated inflammatory disease, is characterized by airway hyperresponsiveness and inflammation. Growing evidence suggests that increased expression of acidic mammalian chitinase (AMCase) may play a role in the pathogenesis of asthma. In the present study, we sought to develop an RNA interference approach to suppress allergic asthma in mice through silencing of AMCase expression. Mice sensitized with ovalbumin (OVA) were intratracheally administered a recombinant adeno-associated virus expressing short hairpin RNA (rAAV-shRNA) against AMCase. In OVA-sensitized mice, the development of allergic symptoms was significantly associated with elevated AMCase expression. After administration of rAAV-shRNA, there was a significant reduction of AMCase expression in the lung and in bronchoalveolar lavage fluid (BALF) cells of sensitized mice. Sensitized mice receiving rAAV-shRNA showed a significant improvement in allergic symptoms, including airway hyperresponsiveness (AHR), eosinophil infiltration, eotaxin, interleukin-13 secretion in BALF, and serum OVA-specific IgE level. Our data suggest the hyperexpression of AMCase in asthma can be suppressed by rAAV-mediated shRNA. Silencing AMCase expression by shRNA may be a promising therapeutic strategy in asthma.

Introduction

THE PREVALENCE AND MORTALITY of asthma have been increasing worldwide, and the World Health Organization (WHO, Geneva, Switzerland) recognizes that asthma is of major public health importance. Chronic airway inflammation with pathological immune response is the hallmark of asthma. The key feature of asthma is the production of allergen-specific IgE, resulting in immediate-type hypersensitivity reactions followed by the development of late-phase responses such as eosinophil recruitment, mucus production, and airway hyperresponsiveness (AHR). Eosinophilic inflammation driven by helper T cell type 2 (Th2) cytokines is deemed to play a crucial role in the pathogenesis of asthma (Passalacqua and Ciprandi, 2008). Elevation of acidic mam-

malian chitinase (AMCase) (Boot *et al.*, 2001), the prototypic chitinase, appears to associate with Th2-mediated inflammation through an interleukin (IL)-13-dependent mechanism (Zhu *et al.*, 2004; Ramanathan *et al.*, 2006; Kawada *et al.*, 2007; Bucolo *et al.*, 2008; Musumeci *et al.*, 2008). Accordingly, AMCase is hyperexpressed in the lungs of patients with asthma and in animal models of asthma (Zhu *et al.*, 2004; Bierbaum *et al.*, 2005; Chupp *et al.*, 2007). Such induction of AMCase was found not only in lung epithelial cells but also in alveolar macrophages in ovalbumin (OVA)-stimulated mice (Zhu *et al.*, 2004). Moreover, Zhu and colleagues have shown that inhibition of AMCase activity with specific antibodies was able to reduce the inflammatory responses in bronchoalveolar lavage fluid (BALF) and lung tissues. It was suggested by Donnelly and Barnes that inhibition of AMCase ameliorates asthmatic

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symptoms, the result of inhibitory effects on the expression of several proinflammatory chemokines including macrophage inflammatory protein (MIP)-1 β , macrophage chemoattractant protein (MCP)-1, and eotaxin (Donnelly and Barnes, 2004).

RNA interference (Fire *et al.*, 1998) has become a powerful tool for modulating gene expression in cells (Cullen, 2006). Because short hairpin RNA (shRNA) has been shown to be efficiently processed into small interfering RNA (siRNA) in mammalian cells, strategies for producing siRNAs have been focused on transcription of shRNA, using RNA polymerase III promoters such as U6 and H1 (Storvold *et al.*, 2007; Lee *et al.*, 2008b). Moreover, viral vectors appear to have the highest efficiency in delivering siRNA into mammalian cells (Li *et al.*, 2006; ter Brake *et al.*, 2008). Despite potential immunogenicity problems generated by some viral vectors (Zaiss and Muruve, 2008), adeno-associated viral (AAV) vectors offer a compromise between an adequate level of transduction and an acceptable safety profile (Leung *et al.*, 2007). Hence, several reports have successfully used an AAV-mediated shRNA therapeutic system to control viral infections (Moore *et al.*, 2005; Fechner *et al.*, 2008) and genetic disorders (Franich *et al.*, 2008). In the present study, we hypothesized that specific suppression of elevated AMCase can be achieved by AAV-mediated shRNA production, which would therefore result in reduced eosinophilia and Th2-mediated airway inflammation in a mouse model of asthma. We further investigated whether the inhibition of AMCase may be associated with reduced expression of IL-13, eotaxin, and other proinflammatory molecules.

Materials and Methods

Cell culture

3T3 and 293T cell lines were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT) with 5% CO₂ in humidified air at 37°C.

Cloning of AMCase and setup of AMCase-overexpressing cell line

RNA was extracted from mouse lung tissues, using the TRIzol reagent (Invitrogen) and reverse-transcribed (RT) with a two-step RT-polymerase chain reaction (PCR) kit (Invitrogen). The full-length AMCase sequence was amplified with the following primers: forward, 5'-ATCAGAATTC TATGGCCAAGCTACTTCTC-3'; and reverse, 5'-TTTCTGC GGCCGCATGGCATTAGGTTTCATGGC-3'. The AMCase-overexpressing cell line was established by transfection into 3T3 cells of a pTriEx-neo vector carrying the AMCase sequence. A dilution series under G418 selection pressure was applied.

