Carboxymethyl chitosan prolongs adenovirus-mediated expression of IL-10 and ameliorates hepatic fibrosis in a mouse model

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Funding information
2019 Chongqing Support Program for Entrepreneurship and Innovation, Grant/ Award Number: cx2019113; 2019 Funding for Postdoctoral Research, Chongqing Human Resources and Social Security Bureau, Grant/ Award Number: 298; 2019 Science and Technology Research Plan Project of Chongqing Education Commission, Grant/ Award Number: KJQN201900410; 2019 Youth Innovative Talent Training Program of Chongqing Education Commission, Grant/

Abstract
Effective and safe liver-directed gene therapy has great promise in treating a broad range of liver diseases. While adenoviral (Ad) vectors have been widely used for efficacious in vivo gene delivery, their translational utilities are severely limited due to the short duration of transgene expression and solicitation of host immune response. Used as a promising polymeric vehicle for drug release and nucleic acid delivery, carboxymethyl chitosan (CMC) is biocompatible, biodegradable, anti-microbial, inexpensive, and easy accessible. Here, by exploiting its biocompatibility, controlled release capability and anti-inflammatory activity, we investigated whether CMC can overcome the shortcomings of Ad-mediated gene delivery, hence improving the prospect of Ad applications in gene therapy. We demonstrated that in the presence of optimal concentrations of CMC, Ad-mediated transgene expression lasted up to 50 days after subcutaneous injection, and at least 7 days after intrahepatic injection. Histologic evaluation and immunohistochemical analysis revealed that CMC effectively alleviated Ad-induced host immune response. In our proof-of-principle experiment using
1 | INTRODUCTION

Liver is a key organ in the human body and carries out a variety of essential functions including digestion, metabolism, detoxification, immunity and blood clotting. At the cellular level, hepatocytes constitute the vast majority of cells in the liver parenchyma and are implicated in the majority of monogenic liver inherited disorders, metabolic disorders, viral infections, and malignancies. Even though conventional treatments can alleviate symptoms of some liver disorders, very few curative treatments currently exist. Therefore, effective and safe liver-directed gene therapy holds great promise in treating a broad range of liver diseases, such as cancer, metabolic disorders, and certain monogenic disorders.

Liver is an ideal target for gene therapy since it is one of the largest organs in the human body, and contains 10%–15% of the total blood volume of the body. For the past several decades, numerous efforts have been devoted to the development of liver-directed gene delivery systems. The gene delivery systems are in general divided into two categories: viral vector-based and non-viral vector-based delivery systems. The viral vector systems include a large group of recombinant, replication-deficient viruses; and the most commonly-used ones are adenoviral (Ad) vectors, adeno-associated viral (AAV) vectors, lentiviral vectors, as well as certain less frequently used ones such as foamy viral vectors and herpes simplex viral vectors. While viral vectors are highly effective in gene delivery and have been used in approximately 70% clinical trials, the major hurdles for viral vectors include potential carcinogenesis and/or immunogenicity. Non-viral vector-based delivery systems take advantage of receptor-mediated endocytosis and/or membrane fusion functions via the use of lipids, polymers, proteins, and peptides, as well as by physical forces such as needle injection, gene gun, electroporation, sonoporation, and hydrodynamic delivery. While the major challenge for non-viral vector-mediated gene delivery is its relatively low efficiency, an increasing number of non-viral vectors are emerging as valid vehicles for the delivery of genetic materials, especially the use of lipid-based nanoparticles for RNA delivery.

Nonetheless, viral vectors remain as one of the most preferred approaches to target hepatocytes. Among them, Ad vector represents the prototype viral vector with high gene transfer efficiency, well-understood virology and pathogenicity, and ease to mass production. The major drawbacks for Ad vector as a liver-targeted delivery system are a relatively short-term of transgene expression and the solicitation of host immune response. Therefore, it is conceivable that overcoming the above shortcomings should make Ad vector a more desirable gene delivery vector for liver-directed gene therapy.

