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Efficient Attenuation of NK Cell–Mediated Liver Injury through Genetically Manipulating Multiple Immunogenes by Using a Liver-Directed Vector

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Adenovirus or adenoviral vectors were reported to induce serious liver inflammation in an NK cell–dependent manner, which limits its clinical applicability for liver gene therapy. We tried to develop an efficient liver-directed therapeutic approach to control hepatic NK cell function via simultaneously manipulating multiple immune genes. Based on our previous study, we found that CCL5 knockdown synergistically enhanced the attenuating effect of silencing CX3CL1 (fractalkine [FKN]) in adenovirus-induced acute liver injury. In addition, the combined treatment of human IL-10 expression with FKN knockdown would further strengthen the protective effect of silencing FKN. We used a hepatocyte-specific promoter to construct a hepatocyte-specific multiple function vector, which could simultaneously overexpress human IL-10 and knock down CCL5 and FKN expression. This vector could attenuate adenovirus-induced acute hepatitis highly efficiently by reducing liver NK cell recruitment and serum IFN-γ and TNF-α. The multiple function vectors could be delivered by nonviral (hydrodynamic injection) and viral (adenovirus) approaches, and maintained long-term function (more than 1 month in mice). Our results suggest a possible strategy to ameliorate the acute liver injury induced by adenovirus by modulating multiple immune genes. The novel multifunction vector has an extensive and practical use for polygenic and complex liver diseases such as malignancies and hepatitis, which correlate with multiple gene disorders. The Journal of Immunology, 2013, 190: 4821–4829.

The liver is the largest solid organ in the body, and its efficient protein synthetic capacity makes the liver an attractive target organ for gene therapy (1, 2). Although exciting results were obtained in animal models and in some clinical trials, many obstacles remain in clinical practice. Among these obstacles, liver-specificity and immune injury are the most challenging (3). Due to its liver tropism feature, ability to infect quiescent hepatocytes, and high capacity for transgenes, the adenoviral vector is now the most widely used vector in clinical trials (2). Systemic infection of high-dose adenoviruses will initiate innate and adaptive immune responses against adenovirus that causes severe allergic reaction and inactivation of adenoviral vectors, which is the major drawback of adenovirus-mediated gene therapy (4).

A variety of strategies have been used to reduce the immunogenicity of adenovirus and inhibit the liver injury induced by adenovirus. First, gutless adenovirus lacking all viral coding regions has been developed and demonstrated to be an attractive gene therapy vector with highly reduced toxicity and long-term transduction efficiency (5, 6). Second, chemically engineered vectors such as polyethylene glycol modified adenoviruses exhibit receptor-specific cell transduction and reduced vector toxicity (7). Third, because adenovirus-induced strong innate responses are mainly mediated by innate immune cells such as NK cells, cell activities and functions can be regulated to ameliorate the liver injury. Blocking of NKG2D receptor inhibited NK cell activation upon adenovirus infection, leading to a delay in adenovirus clearance and NK cell–mediated cytolysis (8). Knockdown of chemokines such as fractalkine (FKN) to inhibit NK cell recruitment and activity could protect the acute liver injury induced by adenoviral vector (9). Inflammatory cytokines such as IFN and TNF had important roles in adenovirus-induced acute liver injury; therefore, overexpression of inhibitory cytokines IL-10 has also been used to modulate the injury (10, 11). Finally, to achieve liver specificity in gene therapy, liver-specific promoters cannot only achieve prolonged transgene expression but also reduce side effects caused by transgene expression in non–liver cells (12, 13). Various liver-specific promoter elements and chimeric promoters have been evaluated for high liver specificity and activity (14).

Because liver diseases, such as malignancies and hepatitis, correlate with several immune gene disorders, a vector containing combinational gene intervention (e.g., simultaneous overexpression and knockdown of multiple genes) is urgently needed to modulate the inflammatory liver microenvironment during disease (15). Adenovirus-mediated coexpression of TRAIL and a short hairpin RNA (shRNA) containing a sequence to silence COX-2 exhibited great synergistic antitumor activity in experimental hepatocellular carcinoma (16). Thus, development of vectors that can handle multiple genes is practically needed.