Real-time PCR

Expression of AMCase, humanized *Renilla* green fluorescent protein (hrGFP), and actin was analyzed by real-time PCR, using a LightCycler PCR system (Roche, Indianapolis, IN). Actin 300 (forward primer, 5'-GAAACTACATTCAAT TCCATC-3'; reverse primer, 5'-CTAGAAGCACTTGCGGT GCAC-3') was used as a housekeeping gene. The reaction parameters for actin 300 and hrGFP amplification (forward

primer, 5'-ATGGTGAGCAAGCAGATCCTG-3'; reverse primer, 5'-GGTGCCTCGTACACGAAGCC-3') were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 10 sec, 50°C for 10 sec, and 72°C for 10 sec. AMCase was amplified using the following primers: forward, 5'-TGGACACACCTTCATCCTGA-3'; and reverse, 5'-AACA AGCCCTGCTTGACAAT-3'. After initial denaturation at 95°C for 10 min, 45 cycles were performed at 95°C for 10 sec, 50°C for 10 sec, and 72°C for 10 sec.

Design and construction of shRNA

Three sequences specifically targeting murine AMCase were designated as siRNA185, siRNA897, and siRNA1336 (siRNA185: top, GCAGAACAAUGAGAUCACCACCAUA; bottom, UAUGGUGGUGAUCUCAUUGUUCUGC; siRNA897: top, GGGUUCUGGGCCUACUAUGAGAUUU; bottom, AAA UCUCAUAGUAGGCCCAAGACCC; siRNA1336: top, GGCA GUGCAUCAUUGGAAUCACAUA; bottom, UAUGUGAUU CCAUUGAUGCACUGCC). shRNA molecules with the same sequences as siRNA, but containing a random eight-nucleotide (CAAGCTTC) loop structure and a four-nucleotide terminator (3'-TTTT) overhanging the 3' end, were inserted into a pCI-neo plasmid vector with an mU6 promoter. Here an shRNA-targeting luciferase (Luciferase: top, 5'-TCCAATTCAGCGGG AGCCTTCAAGAGAGGCTCCCGCTGAATTGGA-3'; bottom, 5'-GGATTCCAATTCAGCGGGAGCCTCTCTTGAAGGCTCC CGCTGAATTGGA-3') served as the shRNA control.

Cloning and generation of recombinant adeno-associated virus

The shRNA1336 and shRNA controls were subcloned into adeno-associated viral vector pAAV2-IRES-hrGFP. Virus production was performed with the AAV2 helper system (Stratagene, La Jolla, CA). Briefly, plasmid DNA (rAAV-shRNA1336-IRES-hrGFP plasmid plus the pRC vector encoding Rep and Cap proteins and the pHelper vector encoding adenoviral gene products) was used to transfect 293T cells at the 80% confluence stage. Cell lysates were collected 48 hr posttransfection and purified by CsCl density gradient centrifugation. Titers of rAAV-shRNA1336-IRES-hrGFP were determined by RT-PCR analysis by calculating the viral genome copy number.

5'-RACE analysis

5'-RACE (rapid amplification of cDNA ends) analysis was performed with a GeneRacer kit (Invitrogen). Briefly, total RNA (5 μ g) was extracted from 3T3/AMCase cells treated with or without siRNA185 and siRNA1336 and ligated to GeneRacer Oligo. The resultant product was reverse-transcribed into cDNA, using a random primer. To detect cleavage products, PCR was performed with a 5'-nested primer and an AMCase-specific primer (5'-TTTCTGCGGCCGCATGGCAT TAGGTTTCATGGC-3' and 5'-AACAAGCCCTGCTTGACA AT-3', respectively) and then analyzed by electrophoresis and gene sequencing.

Animal immunization protocol

Animal studies were approved by the Institutional Animal Care and Use Committee of Chang Gung University (Taoyuan, Taiwan). Pathogen-free wild-type female BALB/

cByJNarl mice from the National Laboratory Animal Center (Taipei, Taiwan) were used. Mice were 6–8 weeks old at the beginning of the experiment. Mice were injected intraperitoneally with chicken OVA (20 μ g) complexed to alum or with normal saline alone. The process was repeated 3 days later. Thirteen days after the first immunization, animals were reinjected intraperitoneally with chicken OVA. Moreover, mice received by inhalation either an aerosol of OVA (2%, w/v) in normal saline or normal saline solution alone. Mice were thereafter challenged four times with OVA on days 16, 20, 23, and 27. On day 25, 1×10^{11} genome copies of rAAV-shRNA1336-IRES-hrGFP, rAAV-shRNAcontrol-IRES-hrGFP, rAAV-IRES-hrGFP (rAAV-mock), or normal saline solution was administered intratracheally. Mice were sacrificed on day 28.

Measurement of airway hyperresponsiveness

On day 28, 24 hr after the last challenge, airway responsiveness was measured in unrestrained animals by whole body plethysmography (Buxco Electronics, Wilmington, NC) (Lee *et al.*, 2008a). Briefly, mice were administered aerosolized normal saline or methacholine at increasing concentrations (0, 25, and 50 mg/ml) for 3 min. Recordings were taken and averaged for 3 min after each nebulization. Airway reactivity was expressed as the mean enhanced pause (Penh).

Determination of OVA-specific IgE

Serum levels of OVA-specific IgE were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Nunc MaxiSorp; Thermo Fisher Scientific, Waltham, MA) were coated with OVA (10 μ g/ml) and blocked. After addition of serum samples at proper dilutions, biotin-conjugated rat anti-mouse IgE was added to the wells. The reaction was developed with streptavidin–horseradish peroxidase (HRP). Substrate solution was then added to each well and the plates were incubated for 30 min at room temperature in a dark room. After the addition of stop solution, the absorbance was read with an ELISA plate reader at 450 nm.

Assay of eotaxin and IL-13 levels

Eotaxin and IL-13 levels in BALF were assayed with commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Western blot analysis

Cell and tissue samples for AMCase analysis were prepared with lysis buffer. After separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the samples were identified by incubation with rabbit anti-mouse AMCase antibodies and developed with an enhanced chemiluminescence (ECL) detection kit (GE Healthcare Life Sciences, Piscataway, NJ). A monoclonal antibody against β -actin was used as control.