As a product of the deacetylation of chitin, chitosan is a linear polysaccharide, multi-functional, and eco-friendly anti fouling polymer. Chitosan is nontoxic, biocompatible, and biodegradable, which makes it a polymer of choice for many biomedical and pharmaceutical applications since it was first reported in late 1990s. The chemical versatility of chitosan and its derivatives is reflected by its chemical (IHC) analysis revealed that CMC effectively alleviated Ad-mediated transgene expression lasting up to 50 days after subcutaneous injection, and at least 7 days after intrahepatic injection. Histologic evaluation and immunohistochemical (IHC) analysis revealed that CMC effectively alleviated Ad-induced host immune response. Using the CCl4-induced experimental mouse model of chronic liver damage, we demonstrated that repeated intrahepatic administrations of Ad-IL10 mixed with CMC effectively mitigated the development of hepatic fibrosis. Collectively, these results indicate that CMC can improve the prospect of Ad-mediated gene therapy by diminishing the host immune response while allowing readministration and sustained transgene expression.

KEYWORDS
adenovirus vector, carboxymethyl chitosan (CMC), chitosan, gene delivery, gene therapy, hepatic fibrosis, host immune response
host immune response while allowing readministration and sustained transgene expression.

2 | MATERIALS AND METHODS

2.1 | Cell culture and chemicals

HEK-293 derivative 293pTP and RAPA cells were previously characterized and used for adenovirus packaging and subsequent amplification. These cells were cultured in high glucose complete Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (Cat# S711-001S, Lonsera), 100 units of penicillin, and 100 μg of streptomycin at 37°C in 5% CO₂ as described. CMC (cat# sc-358091, CAS 83512-85-0) was purchased from Santa Cruz Biotechnology. CMC was dissolved in sterile PBS to prepare for a stock solution of 5% (wt/vol) and was kept at 4°C. Unless indicated otherwise, all other chemicals were purchased from Sigma-Aldrich, Thermo Fisher Scientific, or Solarbio.

2.2 | Construction, amplification, and purification of the recombinant adenoviruses Ad-FLuc, Ad-IL10, and Ad-GFP

All recombinant adenoviruses were constructed by using the AdEasy technology. The Ad-FLuc, which co-expresses firefly luciferase (Fluc) and GFP, was generated as described in our previous studies. For the construction of Ad-IL10, the coding region of human IL-10 was PCR amplified and subcloned into an adenoviral shuttle vector, pAdTrack-CMV. The resulting vector was used to generate the recombinant adenoviral plasmid pAd-IL10 through homologous recombination with an adenoviral backbone vector in bacterial BJ5183 cells. The adenoviral plasmid pAd-IL10 was subsequently linearized and used to generate recombinant adenovirus Ad-IL10 in 293pTP or RAPA packaging cells. The Ad-IL10 also co-expresses GFP as a marker for tracking infection efficiency as described. An analogous adenovirus expressing GFP only, Ad-GFP, was used as a mock virus control.

For direct in vivo injection studies, Ad-FLuc, Ad-IL10, and Ad-GFP were amplified in large scale and purified through CsCl gradient ultracentrifugation, as described. The purified high titer adenovirus stocks were titered, aliquoted, and stored at −20°C. Desalting dialysis against sterile PBS was performed immediately prior to use.

2.3 | Determination of the effect of CMC on Ad-directed acute and chronic host immune response

The use and care of animals in this study was approved by the Animal Care and Use Committee of The University of Chicago, Illinois, USA, and the Ethics Committee for Research and Experimental Animal Use of Chongqing Medical University, Chongqing, China. All experimental procedures followed the approved guidelines.

Subcutaneous or intrahepatic injection of Ad-GFP mixed with or without CMC was carried out to assess Ad-induced host immune response. For the acute response, two routes of Ad delivery were assessed. In the first route, C57BL/6J mice (n = 9, male, 6-week old) were subcutaneously injected into both flanks with 10^{10} pfu (plaque forming unit) of Ad-GFP mixed with either PBS alone or 1.3% CMC (wt/vol, in PBS) in 30 μl total volume. Three mice were sacrificed at 6, 24, and 72 h after injection. The injection sites were retrieved for histologic and IHC analyses. In the second route, C57BL/6J mice (n = 30, male, 6-week old) were subjected to intrahepatic injection with 10^{10} pfu of Ad-GFP mixed with either PBS alone (Ad-GFP, control group) or 1.3% CMC (wt/vol, in PBS) (Ad-GFP + CMC, treatment group) in 30 μl total volume. At 6, 24, and 72 h after injection, five mice from each group were sacrificed at each time point. Both serum and liver samples were retrieved for blood analysis and histologic evaluation, respectively.