In the current study, we found that synergistically interfering CCL5 and FKN or the combined overexpression of human IL-10 (huIL-10) with FKN knockdown could enhance the protective ef-
fect of silencing FKN alone in adenovirus-induced acute liver injury. Next, we constructed a novel multifunction plasmid vector using an efficient hepatocyte-specific promoter, which could simultaneously overexpress and knock down multiple immune genes in a liver-directed manner. Our data show that simultaneous overexpression of huIL-10 and knockdown of endogenous CCL5 and FKN highly attenuated adenovirus-mediated acute liver injury by inhibiting NK cell recruitment and activation and inflammatory cytokines production. This vector could be delivered by viral and nonviral approaches and exert function in vivo for a long time.

Materials and Methods

Mice and i.v. injection

C57BL/6 mice were purchased from the Experimental Animal Center (Chinese Academy of Science, Shanghai, China). All mice were maintained under specific pathogen-free and controlled conditions (23°C, 55% humidity, and 12-h day/night rhythm), and received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. Mice were used between 6 and 8 wk of age. In high-dose adenovirus-induced liver acute injury model, mice were injected with 1 × 10^11 viral particles (VP) Ad5-EGFP or mixed adenoviruses into the tail vein in a 300-μl volume. Plasmid vectors were hydrodynamically injected into the tail vein in a 2-ml volume, as described previously (17). Generally, a total of 50 μg plasmid vector was injected into the tail veins of 6–8-wk-old C57BL/6 mice in a volume of saline equivalent to 10% of the body mass of the mouse (e.g., 2 ml for mouse of 20 g). The total volume was delivered within 5–8 s.

Vector construction and adenoviral vector preparation

pLIVE vector (Mirus Bio, Madison, WI) was modified to delete original restricted enzyme sites Spe I and Xba I and introduce new sites into the intron 2 region of the vector. Template 97mer oligonucleotides containing microRNA (miRNA)-30-based shRNA structure (Supplemental Table I) was ligated into the shuttle vector MSCV-LMP (Open Biosystems, Huntsville, AL) and then constructed into pLIVE vector. More cassettes could be assembled in a multiple-target manner, as Xba I and Spe I were isocaudomers. The IRES element and DsRed2 were amplified from pIRE52-DsRed2 (Clontech, Mountain View, CA), whereas huIL-10 was obtained from pMD18T–huIL-10 (Sino Biological, Beijing, China). All constructs were confirmed by DNA sequencing (Invitrogen, Shanghai, China).

The recombinant replication-deficient E1, E3-deleted type 5 Adeno-X expression system was purchased from Clontech. shNeg-Ad and Multi-Ad were constructed by homologous recombination. Recombinant adenoviruses were packaged and propagated in HKE293 cells and purified by CsCl discontinuous density gradient centrifugation. Typical vector titers ranged between 8–20. Replication-deficient adenoviruses containing the EGFP gene (Ad5-EGFP) were purchased from 5+ MMI (5+MMI, Beijing, China). Recombinant adenoviruses containing the EGFP gene (Ad5-EGFP) were purchased from 5+ MMI (5+MMI, Beijing, China).

Whole-body imaging of adenovirus infection

Mice infected with multifunction adenoviral vector or control vector were placed into an IVIS imaging chamber (Caliper Life Sciences) when fully embedded in Tissue-Tek OCT compound and snap frozen in liquid nitrogen. Cryostat sections (6 mm thick) were prepared using a Leica CM1950 cryostat. EGFP and DsRed2 fluorescence protein expression was analyzed with a fluorescence microscope (Carl Zeiss AXIOSCOP2, Oberkochen, Germany).

Mouse hepatic cell isolation

Liver leukocytes were isolated similar to what has been described previously (18). Livers were passed through a 200-gauge stainless steel mesh. Cells were resuspended in 40% Percoll (Life Technologies BRL) and centrifuged at 1260 × g for 15 min at room temperature. Pelleted leukocytes were washed in PBS and used to evaluate cellularity and for flow cytometry (FCM) staining.

NK cell depletion

Mice were injected i.v. with 50 μg anti-asialo-GM1 Ab (Wako, Tokyo, Japan) or 50 μg rabbit IgG control (Calbiochem, La Jolla, CA) per mouse 24 h before adenovirus infection. The elimination of NK cells was confirmed with FCM.