AMCase activity

Chitinase bioactivity in BALF was measured with a fluorogenic substrate according to previous methodology (Zhu *et al.*, 2004). Briefly, samples were incubated with 4-methylumbelliferyl β -D-N,N'-diacetylchitobioside (4-MU;

Sigma-Aldrich, St. Louis, MO) in citrate–phosphate buffer (0.1 M–0.2 M), pH 5.2, at 37°C for 1 hr. The activity was then measured with a fluorometer at an excitation of 350 nm and an emission of 450 nm.

Lung histology

Lung tissues were removed and fixed with 4% formaldehyde. Paraffin sections, 10 μ m thick, were stained with hematoxylin and eosin.

Immunohistochemistry

AMCase, hrGFP, and Ki67 expression in lung tissues was analyzed by immunohistochemistry (IHC). Lung tissue samples were prepared as 10- μ m-thick paraffin sections and incubated with rabbit anti-AMCase, anti-hrGFP, or anti-Ki67 as the primary antibody. Sections were then counterstained with hematoxylin.

Statistical analysis

Results are presented as means $\pm$ SEM. Data were analyzed by nonparametric Mann–Whitney *U* test. A two-tailed *p* value less than 0.05 was considered statistically significant.

Results

Reduction of AMCase expression levels by RNA interference

RNA interference (RNAi) technology was used to evaluate whether the reduction of AMCase expression may affect asthma symptomatology in an OVA-sensitized mouse model. First, three shRNA expression vectors containing the mouse U6 promoter were constructed (pCI-shRNA185, pCI-shRNA897, and pCI-shRNA1336). Their ability to suppress AMCase expression was examined in a stable cell line overexpressing AMCase. All three constructs were able to reduce AMCase expression to various extents (Fig. 1a). AMCase expression was reduced by 85% with shRNA1336, by 50% with shRNA185, and by 20% with shRNA 897. Second, rAAV vectors encoding luciferase and shRNA1336, rAAV-shRNAcontrol-hrGFP and rAAV-shRNA1336-hrGFP (rAAV-shRNAcontrol and rAAV-shRNA1336, in brief) were cloned and investigated for their inhibitory effects on AMCase expression. Figure 1b shows that infection with rAAV-shRNA1336 significantly suppressed AMCase expression in the stable cell line between days 2 and 8. These findings indicate that rAAV-shRNA1336 had the better inhibitory effect on the expression of AMCase. To confirm that reduction of AMCase expression was mediated by specific cleavage of AMCase mRNA after transduction with rAAV-sh1336, 5'-RACE PCR was performed with stable cell lines overexpressing AMCase (see Supplementary Fig. 3 at www.liebertonline.com/hum).

Levels of AMCase in BALF and lungs of OVA-sensitized mice

To investigate whether elevated levels of AMCase expression were specifically located in the airways of OVA-sensitized mice, we harvested and analyzed lung, BALF, and peritoneal cells 24 hr after the last OVA challenge. Notably, 17- and 37-fold increases in AMCase mRNA level were

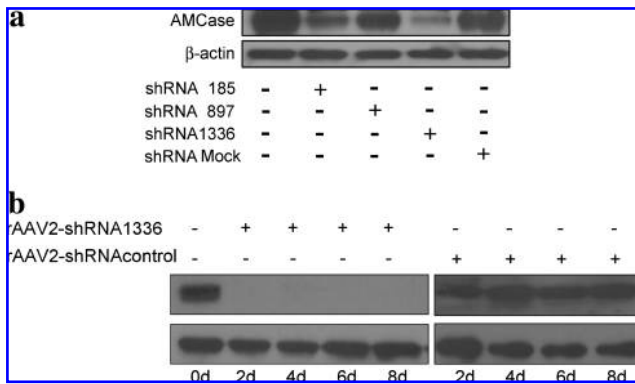


FIG. 1. Inhibitory effects of pCI-shRNA and recombinant adeno-associated virus (rAAV)-mediated short hairpin RNA (shRNA) on acidic mammalian chitinase (AMCase) expression. (a) Representative Western blot of an AMCase-overexpressing stable cell line treated with pCI-shRNA185, pCI-shRNA897, and pCI-shRNA1336 for 48 hr (shRNA mock: negative control of shRNA). (b) AMCase expression of the same cell line after infection with rAAV vectors expressing shRNA control or shRNA1336, and harvested on days 0, 2, 4, 6, and 8. Western blots using anti-AMCase and anti- β -actin antibodies are shown.

found in the lung tissues and BALF of OVA-sensitized mice (Fig. 2a), respectively. No major differences in AMCase expression were seen in peritoneal cells from sensitized animal compared with control mice treated with normal saline. Similar findings were obtained when examining AMCase protein levels (Fig. 2b). Hence, hyperexpression of AMCase was confined chiefly to the airways. We also investigated whether the clinical symptoms in mice were associated with AMCase expression in the airways. As shown in Fig. 2c, airway hyperresponsiveness (AHR) was positively correlated with AMCase mRNA in lung cells as determined by real-time PCR. On the basis of using the 4-MU substrate system to measure AMCase activity in the airways, an association between AMCase activity and eosinophil infiltration was identified (Fig. 2d). In addition, we also found a positive correlation between AMCase expression and the total number of eosinophils in BALF (data not shown).