For long-term chronic immune response, C57BL/6J mice (n = 20, male, 6-week old) were subjected to intrahepatic injection with 10^{10} pfu of Ad-GFP mixed with either PBS alone (Ad-GFP, control group) or 1.3% CMC (wt/vol, in PBS) (Ad-GFP + CMC, treatment group) in 30 μl total volume. The intrahepatic injections were repeated once every 10 days. Five mice from each group were sacrificed at 4 and 8 weeks after the first injection. Both serum and liver samples were retrieved for blood analysis and histologic evaluation, respectively.

2.4 | Subcutaneous and intrahepatic injection of Ad-FLuc encapsulated with CMC

Subcutaneous injection of the adenovirus Ad-FLuc was used to determine the effect of various concentrations of CMC on adenovirus-mediated transgene expression as described. Specifically, C57BL/6J mice (male, 6-week old, n = 4) were subcutaneously injected into both flanks with 10^{10} pfu of Ad-FLuc, which was premixed with 0% (wt/vol, in PBS), 1.25% (wt/vol, in PBS), 2.5% (wt/vol, in PBS), and 5% (wt/vol, in PBS) CMC (in 30 μl total volume) at each injection site, as shown in Figure 1a. Whole body optical bioluminescence imaging was performed at 3, 9, 15, 21, 28, 35, 43, and 50 days after injection by using the IVIS Spectrum In Vivo Imaging System (Perkin Elmer) with D-Luciferin potassium (Gold Biotechnology, Inc.) as luciferase substrate as described.

For intrahepatic injection of Ad-FLuc, C57BL/6J mice (male, 6-week old, n = 4 each group) received intrahepatic injection of 10^{10} pfu of Ad-FLuc, which was premixed with 0% CMC (wt/vol, in PBS), or 1.3% CMC (wt/vol, in PBS), all in 30 μl total volume, as shown in Figure 1b. Whole body optical bioluminescence imaging was performed at 3, 7, and 12 days after injection by using the IVIS Spectrum in vivo imaging system as described.

2.5 | Establishment of the mouse model of chronic hepatic injury and fibrosis

The hepatic fibrosis model was established as described. Briefly, C57BL/6J mice (male, 6-week old) were intraperitoneally injected with 2.0 μl/g body weight (g/b.w.) of 20% CCl₄ solution (wt/vol, in olive oil) twice a week for up to 8 weeks as described. The control mice received...
intraperitoneal injections of 2.0 μl/g.b.w. olive oil twice a week. At each endpoint, serum levels of liver enzymes and liver histology were analyzed.

2.6 | The effect of CMC-encapsulated Ad-IL10 on the mouse model of hepatic fibrosis

A total of 40 C57BL/6J mice (male, 6 weeks old) were randomly divided into four groups (10 mice each group). Ten mice were subjected to intraperitoneal injection of olive oil twice a week at 2.0 μl/g. b.w. and served as the control group. The other 30 mice were intraperitoneally injected twice a week with 2.0 μl/g 20% CCl4 solution (wt/vol, in olive oil) to establish experimental liver fibrosis. Concurrently with the first injection of CCl4 solution, 10 mice were intrahepatically injected with 1010 pfu of Ad-GFP (in PBS, each mouse in 30 μl total volume; aka, Fibrosis model group), 1010 pfu of Ad-IL10 (in PBS, each mouse in 30 μl total volume; aka, IL10 group), or 1010 pfu of Ad-IL10 (in 1.3% CMC/PBS, each mouse in 30 μl total volume; aka, IL10 + CMC group). The Ad injections (mixed with or without CMC) were repeated once every 10 days. Animal body weight changes were recorded weekly. At weeks 4 and 8 after the first injection, five mice from each group were sacrificed. Both serum and liver samples were retrieved for blood analysis and histologic evaluation, respectively.
2.7 | Determination of the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), and albumin (Alb)