Flow cytometric analysis

The mAbs and isotypes used in this study included (all from BD Pharmingen): FITC-labeled anti-CD11b, RatIgG2b, anti-CD19, RatIgG2a, anti–TCR-γδ, ArHlgG, anti-NK1.1, MsIgG2a κ, and anti-CD3ε; PE-labeled anti-CD69, ArHlgG λ3, anti-I-66G, and RatIgG2a κ; PerCP-Cy5.5-labeled anti-CD54, MsIgG2a κ, anti-F4/80, RatIgG2a κ, and anti-NK1.1; and APC-labeled anti-CD3, ArHlgG1 κ, anti-CD11c, ArHlgG λ2, anti-Gr-1, and RatIgG2b κ. Cells were stained with the indicated fluorescence-labeled mAbs according to standard protocol and analyzed using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed with FlowJo software version 7.6.

Analysis of liver injury

Liver injury was assessed at the indicated time points after adenovirus infection by measuring serum alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (GGT), creatinine (CRE) and bilirubin (BILI). The normal serum levels of each parameter are as follows: ALT, <40 IU/L; AST, <40 IU/L; ALP, 53–128 IU/L; GGT, 7–32 IU/L; CRE, 53–108 μmol/L; and BILI, 5.1–19 μmol/L. All these liver injury parameters were measured using a test kit (Rong Sheng, Shanghai, China) following the manufacturer’s instructions.

H&E staining

For histologic analysis, liver sections were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were affixed to slides, deparaffinized, stained with H&E, and examined using light microscopy.

ELISA for cytokine detection

Mouse serum was harvested at indicated time points after hydrodynamic injection of plasmids or adenovirus infection, huIL-10 was analyzed using an huIL-10 sandwich ELISA kit (CUSABIO, Wuhan, China). Mouse IFN-γ and TNF-α were analyzed using mouse IFN-γ and TNF-α sandwich ELISA kits (Dawei, Shenzhen, China).

RNA preparation and real-time quantitative PCR

Total RNA was isolated from Hepa1–6 cells or mouse hepatocytes using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. The same quantity of total RNA was reverse-transcribed to cDNA using an M-MMLV transcriptase (Invitrogen). Quantitative PCR was performed using SYBR Green I (Takara) for 45 cycles at 95°C for 15 s and 60°C for 60 s with ABI-Prism 7000 (Applied Biosystems) according to the manufacturer’s instructions. Primer sequences were as follows: β-actin: sense, 5'-CACGCTTCTTTGACAGCTCTTT-3'; antisense, 5'-ATGCCGGAGCCTGTGTC-3'; FKN: sense, 5'-ACACCCCAACTCCAGTAAGC-3'; antisense, 5'-CGGAAGACCTCAGGAACACAC-3'; CCL5: sense, 5'-ATAGGCCTCGACCACACTC-3'; antisense, 5'-GATGCCCATTTCCTCCAGGAC-3'. Results were analyzed using the ΔΔCt method as described previously (19). Values were expressed as fold change compared with control.

Statistical analysis

Results were analyzed by Student t test when appropriate. All data are expressed as mean ± SEM. A p value <0.05 was considered statistically significant.
Results

Adenovirus induces acute liver injury by recruiting NK cells

Systemic delivery of high-dose, replication-defective recombinant adenovirus causes acute liver injury, as demonstrated in mice and in clinical trials (20, 21). Toxicity associated with adenovirus infection has been linked to the activation of both innate and adaptive immune responses (22–24). In the present study a robust, high level of ALT was detected after systemic infection with high-dose adenovirus, which was accompanied by an increase in liver leukocytes and liver NK cell proportion (Fig. 1A–C). Depletion of NK cells before adenovirus administration could significantly prevent acute liver injury, with a much lower serum ALT level and fewer liver leukocytes (Fig. 1B, 1C), and produce less lymphocyte infiltration and tissue necrosis (Fig 1D). NK depletion also led to