Reduction of AHR and AMCase expression in the airways of OVA-sensitized mice after rAAV-shRNA1336 administration

We then examined whether rAAV-shRNA was able to suppress airway hyperresponsiveness and AMCase hyper-

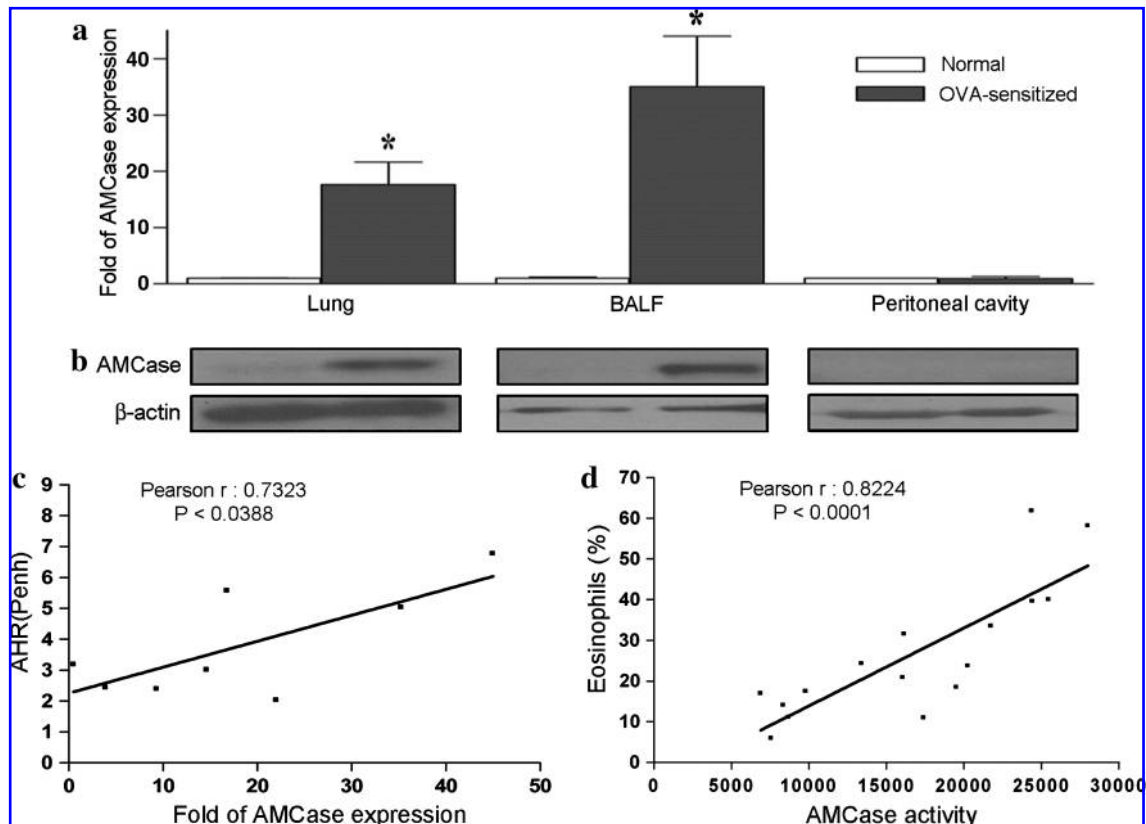


FIG. 2. Quantification of AMCase expression and its correlation with asthmatic symptoms. Cell samples from lung tissues, bronchoalveolar lavage fluid (BALF), and the peritoneal cavity were harvested from normal saline controls ($n = 5$) and OVA-sensitized mice ($n = 9$), and their AMCase expression was quantified via (a) real-time PCR and (b) Western blot. In (a), mRNA levels of AMCase expression detected by real-time RT-PCR were normalized to actin and compared with normal controls ($*p < 0.05$, nonparametric Mann-Whitney test). In (b), AMCase protein levels were analyzed by Western blot. In (c) and (d), airway hyperresponsiveness (AHR) and eosinophil infiltration were correlated with mRNA levels of AMCase expression and AMCase activity by enzymatic assay (Pearson correlation, $p < 0.05$). Penh, enhanced pause.