Mouse cardiac blood collection was carried out as previously described.\(^\text{37,44}\) Briefly, mice were first anesthetized by intraperitoneal injection of 3% sodium pentobarbital at 50 mg/kg body weight. An incision was made in upper-middle abdomen across the abdominal and chest cavity to expose the liver and heart, and phlebotomized slowly from the left ventricle until reaching 500 μl for each mouse. The mice were then euthanized. The collected blood samples were centrifuged at 3500RPM for 10 min at RT. The upper portion of the serum samples was collected for assessing liver function parameters: ALT (Cat# C009-2-1, Nanjing Jianscheng Bioengineering Institute, China), AST (Cat# C010-2-1, Nanjing Jianscheng Bioengineering Institute), TBIL (Cat# C019-1-1, Nanjing Jianscheng Bioengineering Institute), and DBIL (Cat# C019-2-1, Nanjing Jianscheng Bioengineering Institute), and Alb (Cat# A028-2-1, Nanjing Jianscheng Bioengineering Institute).

2.8 | Total RNA isolation and touchdown-quantitative real-time PCR (TqPCR) analysis

Total RNA from freshly-prepared liver tissues was isolated by using the TRIZOL Reagent (Invitrogen) as described.\(^\text{45,46}\) Total RNA was subjected to reverse transcription with hexamer and M-MuLV reverse transcriptase (New England Biolabs). The cDNA products were further diluted and used as PCR templates. Gene-specific qPCR primers were designed by using the Primer3 program (Table S1). TqPCR was carried out by using 2x SYBR Green qPCR Master Mix (Bimake) on a CFX-Connect unit (Bio-Rad Laboratories) as described.\(^\text{37}\) All TqPCR reactions were done in triplicate. Gapdh was used as a reference gene. Quantification of gene expression was carried out by using the \(2^{-\Delta\Delta Cq}\) method as described.\(^\text{48}\)

2.9 | Hematoxylin and eosin (H&E) analysis and Sirius red staining

The retrieved skin and liver samples were fixed with 4% paraformaldehyde and subjected to paraffin embedding, followed by sectioning. The slides were deparaffinized and used for H&E staining as previously described.\(^\text{46,49,50}\) The sections from the retrieved liver samples were deparaffinized and subjected to Sirius red staining (Picro Sirius Red solution, G1471, Solarbio) as previously reported.\(^\text{43}\)

2.10 | IHC staining

The sections from the above paraffin-embedded skin and liver samples were also deparaffinized and subjected to IHC staining as described.\(^\text{45,48,51,52}\) Specifically, the sections were deparaffinized and rehydrated. After antigen retrieval, the sections were subjected to immunostaining with antibodies against CD45 (1:100 dilution; Wanleibio; Cat# WL00922), CD54 (1:100 dilution; Wanleibio; Cat# WL025268), CD40L (1:50 dilution; Bimake; Cat. No. A5778), CD3D (1:50 dilution; Bimake; Cat. No. A5886), TNFα (1:200 dilution; Wanleibio; Cat# WL01896), IL1β (1:200 dilution; Wanleibio; Cat# WL00891), CD20 (1:200 dilution; Wanleibio; Cat# WL02883), IL10 (1:200 dilution; Wanleibio; Cat# WL03088), Collagen I (1:100 dilution; Wanleibio; Cat# WL0088), α-SMA (1:50 dilution; Wanleibio; Cat# WL02510), or TIMP1 (1:100 dilution; Wanleibio; Cat# WL02342). The proteins of interest were detected with the biotin labeled goat anti-rabbit IgG or anti-mouse IgG streptavidin-HRP kit (SP Kit, SP-9000, ZSGB-Bio). Minus primary antibody and/or rabbit IgG and mouse IgG were used as negative controls. Staining results were recorded under a bright field microscope (Leica, DM4B).
When the intrahepatic injection was carried out as illustrated in Figure S1B, we found that injections with or without 1.3% CMC exhibited strong FLuc signals at Day 3 (Figure 1b). However, the Ad-FLuc+1.3% CMC group maintained relatively strong signals at Day 7 (although the signals were not readily detectable at Day 12), while the Ad-FLuc alone group failed to display any FLuc activity under the same imaging condition (Figure 1b, Panel i vs. ii). Collectively, these results demonstrate that optimal concentrations of CMC (e.g., between 1.25% and 2.5%) can effectively prolong adenovirus-mediated transgene expression in vivo.