FIGURE 1. Acute liver injury induced by systemic infection of high dose adenovirus is mainly mediated by NK cells. (A) C57BL/6 mice were injected i.v. with saline or a high dose of replication-deficient adenovirus (Ad5-EGFP, $1 \times 10^{11}$ VP/mouse). Serum ALT levels were measured at indicated time points. Values are expressed as the mean $\pm$ SEM ($n = 3$). (B) C57BL/6 mice were administered 50 μg anti-asialo-GM1 or Rabbit IgG control i.v. 1 d before adenovirus infection. Mice were subsequently infected with $1 \times 10^{11}$ VP Ad5-EGFP i.v. Serum ALT levels were measured 24 h later. Absolute leukocyte (CD45.2+) numbers per liver were counted (B) and liver NK cell (NK1.1+CD3$^+$) proportion in total mononuclear cells (MNCs) was analyzed with FCM (C). Values are expressed as the mean $\pm$ SEM ($n = 4$). *$p < 0.05$, **$p < 0.001$ versus mice treated with rabbit IgG control. (D) Liver paraffin sections were prepared and stained with H&E to detect lymphocyte infiltration and hepatocyte necrosis (original magnification $\times 200$; scale bar, 50 μm). (E) Mice were sacrificed, and frozen liver sections were made 1 d after infection. EGFP expression was examined by fluorescence microscopy (original magnification $\times 100$; scale bar, 100 μm). A representative experiment is shown. (F) Total genomic DNA was isolated from the liver and analyzed for adenovirus DNA copies by quantitative real-time PCR. Values are expressed as the mean $\pm$ SEM ($n = 4$). *$p < 0.05$ versus mice treated with rabbit IgG control.
higher levels of EGFP expression and larger amounts of adenoviral DNA in the liver (Fig. 1E, 1F), which indicated that NK cells might have a critical role in innate immune elimination of adenoviral vectors and acute hepatitis.

**Simultaneously silencing CCL5 and FKN enhances the protective effect of silencing FKN alone in adenovirus-induced acute liver injury**

Chemokines are important for NK cell recruitment into the liver; indeed, we previously found that knocking-down FKN prevented NK cell-mediated acute liver injury from adenovirus infection in mice. Expression of multiple chemokines such as FKN and CCL5 was enhanced after adenovirus infection (9, 24) both in vitro and in vivo, which was also demonstrated in this study (Supplemental Fig. 1A, 1D). Therefore, we speculated whether the protective effect would be more significant when more chemokines were silenced. Using a hepatocyte-specific promoter and miRNA-based shRNA structure, we constructed RNA interference (RNAi) vectors targeting CCL5 or FKN (each containing three different RNAi sequences to enhance the interference efficiency) and test their silence effects in vitro (Supplemental Fig. 1A). Consistent with liver injury, a robust, high level of ALT was detected upon high-dose adenovirus infection, and hydrodynamic injection of FKN silence vector pLIVE-(shFKN*3)mir decreased ALT level and absolute number of liver leukocytes. Synergistically, silence CCL5 and FKN exerted a stronger protective effect, with lower ALT level, liver leukocytes numbers, and NK cell proportion (Fig. 2A–C). High-dose adenovirus infection also caused severe pathologic damage, whereas simultaneous knockdown of CCL5 and FKN exhibited the least lymphocyte infiltration and tissue necrosis (Fig. 2D). Mice treated with both CCL5 and FKN RNAi vectors had a lower level of serum IFN-γ than did mice with only FKN knockdown, although the reduction of serum TNF-α was not significant (Fig. 2E).