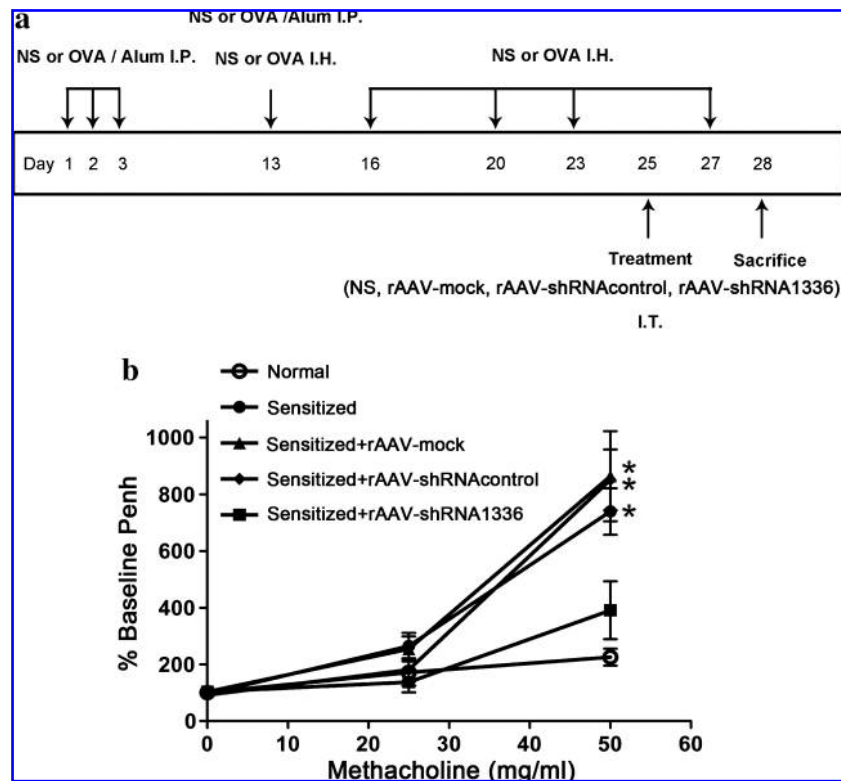


FIG. 3. Reduction of airway hyperresponsiveness by treatment with rAAV-shRNA. (a) Schematic protocol for ovalbumin (OVA) sensitization and rAAV treatment. Mice were sensitized with OVA and treated with rAAV as described in Materials and Methods. In (b), airway hyperresponsiveness was measured by whole body plethysmography. Data are expressed as enhanced pause (Penh) values. Shown are data for normal saline controls ($n = 4$); sensitized mice ($n = 10$); and OVA-sensitized mice receiving rAAV-mock ($n = 4$), rAAV-shRNAcontrol ($n = 5$), or rAAV-shRNA1336 ($n = 7$) ($*p < 0.05$ vs. rAAV-shRNA1336, nonparametric Mann-Whitney test). IH, inhalation; IP, intraperitoneal; IT, intratracheal; NS, normal saline treated.

expression in our mouse model. Figure 3a depicts a schematic representation of the rAAV treatment protocols. Mice were sensitized with a mixture of OVA and alum, and then challenged by hyperexposure to aerosolized OVA. Three days after the intratracheal delivery of 10^{11} rAAV encoding LacZ (rAAV-LacZ), rAAV infection was evident in lung and BALF cells (data not shown). At this time, airway hyperresponsiveness was measured by methacholine challenge. Figure 3b shows that methacholine induced a significant increase in AHR in OVA-sensitized mice either with or without control viral infection (rAAV-mock and rAAV-shRNAcontrol). In contrast, sensitized mice receiving rAAV-shRNA1336 against AMCase showed significantly decreased Penh values in response to methacholine.

We subsequently investigated whether such treatment resulted in reduced expression of AMCase. Figure 4a shows that AMCase expression was reduced by 2-fold in lungs and by 3-fold in BALF cells of mice treated with rAAV-shRNA1336 compared with mice receiving rAAV-shRNAcontrol or rAAV-mock. We also measured AMCase protein levels and activity in lung and BALF cells by immunohistochemical or enzymatic assay, using a commercial fluorescence system (4-MU). Figure 4b and c shows elevated expression of AMCase in the airway epithelial cells and BALF cells of OVA-

sensitized animals receiving rAAV-shRNAcontrol or vehicle. In contrast, markedly reduced AMCase expression was seen in rAAV-shRNA1336-treated animals. Immunohistochemical analysis of the adjunct sections revealed that most of the airway cells such as epithelial cells expressed high levels of AMCase in sensitized animals (Fig. 4d). With the coordinated detection of hrGFP expression, these epithelial cells appeared to be the main target of AAV. Furthermore, by analyzing rAAV-shRNA1336-infected specimens, we actually found that high hrGFP-staining regions had lower levels of AMCase expression, indicating that administration of rAAV-shRNA1336 indeed reduced AMCase expression in lung tissues of OVA-sensitized mice. Moreover, 5'-RACE PCR was performed to confirm that reduction of AMCase was mediated by specific cleavage of AMCase mRNA after transduction with rAAV-shRNA1336, using RNA samples from the lungs of OVA-sensitized mice treated with rAAV-shRNA (Supplementary Fig. 3).

rAAV-shRNA1336 reduces immunopathological reactions in OVA-sensitized mice

Because treatment with rAAV-shRNA1336 significantly reduced AHR of OVA-sensitized mice, we further examined