### 3.2 CMC mitigates adenoviral vector-elicited host immune and inflammatory responses in vivo

We further analyzed the effect of CMC on adenovirus-induced acute and chronic host immune response. When Ad-GFP vector mixed with or without 1.3% CMC was subcutaneously injected into immunocompetent mice, papules appeared at the injection sites of the Ad-GFP group, but not the Ad-GFP + CMC group at 24 h after injection (Figure 2a). H&E staining revealed that while a large number of inflammatory cells were presented in the whole skin layer, especially around the injection site under the dermis in the Ad-GFP group at 6 and 24 h after injection, the Ad-GFP + CMC group exhibited lower numbers of inflammatory cells in the skin, especially around the injection site at all three time points (Figure 2b, Panel i). Since mature dendritic cells (DCs) are important antigen presenting cells to activate T lymphocytes and secrete TNFα and IL1β to regulate the acute immune response, we performed IHC analysis to assess the presence of these inflammatory response cells, and found that the presence of CMC in Ad-GFP subcutaneous injection effectively decreased the number of inflammatory cells (marked by CD45), mature DCs (marked by CD54), mature T lymphocytes (marked by CD40L and CD3D), and secreted TNFα and IL1β at Week 4, compared with that in the Ad-GFP only group (Figure S2E), no discernible differences in the gross appearance of the liver samples in both groups, compared with that of the normal control, were observed (Figure S2F). H&E staining revealed that the number of inflammatory cells around the central veins and portal areas in the Ad-GFP + CMC group was lower than that in the Ad-GFP only group at both Week 4 and Week 8 time points, respectively (Figure 4b).

Since chronic inflammatory injury usually activates both cellular immunity and B cell-dependent humoral immunity,

3.3 CMC alleviates adenovirus-induced chronic inflammatory injury of liver

We next examined the effect of CMC on chronic inflammatory injury after repeated long-term administration of adenovirus to the liver. Specifically, Ad-GFP mixed with or without 1.3% CMC (wt/vol, in PBS) was intrahepatically injected once every 10 days for 4 and 8 weeks, respectively (Figure 4a). While the serum ALT and AST activities were elevated in the Ad-GFP only group at Week 4, compared with that in the Ad-GFP + CMC group (Figure S2E), no discernible differences in the gross appearance of the liver samples in both groups, compared with that of the normal control, were observed (Figure S2F). H&E staining revealed that the number of inflammatory cells around the central veins and portal areas in the Ad-GFP + CMC group was lower than that in the Ad-GFP only group at both Week 4 and Week 8 time points, respectively (Figure 4b).

Since chronic inflammatory injury usually activates both cellular immunity and B cell-dependent humoral immunity,

3.4 Intrahepatic administration of CMC-encapsulated Ad-IL10 effectively alleviates hepatic fibrosis in a mouse model

While the exact mechanism remains to be fully understood, liver fibrosis is considered as a fibrotic healing response against a chronic injury or insult to the liver. Here, we sought to establish an experimental hepatic fibrosis mouse model by intraperitoneal injection of carbon tetrachloride (CCl4). Briefly, C57BL/6J mice were intraperitoneally injected with 2.0 μl/g.b.w. of 20% CCl4 solution (in olive oil) twice a week for up to 8 weeks as described. The control mice received intraperitoneal injections of 2.0 μl/g.b.w. olive oil twice a week. Mouse body weight was monitored and exhibited no significant difference between the model group and control group (Figure S4A). At
FIGURE 2  CMC effectively mitigates host acute immune response to subcutaneous adenovirus infection. (a) Ad-GFP with (red cycle) or without (yellow cycle) 1.3% (wt/vol, in PBS) CMC in 30 μl total volume of PBS were injected subcutaneously into the back of C57BL/6J mice as shown. Mice were sacrificed at 6, 24, and 72 h after injection, respectively. The appearance of skin at injection site was documented. Representative images are shown. (b) Histologic evaluation and immunohistochemical staining. The retrieved skin tissues from injection sites were subjected to H&E staining and inflammatory cells were indicated by yellow boxes (×100) and arrows (×200) (i). The retrieved tissues were further subjected to IHC staining assays with antibodies for CD45, CD54, CD40L, CD3D, TNFα, and IL1β (ii). Representative positively stained cells are indicated with red arrows (×400). Representative results are shown.
4 and 8 weeks after injection, serum levels of liver enzymes were analyzed and found that ALT and AST (except Week 8) activities, but not TBIL, DBIL, and Alb (except for Week 4), were significantly elevated in the fibrosis model group (Figure S4B). Gross images of the retrieved liver samples revealed that fibrotic nodules were readily found in the model group at both Week 4 and Week 8, respectively, compared with that in the control group (Figure S4C, Panel a). H&E staining showed that inflammatory cells and balloon-like changes of hepatocytes were presented in the liver tissues of the fibrosis model group, but not in the control group, at Weeks 4 and 8, respectively (Figure S4C, Panel b). Sirius red staining revealed that collagen was deposited around the central veins and portal areas of liver tissues in the fibrosis model group at Week 4; and more fibrous septa and pseudolobuli were readily observed in the fibrosis model group, but not in the control group, at Weeks 4 and 8, respectively (Figure S4C, Panel c). These results demonstrate that the mouse model of CCl₄-induced experimental hepatic fibrosis was successfully established.