**FIGURE 2.** CCL5 silence synergistically enhances the attenuating effect of silencing FKN in adenovirus-induced acute liver injury. Total vectors (50 μg; 25 μg per vector when two vectors were mixed) was delivered to the liver of C57BL/6 mice by hydrodynamic tail-vein injection 3 d before adenovirus infection. Mice were injected i.v. with a high dose of replication-deficient adenovirus (Ad5-EGFP, 1 × 10^{11} VP/mouse). Liver injury was analyzed 1 d after infection. (A) Serum ALT levels of C57BL/6 mice, treated with the indicated vectors and infected by adenovirus for 24 h, were measured. Values are expressed as the mean ± SEM (n = 5). ***p < 0.001 versus mice treated with pLIVE vector and adenovirus, *p < 0.05 versus mice treated with pLIVE-(shFKN*3)mir, pLIVE, and adenovirus. (B) Absolute leukocyte numbers per liver were counted (**p < 0.01 versus mice treated with pLIVE vector and adenovirus), and liver NK cell proportion in total mononuclear cells (MNCs) was analyzed by FCM (C). *p < 0.05 versus mice treated with pLIVE vector and adenovirus. (D) Liver paraffin sections were prepared and stained with H&E to detect lymphocytes infiltration and hepatocytes necrosis. Arrows indicate areas of hepatocyte necrosis (original magnification ×200; scale bar, 100 μm). (E) Serum IFN-γ and TNF-α were measured with ELISA. Values are expressed as the mean ± SEM (n = 5). **p < 0.01 versus mice treated with pLIVE vector and adenovirus.
Simultaneous huIL-10 overexpression and FKN knockdown enhance the attenuating effect of knocking down FKN alone in adenovirus-induced acute liver injury

IL-10 functions as a critical regulator of NK cell–related inflammation (11, 25, 26). We tried to determine whether overexpression of huIL-10 would further enhance the attenuating effect of silencing FKN in adenovirus-induced acute liver injury. The combination of huIL-10 overexpression with FKN knockdown showed a stronger protective effect than FKN knockdown alone, as evidenced by a much lower ALT level and significantly reduced liver leukocytes and NK proportion (Fig. 3A–C). Lymphocyte infiltration and tissue necrosis were consistent with the serum ALT level (Fig. 3D). huIL-10 was detected only in mice treated with pLIVE–huIL-10, which also had the lowest serum IFN-γ and TNF-α in these mice (Fig. 3E, 3F).

Simultaneous manipulation of FKN, CCL5, and huIL-10 using a hepatocyte-directed vector highly efficiently prevents against adenovirus-induced acute hepatitis via downregulating NK cells

As expected, when the three vectors silencing CCL5 or FKN or over-expressing huIL-10 were mixed together and used for treating acute liver injury induced by high-dose adenovirus, the liver inflammation was greatly inhibited with the lowest ALT level (Fig. 4A). As a result, we constructed a liver-directed multifunction vector, named Multi-Vector, by simultaneously overexpressing huIL-10 and knocking down CCL5 and FKN (Supplemental Fig. 1B). To test the ability of the Multi-Vector to control gene expression under acute hepatic injury conditions in vivo, it was hydrodynamically injected into C57BL/6 mice 3 d before adenovirus infection. The function of Multi-Vector was verified 1 d later; it could overexpress both huIL-10 and DsRed2 (as a report gene) while simultaneously silencing CCL5 and FKN expression in vivo (Supplemental Fig. 1C–E). Consistent with liver injury, hydrodynamic injection of control shNeg-Vector resulted in a robust high level of ALT, AST, GGT, and ALP upon high-dose adenovirus infection (Fig. 4B). Using the therapeutic vectors, huIL-10 overexpression, CCL5 knockdown, or FKN knockdown alone decreased the liver injury level to some extent, but the Multi-Vector treatment exhibited the most significant protective effect (Fig. 4B). Although adenovirus infection markedly increased the absolute leukocyte number in control mice livers, the number only mildly increased in the therapeutic Multi-Vector–treated mice. For NK cells in particular, Multi-Vector

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treatment exhibited the strongest inhibition of NK recruitment (Fig. 4C); no significant differences in recruitment were observed among other lymphocytes, including CD3+ T cells, NKT cells, γδ T cells, neutrophils, and Kupffer cells (Supplemental Fig. 2). Interestingly, mice treated with shNeg-Vector had the lowest dendritic cell (DC) populations, whereas mice treated with huIL-10–Vector and adenovirus, Multi-Vector could inhibit NK cell recruitment to the liver, and the inhibition effect lasted for at least 1 wk. Liver functionality was assessed at longer time points following multifunction vector administration and adenovirus infection. Different liver injury parameters (e.g., serum ALT, AST, ALP, GGT, CRE, BILI) were measured at weekly intervals. Multi-Vector was able to suppress the adverse consequences measured at 1–35 d after infection (Fig. 6A, Supplemental Fig. 3A). There were no significant adverse effects.