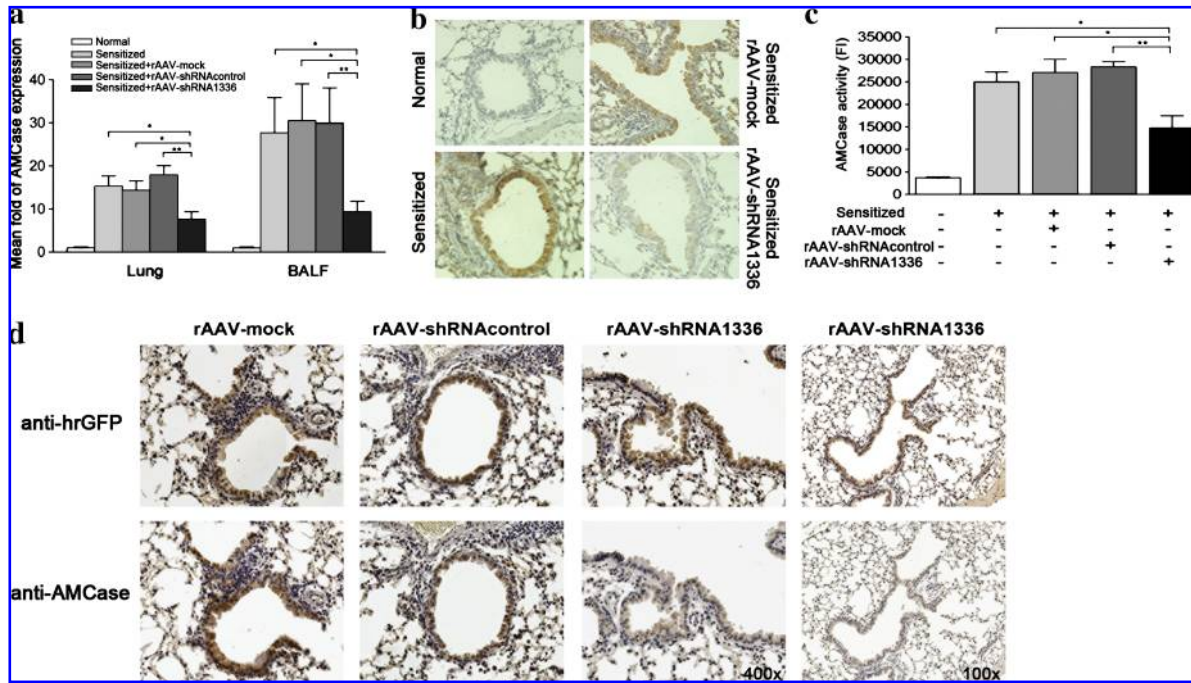


FIG. 4. Suppression of AMCase in lung tissues and BALF cells of rAAV-shRNA-treated mice. In (a), real-time PCR was performed to quantify AMCase mRNA in lung tissues and BALF cells. Data are normalized to actin and compared with normal controls. (b) Expression of AMCase in the lung as detected by immunohistochemistry (IHC) (original magnification, $\times 400$). (c) AMCase activity in BALF cells as measured by fluorogenic chitin substrate. FI, fluorescence intensity. (d) Expression of AMCase and humanized *Renilla* green fluorescent protein (hrGFP) in the lung was detected by IHC. Normal mice ($n = 7$), sensitized mice ($n = 7$), and sensitized mice treated with rAAV-mock ($n = 8$), rAAV-shRNAcontrol ($n = 5$), or rAAV-shRNA1336 ($n = 10$) were investigated; results are shown for sensitized, vector-treated mice ($*p < 0.05$ and $**p < 0.01$, non-parametric Mann-Whitney test).

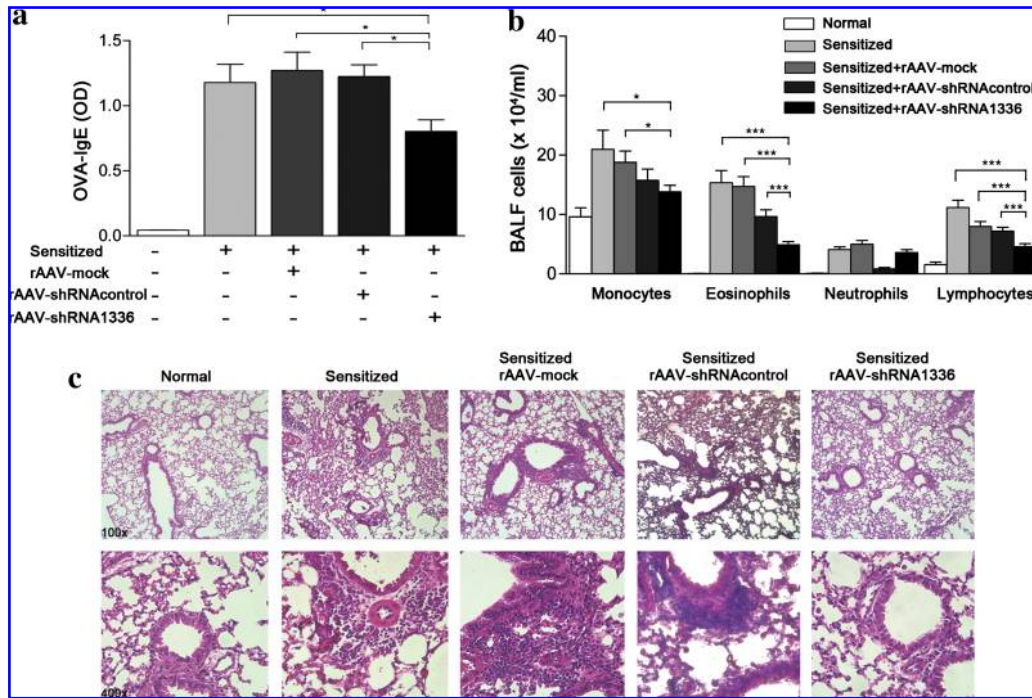


FIG. 5. Reduction of immunopathological responses in rAAV-shRNA-treated mice. In (a), serum levels of OVA-specific IgE were measured by ELISA. OD, optical density. In (b), the number of eosinophils within BALF was calculated by microscopy at an original magnification of $\times 400$. (c) Hematoxylin-eosin (H&E) histopathology of the lung as analyzed by microscopy at original magnifications of $\times 100$ (top) and $\times 400$ (bottom). Normal mice ($n = 7$); sensitized mice ($n = 7$); and sensitized mice treated with rAAV-mock ($n = 10$), rAAV-shRNAcontrol ($n = 5$), or rAAV-shRNA1336 ($n = 11$) were investigated ($*p < 0.05$ and $**p < 0.01$, nonparametric Mann-Whitney test).

whether the other essential features of immunopathological allergic responses among these treated OVA-sensitized mice were affected. The OVA-specific IgE serum level was significantly lowered in sensitized mice treated with rAAV-shRNA1336 compared with animals treated with rAAV-shRNAcontrol (Fig. 5a). Moreover, infection with rAAV-shRNA1336 not only reduced eosinophil infiltration in BALF (Fig. 5b), but also decreased the infiltration of inflammatory cells into the lung tissues of sensitized mice (Fig. 5c). Altogether, these results indicate that asthmatic mice receiving rAAV-shRNA1336 showed a significant improvement in allergic inflammatory responses.