Interleukin-10 (IL10) is a cytokine produced by numerous activated immune cells such as B cells, mast cells, granulocytes, macrophages, DCs, and multiple T cell subsets, with plural and diverse
cellular functions, and may play a beneficial preventive role in hepatic fibrosis.\textsuperscript{55,57,62} As a proof-of-concept experiment, we sought to investigate whether the intrahepatic administration of CMC-encapsulated Ad-IL10 would prevent and/or alleviate hepatic fibrosis in the mouse model of CCl\textsubscript{4}-induced experimental hepatic fibrosis. As shown in Figure 5a, three groups were set up: the fibrosis model only, the Ad-IL10 group, and Ad-IL10 + CMC group, in which all mice were treated with CCl\textsubscript{4} twice a week for 4 and 8 weeks, while the mice in the Ad-IL10 and Ad-IL10 + CMC groups also received intrahepatic injections of the Ad-IL10 and Ad-IL10 + CMC, respectively, once every 10 days for 4 and 8 weeks.

While the mouse body weight of the three groups did not show any significant difference (Figure 5S A), the serum ALT of the IL-10 + CMC group increased at Week 4, and the serum DBIL level of the IL-10 group increased compared with that in the fibrosis group at Week 4 (Figure 5S B). The gross images indicated that while the liver surface in all three groups was rough and granular at both Weeks 4 and 8, the number of white nodules on the liver surface in the Ad-IL10 group and the Ad-IL10 + CMC group was less than that in the fibrosis model group at Week 8 (Figure 4b).

The retrieved liver tissues were subjected to H&E staining (b). Representative inflammatory cells are indicated with yellow arrows (×200). The retrieved liver tissues were also subjected to IHC staining with antibodies against CD45 and CD20 (c). Representative positively stained cells are indicated with red arrows (×400) (c). Representative results are shown...
at both Weeks 4 and 8 (Figure 5d). These results demonstrate that adenovirus-mediated delivery of IL10, especially when Ad-IL10 was encapsulated with CMC, could effectively alleviate CCl4-induced hepatic fibrosis.

It has been well established that hepatic staller cells (HSCs) are the main myofibroblast progenitor cells and key effectors of fibrogenic response. During liver injury, activated HSCs progressively lose their star-shaped morphology and their lipid droplets, and produce abundant extracellular matrix components such as Type I, III, and IV collagens, fibronectin, laminin, and proteoglycans, and pro-inflammatory mediators, as well as expressing high levels of alpha smooth muscle actin (α-Sma) and tissue inhibitor of metalloproteinase 1 (Timp1) that contribute to the change from adipocytic phenotype to profibrogenic and inflammatory phenotype. The qPCR analysis indicated that while the expression of IL-10 increased, the expression of Type I collagen, α-Sma, and Timp1 significantly decreased both in the Ad-IL10 group and the Ad-IL10 + CMC group, compared with that in the fibrosis model group, although the decrease in the Ad-IL10 + CMC group was more pronounced than that in the Ad-IL10 group (Figure S5C). Furthermore, the IHC staining analysis revealed the high expression of IL-10 in the Ad-IL10 + CMC group, compared with that in other two groups (Figure 6a). As expected, the expression of collagen I, α-Sma, and Timp1 was most profoundly down-regulated in the Ad-IL10 + CMC group, compared with that in the fibrosis model group and the Ad-IL10 group (Figures 6b–d and S5D). Collectively, the above results strongly suggest that CMC-encapsulated Ad-IL10 may effectively control the release and production of IL-10 through intrahepatic injection and subsequently alleviate CCl4-induced hepatic fibrosis in a mouse model.