Multifunction vector has a long-lasting effect on NK cell recruitment and protects against liver injury induced by high-dose adenovirus infection

We tested whether the multifunction vector had a long-lasting effect on NK cell recruitment after adenovirus infection. As shown in Fig. 5, NK cells were recruited to the liver shortly after adenovirus infection. Multi-Vector could inhibit NK cell recruitment to the liver, and the inhibition effect lasted for at least 1 wk. Liver functionality was assessed at longer time points following multifunction vector administration and adenovirus infection. Different liver injury parameters (e.g., serum ALT, AST, ALP, GGT, CRE, BILI) were measured at weekly intervals. Multi-Vector was able to suppress the adverse consequences measured at 1–35 d after infection (Fig. 6A, Supplemental Fig. 3A). There were no significant adverse effects.
Mice treated with shNeg-Vector and adenovirus. Values are expressed as mean (MNCs) was analyzed by FCM (A). Absolute leukocyte numbers per liver were counted (A), and liver NK cell proportion in total mononuclear cells (MNCs) was analyzed by FCM (B) at the indicated time after adenovirus infection. Values are expressed as mean ± SEM (n = 3). *p < 0.05 versus mice treated with shNeg-Vector and adenovirus.

Moreover, in mice pretreated with shNeg-Vector or Multi-Vector for 30 d and then infected with high-dose adenovirus, Multi-Vector still protected against high-dose adenovirus-induced acute hepatitis with a lower ALT levels and NK cell proportion in the liver (Fig. 6B, 6C), indicating that the protective effects mediated by the Multi-Vector could last for at least 1 mo.

To explore whether the use of our multifunction vector can be delivered by virus but not only by hydrodynamic injection, we packaged the vector into an adenoviral vector by homologous recombination (named shNeg-Ad or Multi-Ad) and infected C57BL/6 mice with a mixture of vector-free adenovirus (Ad5-EGFP) and a high dose of $1 \times 10^{11}$ VP/mouse of adenovirus at different ratios. Adenovirus infection and function was monitored in real time with the DsRed2 expression by whole body imaging (Fig. 7A). There is no significant difference of adenoviral burden between different groups (Fig. 7B). Liver inflammation was examined 24 h later. As shown in Fig. 7C–E, Multi-Ad significantly attenuated adenovirus-induced liver injury, even at the low dose of vector-containing adenovirus, by decreasing leukocyte and NK cell recruitment into the liver as well as serum IFN-γ and TNF-α (Fig. 7D, 7E). Similarly, Multi-Ad was able to suppress the adverse consequences measured at 1–28 d after infection (Supplemental Fig. 3B). These data suggested that a multifunction vector can be delivered by viral vectors and that this multifunction vector can control inflammation that occurs upon adenovirus administration when given at the same time.

Discussion

Normally, the administration of replication-deficient recombinant adenovirus elicits NK cell-mediated innate immunity and causes acute inflammation, which is a major drawback when used for gene therapy. In this study, we successfully prevented and treated adenovirus-induced acute hepatitis using a liver-directed multifunction vector that simultaneously knocked down and overexpressed multiple immune genes. By using this multifunctional vector, recruitment of NK cells to the liver was successfully inhibited by synergistically silencing CCL5 and FKN while NK cytotoxicity was elaborately ameliorated by overexpressing inhibitory cytokine IL-10.

Adenoviruses are among the most commonly used vectors for liver gene therapy because of their liver tropism, high capacity for therapeutic genes, high operability, and productivity; however, adenovirus activate host innate immunity and result in acute lethal inflammation (4). Strategies to eliminate the side effect are therefore important. Besides vector modification, such as deletion of viral coding sequences and PEGylation (covalent attaching polyethylene glycol polymers to adenoviral vector), some strategies have been used to modify the local immune microenvironment. Restricting transgene expression to hepatocytes decreases the risk of Ab formation against transgene products because of the immunotolerant environment of the liver (12). Although hepatotropic adenovirus preferentially infects hepatocytes, it also might infect other tissues like lung and lymph nodes. Therefore, hepatocyte-specific promoters for cell-specific expression might overcome these obstacles.