Because leukocyte infiltration was found to be reduced after treatment with rAAV-shRNA, we further investigated whether reduction of AMCase activity could affect the expression of Th2-associated cytokines and chemokines. Specifically, we focused on IL-13, as its expression has been associated with AMCase expression, and eotaxin, which is an eosinophil-specific chemoattractant. As shown in Fig. 6a and b, concentrations of IL-13 and eotaxin were reduced in OVA-sensitized mice that received rAAV-shRNA1336 treatment. We subsequently focused on the expression of other Th2-associated cytokines (IL-4 and IL-5) and inflammatory chemokines (MCP-1 and MIP-1 β) according to the various rAAV treatments. As shown in Fig. 6c, treatment with rAAV-shRNA1336 caused a reduction in expression of cytokines and chemokines in the lung, although the inhibition of IL-4 and IL-13 was less prominent compared with that of chemokines (eotaxin, MCP-1, and MIP-1 β).

Discussion

Experimental studies have suggested the potential use of RNAi for targeting asthma (Lee *et al.*, 2008a; Lively *et al.*, 2008). Our current work indicates that AMCase is a potential target. Our findings revealed that AMCase expression was positively associated with asthmatic symptoms, AHR, and eosinophil infiltration. rAAV-mediated shRNA production could effectively reduce AMCase expression in OVA-sensitized mice. Notably, reduction of AMCase expression correlated with a significant improvement of airway inflammation. Altogether, our findings imply that RNA interference targeting AMCase may lead to the development of a potential therapeutic strategy to ameliorate asthmatic symptoms.

AMCase has been hypothesized to associate with Th2-mediated inflammation through an IL-13-dependent mechanism (Zhu *et al.*, 2004), as Zhu and colleagues found significant hyperexpression of AMCase in IL-13-transgenic mice. In fact, IL-13-null mice failed to express AMCase after an allergen challenge (Zhu *et al.*, 2004). Zhu and colleagues showed that inhibition of AMCase activity with specific antibodies reduced the inflammatory response in BALF and lung tissues. However, these authors reported that reduction of AMCase activity did not lead to consequent changes in IL-4 and IL-13 expression. Here we adapted RNAi technology to further knock down AMCase expression, which led to the significant reduction of allergic responses. Importantly, we found that downregulation of AMCase expression was accompanied by suppression of IL-13 in BALF cells. Although lung IL-13 is not entirely suppressed by downregulation of AMCase activity, the existence of other molecular pathways controlling AMCase expression can be estimated.

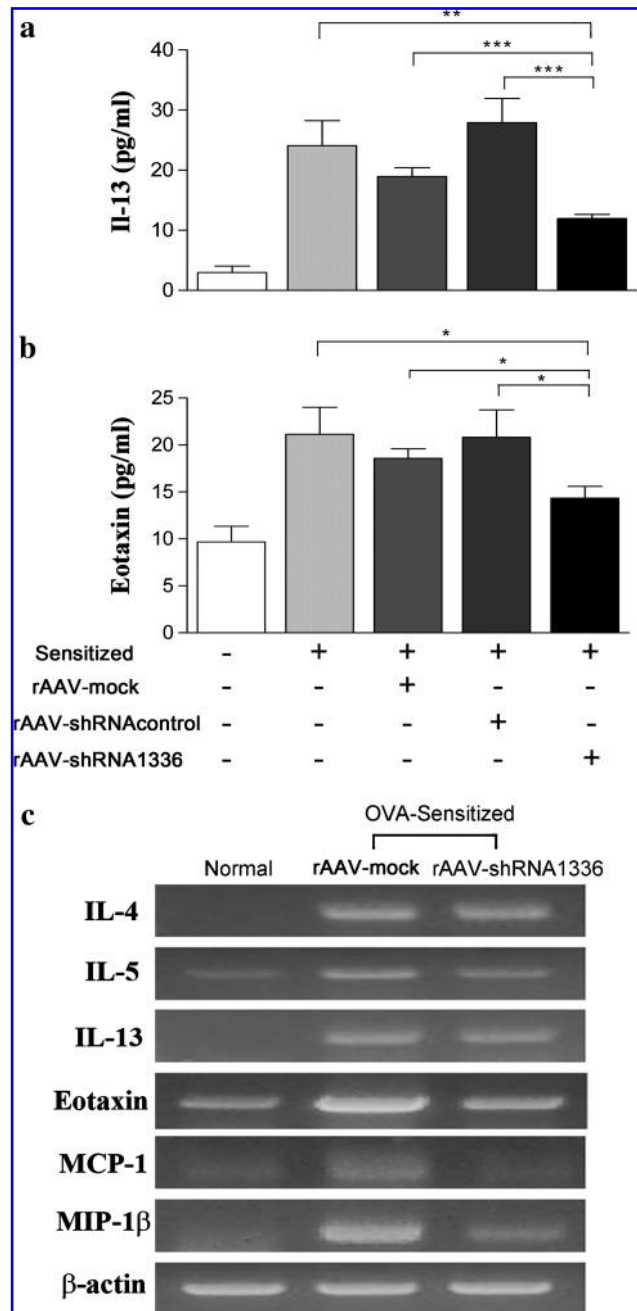


FIG. 6. Expression of cytokines and chemokines in rAAV-shRNA-treated mice. (a and b) Levels of IL-13 and eotaxin in BALF samples, as measured by ELISA. In (c), RNA samples from lung tissues were analyzed for cytokine and chemokine gene expression by means of RT-PCR. Normal mice ($n=3-7$); sensitized mice ($n=5-7$); and sensitized mice treated with rAAV-mock ($n=6-10$), rAAV-shRNAcontrol ($n=5$), or rAAV-shRNA1336 ($n=6-11$) were investigated; results are shown for normal mice and sensitized mice treated with rAAV-mock and rAAV-shRNA1336 (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, nonparametric Mann-Whitney test).