4 | DISCUSSION

Viral vector-mediated gene delivery remains one of the most preferred approaches for liver-directed gene therapy. Among the viral vectors, although AAV-based gene therapy has recently received a great deal of attention due to AAV's favorable biosafety profile and reduced host immune response, Ad vector remains as a vector of choice in many cases including liver-directed gene therapy, because it has at least two advantages over AAV and other viral vectors: it has...
high gene transfer efficiency, and it is easy to scale up for mass production with high titer. However, two major shortcomings for Ad vector as a gene delivery system are the relatively short-term of transgene expression and the solicitation of host immune response. Thus, overcoming these limitations would improve the prospect of using Ad vector as a preferred gene delivery vehicle for gene therapy including liver-directed gene therapy.

In this study, we demonstrated that CMC could effectively prolong Ad-mediated transgene expression in vivo, while reducing Ad-induced host immune response, leading to sustained transgene expression. Using whole body optical imaging analysis, we showed that, in the presence of optimal concentrations (e.g., 1.25%–2.5% wt/vol) of CMC, Ad-mediated firefly luciferase (FLuc) expression lasted up to 50 days after a single subcutaneous injection, and at least 7 days after intrahepatic injection. Histologic evaluation and IHC analysis revealed that CMC effectively alleviated Ad-induced host immune response. Interestingly, we found that CMC at higher concentrations (e.g., 5% wt/vol) seemingly decreased FLuc expression, suggesting that high concentrations of CMC may prevent the timely release of Ad vectors and thus limit Ad-mediated transgene delivery efficiency. While the exact mechanism through which CMC prolongs Ad-mediated transgene expression in vivo remains to be fully understood, it is possible that the complex polymer structure of CMC controls the sustained release of Ad vector, and that the alleviation of Ad-induced host immune response may further contribute to the sustained transgene expression in vivo.

Due to the transient expression nature of Ad-mediated gene delivery, re-administration is a desirable strategy to achieve sustained transgene expression in vivo. However, repeated injections of Ad vector will not only solicit strong host immune response, but also produce neutralizing antibodies to rapidly eliminate Ad vector in vivo. It is conceivable that CMC’s ability to alleviate host immune response may provide an opportunity for the repeated administrations of Ad vector in vivo. To provide a proof-of-concept study, we established a CCl4-induced experimental mouse model of chronic liver damage, and demonstrated that repeated intrahepatic injections, once every 10 days for up to six times, of Ad-IL10 mixed with CMC effectively alleviated the development of hepatic fibrosis. It has been reported that IL10 exhibits anti-hepatic fibrosis activity. Since the pathogenic process of hepatic fibrosis is highly complex, it is conceivable that other contributing factors may be explored and targeted through Ad-mediated gene therapy in combination with CMC. Collectively, our results strongly indicate that chitosan derivatives such as CMC provide apparent benefit for Ad-mediated in vivo gene delivery by diminishing host immune response while allowing sustained transgene expression. Nonetheless, it remains to be investigated whether CMC can prolong the transgene expression of non-secreted proteins mediated by adenoviral vectors in vivo.
In this study, we chose to use CMC as the Ad vector delivery vehicle because CMC is widely available and has been approved for several preclinical and clinical applications such as wound healing dressing, biological imaging, tissue engineering, and controlled drug release.\textsuperscript{10,12,13,16} It is conceivable that other chitosan derivatives may also be used as Ad vector delivery carriers.\textsuperscript{9,11-13} The chemical versatility of chitosan and its derivatives is reflected by its ability to form a poly-cationic charged polymer at physiological pH, and by its modifiable molecular weight and types of surface modifications, which in turn impact chitosan’s chemical and biological properties.\textsuperscript{12} In fact, numerous studies have been devoted to modify and optimize CMC and/or other chitosan derivatives.\textsuperscript{10-12,15} Early studies reported that both linear and branched polyethyleneimine (PEI)-graft-chitosan copolymers functioned as efficient DNA/siRNA delivery vehicles both in vitro and in vivo.\textsuperscript{63,64} Liang et al.\textsuperscript{65} reported that tetradecyl-β-(PEGylated) chitosan nanoparticles, encapsulating β-catenin siRNA, effectively decreased β-catenin protein levels in colon cancer cells in vitro.\textsuperscript{67} Interestingly, a CMC-modified polyamidoamine dendrimer achieved pH-sensitive drug release in response to tumor microenvironment pH changes.\textsuperscript{68} More recently, it has been reported that a reactive oxygen species-sensitive hydrogel with strong free radical scavenging ability was prepared by introducing the thione group into CMC hydrogel, which enhanced CMC’s wound healing efficacy.\textsuperscript{69} Thus, some of these modified CMC and chitosan derivatives may be further explored as potential Ad delivery carriers.