In our previous work, FKN knockdown prevented mice from acute liver injury induced by hydrodynamic adenovirus vector injection (9). However, the protection mediated by FKN knockdown was still not strong enough, likely because of silencing only a single gene and using a non-hepatocyte-specific RNA polymerase III promoter. Indeed, multiple chemokines play critical roles in adenovirus-induced liver injury, including MCP-1, CCL5, and FKN (24). Compared with traditional approaches generating shRNA, miRNA-based shRNA expression from a Pol II promoter was more safe and effective (27, 28). This miRNA-based shRNA expressed from a Pol II promoter-AFP enhancer/albumin promoter can silence genes in a hepatocyte-specific manner, helping to diminish the off-target and other side effects of RNAi. In addition, many more miRNA-based shRNA cassettes can be incorporated into this vector before reaching its capacity. A single transcript containing as many as six miRNA-based sRNAs was tested in our work (Supplemental Fig. 1B).

Inflammation caused by systemic infection of high-dose adenovirus comes from the complex immunopathologic factors, such as the enhanced expression of multiple chemokines, recruitment of innate immune cells, and secretion of several inflammatory cytokines. Therefore, we can target multiple function genes to modulate the liver inflammation. In our study, hepatic CCL5 and FKN expression greatly increased 24 h after adenovirus infection (Supplemental Fig. 1D). This could explain why simultaneous CCL5 and FKN inhibition more significantly protected against liver injury than silencing either single gene alone (Figs. 2 and 4). In addition, because IL-10 exerts strong inhibitory effects on NK cells, the huIL-10 overexpression vector more strongly attenuated adenovirus-induced liver injury and synergized with chemokine inhibition (Figs. 3 and 4). Moreover, our liver-directed multifunction vector can be packaged into adenovirus and other viral vector systems, and the DsRed2 reporter gene can help us to monitor the infection.
distribution, and function of the vectors in a real-time manner. To our knowledge, this report is the first description of a hepatocyte-specific multifunction vector for simultaneously manipulating multiple immune genes, which could modulate the liver immune microenvironment and inhibit acute liver injury induced by high-dose adenovirus.

FIGURE 6. The protective effect of multifunction vector against adenovirus-induced acute liver injury lasts for at least 1 mo. (A) A different vector (50 μg) was delivered to the liver of C57BL/6 mice by hydrodynamic tail-vein injection 3 d before adenovirus infection. Mice were injected i.v. with a high dose of replication-deficient adenovirus (Ad5-EGFP, 1 × 10^{11} VP/mouse). Serum ALT levels were measured at weekly intervals 1–35 d after infection. Values are expressed as the mean ± SEM (n = 4). Mice were pretreated with 50 μg shNeg-Vector or Multi-Vector by hydrodynamic injection. Thirty days later, a dose of 1 × 10^{11} VP/mouse adenovirus was used to infect mice by tail vein injection. (B) Serum ALT level was examined 24 h after infection. Values are expressed as the mean ± SEM (n = 3). **p < 0.01 versus mice treated with shNeg-Vector and adenovirus. (C) The absolute number of liver mononuclear cells (MNCs) was counted, and the liver NK cell proportion was determined by FCM. *p < 0.05 versus mice treated with shNeg-Vector and adenovirus.

FIGURE 7. Multifunction vector delivered by adenoviral vector protects against adenovirus-induced acute liver injury. The adenoviral vector was mixed with vector-free adenovirus in different proportions, and mice were infected by tail vein injection at a dose of 1 × 10^{11} VP/mouse. (A) DsRed2 expression was monitored in real time, and fluorescence signal emission from mice was quantified and analyzed using the IVIS imaging system 24 h after infection. (B) Liver samples were harvested 24 h after infection, and total genomic DNA was isolated from the liver. Adenovirus DNA copies were analyzed by quantitative real-time PCR. Values are expressed as the mean ± SEM (n = 3). (C) Serum ALT levels from C57BL/6 mice treated for 24 h with the adenoviral vector and vector-free adenovirus mixture were measured. Values are expressed as the mean ± SEM (n = 3). ***p < 0.001 versus mice treated with the shNeg-Ad and vector-free adenovirus mixture. (D) Absolute leukocyte numbers per liver were counted, and the proportion of liver NK cells was analyzed with FCM. **p < 0.01, *p < 0.05 versus mice treated with the shNeg-Ad and vector-free adenovirus mixture. (E) Serum levels of IFN-γ and TNF-α were measured by ELISA. Values are expressed as mean ± SEM (n = 3). ***p < 0.001, **p < 0.01, *p < 0.05 versus mice treated with the shNeg-Ad and vector-free adenovirus mixture.
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Disclosures
The authors have no financial conflicts of interest.