Chemokines such as eotaxin, MCP-1, and MIP-1 β have been implicated in airway inflammation (Zimmermann *et al.*, 2003). Eotaxin acts as a chemoattractant for eosinophils in the lung after an allergen challenge (Pope *et al.*, 2005b), whereas expression of MCP-1 and MIP-1 β is enhanced in subjects with

asthma. In the current study, we have shown that inhibition of AMCase in OVA-sensitized mice led to a significant reduction of eotaxin in BALF and to massive suppression of eotaxin, MCP-1, and MIP-1 β in lung tissues. Inhibition of eotaxin-mediated AMCase suppression in turn decreased eosinophil infiltration. An overall improvement in the asthmatic response was achieved by rAAV-shRNA-mediated down-regulation of AMCase. Whether this effect may be ascribed to an IL-13-dependent pathway is currently unclear; further studies are needed to shed more light on this issue. Accordingly, it has been demonstrated that neutralization of IL-13 abrogates the majority of eotaxin activity (Pope *et al.*, 2005a).

Moreover, one study focusing on elaboration of the molecular mechanisms controlling AMCase and its effector responses showed that AMCase physically interacts with epidermal growth factor receptor (EGFR) (Hartl *et al.*, 2008), which has been strongly implicated in the pathogenesis of Th2 tissue inflammation (Davies *et al.*, 1999). In fact, it was demonstrated that EGFR signaling mediated allergen-induced CCL17 chemokine (CCL17/TARC, thymus and activation regulated chemokine) release by lung epithelial cells (Heijink *et al.*, 2007). Indeed, recombinant AMCase was able to induce epithelial cells to produce CCL17, CCL2 (MCP-1), and CXCL8 (IL-8, KC) (Hartl *et al.*, 2008). Indeed, in the current study, we found that reduction of MCP-1 in lung tissues was associated with inhibition of AMCase expression. This result indicates that AMCase plays a positive role in stimulating pulmonary epithelial cell chemokine production.

It is of key importance to determine whether AMCase is a potential therapeutic target, because AMCase is abundantly expressed not only in the airways but also in the gastrointestinal tract (Boot *et al.*, 2001; Paoletti *et al.*, 2007). IL-13 and GATA-3 are known to play a crucial role in the development of asthma (Huebner *et al.*, 2008; Sel *et al.*, 2008), and studies have shown that RNAi targeting IL-13 (Lively *et al.*, 2008) and GATA-3 (Lee *et al.*, 2008a) might serve as a novel therapeutic approach in the treatment of this condition. In the current study, we have shown that AMCase hyperexpression in sensitized mice is likely to be airway specific, being limited to the lung and alveolar macrophages. Local AMCase silencing in the airways could be achieved via an intratracheal administration of rAAV-shRNA.

Further investigation may be needed to determine whether the current strategy for targeting asthma delivers prophylactic and/or therapeutic effects; it could be beneficial if shRNA-mediated reduction of AMCase has prophylactic effects on asthma. Basically, in the current study, allergic responses including BALF eosinophilia as well as the elicitation of serum OVA-IgE appeared on day 18 after inhalation challenge with allergen OVA (see Supplementary Fig. 1 at www.liebertonline.com/hum). AMCase activity was increased after mice received only two inhalation challenges (Supplementary Fig. 1). However, treatment with rAAV-shRNA was applied after mice received four inhalation challenges, suggesting that shRNA-mediated reduction of AMCase possibly has therapeutic effects.

To overcome poor transfection rates *in vivo*, we used the AAV vector as a delivery tool for shRNA, in keeping with similar studies on bronchial diseases (Huang *et al.*, 2008; Lee *et al.*, 2008a). Zavorotinskaya and coworkers have previously compared the efficacy of a single intramuscular injection

versus intratracheal administration of an rAAV vector carrying the IL-4 receptor antagonist in OVA-sensitized mice (Zavorotinskaya *et al.*, 2003). The authors showed that intratracheal administration resulted in significant local effects in the airways, with no systemic or local adverse effects (Zavorotinskaya *et al.*, 2003). Accordingly, the AAV vector achieved a better transfection rate not only *in vivo*, but also *in vitro*. In the present study, liposome-based transfection with pCI-shRNA resulted in approximately 85% AMCase inhibition, whereas almost total suppression was achieved with rAAV-shRNA. These data clearly indicate that rAAV may be regarded as an optimal transfection vector. To investigate the inhibitory effects of RNAi against AMCase, we have used stable cell lines overexpressing AMCase. It may be argued that RNA interference could exert only minor effects on AMCase expression in alveolar macrophages and lung epithelial cells, where AMCase expression is low. To address that issue, we have tested the inhibitory effects of rAAV-shRNA1336 on AMCase expression in lung epithelial cells (MLE-12), and a remarkable reduction of AMCase expression was also observed (data not shown). In addition, we also addressed whether the inhibitory effects of rAAV-shRNA on AMCase expression could be affected by cell division. As shown in Supplementary Fig. 2 (at www.liebertonline.com/hum), most of the proliferating cells (Ki67⁺) were not AMCase-expressing cells, although a few of them appeared to be positively stained for hrGFP, indicating that AAV can still infect some proliferating cells in the lung.

Gene transfer has emerged as a novel powerful therapeutic strategy and many reports have compared the advantages and limitations of various gene delivery vectors. In this study, we have used the rAAV vector as a tool for targeting asthma. Notably, adeno-associated viruses have been investigated in human gene therapy trials. Several studies have pointed out that long-term expression of transgenes may be achieved with little inflammatory response (McPhee *et al.*, 2006; Moss *et al.*, 2007), indicating that gene delivery via rAAV may represent the better option.

In summary, this is the first study to show the potential usefulness of silencing AMCase expression in asthma therapy. Further investigations are warranted to further understand the exact role of AMCase in the pathogenesis of this disabling allergic disorder.

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Author Disclosure Statement

No competing financial interests exist.

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