It is worth noting that many polymers such as PEI, poly(ethylene glycol), poly(l-lysine) (PLL), polyamidoamine dendrimer (PAMAM), and poly(aminoothers) have been exploited to enhance Ad vector-mediated gene delivery, mostly by evading host immune response and/or redirecting tropism.\textsuperscript{70-72} However, our results demonstrate that CMC and potentially other chitosan derivatives may be a superior choice of polymeric helper for Ad gene therapy due to their exceptional properties, including biocompatibility, biodegradability, non-cytotoxicity, antimicrobial and anti-inflammatory activity, low immunogenicity, inexpensiveness, and accessibility.

5 | CONCLUSIONS

We sought to overcome the transient expression nature and strong host immune response associated with Ad-mediated gene therapy by exploiting CMC’s biocompatibility, controlled release capability and anti-inflammatory activity. Our results demonstrated that in the presence of optimal concentrations of CMC, Ad-mediated transgene expression lasted up to 50 days after subcutaneous injection, and at least 7 days after intrahepatic injection, respectively. Histologic evaluation and IHC analysis revealed that CMC effectively alleviated Ad-induced host immune response. In our proof-of-principle study using the CCL\textsubscript{2}-induced experimental mouse model of chronic liver damage, we showed that repeated intrahepatic administrations of Ad-IL10 mixed with CMC effectively alleviated the development of hepatic fibrosis. Collectively, these results indicate that chitosan derivatives such as CMC can provide a beneficial effect for Ad-mediated in vivo gene delivery by diminishing the host immune response while allowing sustained transgene expression.

ACKNOWLEDGMENTS

This study was supported in part by research grants from the 2019 Chongqing Support Program for Entrepreneurship and Innovation (no. cx2019113, Jiaming Fan), the 2019 Science and Technology Research Plan Project of Chongqing Education Commission (KJQN201900410, Jiaming Fan), the 2019 Youth Innovative Talent Training Program of Chongqing Education Commission (no. CY200409, Jiaming Fan), the 2019 Funding for Postdoctoral Research (Chongqing Human Resources and Social Security Bureau no. 298, Jiaming Fan), the Natural Science Foundation of China (82102696, Jiaming Fan), William Wagstaff was supported by the Medical Scientist Training Program of the National Institutes of Health (T32 GM007281). This project was also supported in part by The University of Chicago Cancer Center Support Grant (P30CA014599) and the National Center for Advancing Translational Sciences of the National Institutes of Health through Grant Number UL1 TR000430. Tong-Chuan He was supported by the Mabel Green Myers Research Endowment Fund and The University of Chicago Orthopedics Alumni Fund. Funding sources were not involved in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

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**CONFLICTS OF INTEREST**
The authors declare no conflicts of interest.

**PEER REVIEW**
The peer review history for this article is available at https://publons.com/publon/10.1002/btm2.10306.

**DATA AVAILABILITY STATEMENT**
The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials, or available from the corresponding authors upon reasonable request.

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_How to cite this article:_ Gou Y, Weng Y, Chen Q, et al. Carboxymethyl chitosan prolongs adenovirus-mediated expression of IL-10 and ameliorates hepatic fibrosis in a mouse model. Bioeng Transl Med. 2022;7(3):e10306. doi:10.1002/btm2.10306