References
Figure S1. Evaluation of different vector’s function in vitro and in vivo.

(A) Hepatoma cell Hepa 1-6 was transfected with different RNAi vector using lipofectamine 2000, and cells were infected by Ad5-EGFP at a moiety of infection of 20 infectious unit/cell at the same time. Relative CCL5 and FKN mRNA level were measured by real-time quantitative PCR 48 h later. Data are expressed as relative to β-actin expression. Mean values of triplicate samples from 2 independent experiments are shown (± SEM). **P<0.01, ***P<0.001 versus Hepa 1-6 treated with...
pLIVE-(shNeg*3)mir and Ad5-EGFP. (B) The procedures for plasmid hydrodynamic injection and adenovirus infection are depicted. Five plasmid vectors containing different genes and shRNAmir sequences were constructed. 50 μg of each different vector was delivered to the liver of C57BL/6 mice by hydrodynamic tail-vein injection 3 days before adenovirus infection. Mice were injected intravenously (i.v.) with a high dose of replication-deficient adenovirus (Ad5-EGFP, 1× 10^{11} VP/mouse). Vector function and liver injury were analyzed 1 day after infection. (C) Serum huIL-10 levels were measured 1 day after adenovirus infection. Data are shown as mean ± SEM from 3 mice in each group. (D) Relative CCL5 or FKN mRNA levels in hepatocytes were analyzed by real-time quantitative PCR. Data are expressed as relative to β-actin expression (mean ± SEM, n=3). (E) Mice were sacrificed and frozen liver sections were made 1 day after infection. DsRed2 expression was examined by fluorescence microscopy (original magnification ×200, scale bar: 100 μm). A representative experiment is shown.
Figure S2. Distribution of other cell populations in mice treated with multifunction vector and adenovirus.

50 μg of different vectors was delivered to the liver of C57BL/6 mice by hydrodynamic tail-vein injection 3 days before adenovirus infection. Mice were injected intravenously (i.v.) with a high dose of replication-deficient adenovirus (Ad5-EGFP, 1× 10^{11} VP/mouse). Liver CD3+ T cell (NK1.1’CD3’), NKT cell (NK1.1’CD3’), γδ T cell (γδ TCR’CD3’), neutrophil (CD45.2’CD11b’Ly6G’), kupffer cell (F4/80’) and DC cell (CD3’NK1.1’CD19’CD11c’) proportion was analyzed by FCM 24 h after adenovirus infection. Values are expressed as the mean ± SEM (n=4). **P<0.01, *P<0.05 versus mice treated with shNeg-Vector and adenovirus.
Figure S3. Long-term evaluation the protective effect of Multi-Vector or Multi-Ad against adenovirus-induced acute liver injury.

(A) 50 µg of different vector was delivered to the liver of C57BL/6 mice by hydrodynamic tail-vein injection 3 days before adenovirus infection. Mice were injected intravenously (i.v.) with a high dose of replication-deficient adenovirus (Ad5-EGFP, 1× 10^{11} VP/mouse). Serum AST, ALP, GGT, CRE and BILI levels were measured at 1-35 days post-infection. Values are expressed as the mean± SEM (n=4).

(B) The adenoviral vector shNeg-Ad or Multi-Ad was mixed with vector-free
adenovirus in different proportions, and then mice were infected by tail vein injection at a dose of $1 \times 10^{11}$ VP/mouse. Serum ALT, AST, ALP, GGT, CRE and BILI levels were measured at 1-28 days post-infection. Values are expressed as the mean± SEM (n=4).
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*Underlined letters indicate the flanking miR30 sequences and low case letters represents the miR30 loop structure*