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Vaccination with Platelet-Derived Growth Factor B Kinoids Inhibits CCl_4-Induced Hepatic Fibrosis in Mice

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Running title page

a) PDGF-B vaccines suppress hepatic fibrosis in mice

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ABSTRACT

Platelet-derived growth factor B (PDGF-B) plays an essential role in hepatic fibrosis. Inhibition of the PDGF-B signaling in the chronically injured livers might represent a potential therapeutic measure for hepatic fibrosis. In this study, we assessed the effects of vaccination against PDGF-B on CCl_4-induced liver fibrosis in BALB/c mice. The PDGF-B kinoid immunogens were prepared by cross-linking two PDGF-B–derived B cell epitope peptides (PDGF-B^{16}-[23-38] and PDGF-B^{16}-[72-83]) to ovalbumin and keyhole limpet hemocyanin, respectively. ELISA, Western blotting and NIH3T3 cell proliferation assay verified that immunization with the PDGF-B kinoids elicited the production of high levels of neutralizing anti-PDGF-B auto-antibodies. The vaccination markedly alleviated CCl_4-induced hepatic fibrosis as indicated by the lessened morphological alternations and reduced hydroxyproline contents in the mouse livers. Moreover, immunohistochemical staining for proliferating cell nuclear antigen, α-smooth muscle actin and desmin demonstrated that neutralization of PDGF-B inhibited both the proliferation and activation of hepatic stellate cells in the fibrotic mouse livers. Collectively, this study demonstrated that vaccination with PDGF-B kinoids significantly suppressed CCl_4-induced hepatic fibrosis in mice. Our results suggest that vaccination against PDGF-B might be developed into an effective, convenient and safe therapeutic measure for the treatment of hepatic fibrosis.

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INTRODUCTION

Hepatic fibrosis, independent of the etiology, results mainly from the activation of hepatic stellate cells (HSCs). During the activation, HSCs undergo increased proliferation, altered cellular morphology to a more myofibroblast (MFB) – like cell type and upregulated extracellular matrix (ECM) expression. The activation of HSCs is induced by multiple profibrogenic factors in which transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) are the key stimuli for HSC activation (Friedman, 2008; Pinzani, 2002).

PDGF is a family of pleiotropic cytokines consisting of four polypeptide chains encoded by distinct genes. The PDGF isoforms are expressed as homo- and hetero-dimeric proteins, including PDGF -AA, -AB, -BB, -CC and -DD. The effects of the distinct cytokine dimers are triggered by binding to and dimerizing the cell membrane PDGF receptors alpha (PDGFRα) and beta (PDGFRβ), which have distinct expression patterns in tissues and various cell types. PDGF-BB triggers signaling by both PDGFRα and PDGFRβ, while PDGF-AA binds and dimerizes only PDGFRα. Activation of PDGFR results in phosphorylation of several tyrosine residues in the cytoplasmic domains of the receptors, which allows recruitment of Ras-extracellular-receptor kinase (ERK) and phosphoinositol 3-kinase (PI3 kinase) pathway signaling molecules to modulate the mitogenesis and chemotaxis (Pinzani, 2002; Bonner, 2004).

The relationship between PDGF signaling and hepatic fibrosis has been evidenced by a number of studies. Although all the four PDGF isoforms might be involved in hepatic fibrosis (Campbell, et al., 2005; Czochra, et al., 2006; Borkham-Kamphorst, et al., 2007; Thieringer, et al., 2008). PDGF-B signaling through PDGFRβ is considered to be most closely related to hepatic fibrosis. The expressions of PDGF-B and PDGFRβ are rapidly increased in both the experimental hepatic fibrosis in rats and

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human fibrotic liver (Pinzani, et al., 1994; Wong, et al., 1994; Pinzani, et al., 1996; Borkham-Kamphorst, et al., 2008) as well as in in vitro cultured HSCs (Pinzani, et al., 1994; Pinzani, et al., 1996; Borkham-Kamphorst, et al., 2008). In vitro studies have demonstrated that PDGF-B is the most potent mitogenic factor for HSCs (Pinzani, 2002). Consequently, blockage of PDGF-B signaling inhibits experimental hepatic fibrosis (Borkham-Kamphorst, et al., 2004a; Borkham-Kamphorst, et al., 2004b; Gonzalo, et al., 2007; Chen, et al., 2008).

Because of its pivotal roles in hepatic fibrosis, antagonizing the PDGF-B signaling in HSCs would offer an attractive strategy for the treatment of fibrotic liver diseases. Till now, several approaches have been reported to block the PDGF-B signaling including reducing the synthesis of active PDGF-B (Borkham-Kamphorst, et al., 2004b) or PDGFRβ (Chen, et al., 2008) by gene silencing, neutralizing PDGF-B with specific antibodies (Ab) (Ogawa, et al., 2010), decoying PDGF-B with soluble PDGF-B receptors (Borkham-Kamphorst, et al., 2004a), and suppressing the post-receptor signal transduction pathways (Gonzalo, et al., 2007). Although the efficacies of these measures have been validated in experimental hepatic fibrosis or in cultured HSCs, they are not seemingly possible to be employed in the clinical practice. Some of the agents mentioned above have short half-lives that require repeated administration over a long time period to achieve therapeutic benefits. Measures involving genetic modification are associated with safety concerns. Furthermore, preparing these potential therapeutic agents is costly and arduous. Considering that clinical hepatic fibrosis is a persistent, chronic process, only a safe, effective and convenient measure for the continuous elimination of PDGF-B is feasible for treating hepatic fibrosis. Here we propose that vaccination against PDGF-B might provide a potentially feasible and effective measure for the prevention and retardation of hepatic fibrosis.

One of the recent advances in the therapeutic research field is the “anti-cytokine vaccines” (Zagury, et

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al., 2001; Zagury, et al., 2003). By cross-linking or generating fusion proteins with carrier proteins, the normally non-immunogenic cytokines or growth factors can be converted into immunogens to elicit the production of the specific auto-antibodies (Dalum, et al., 1999) which can further neutralize the abnormally over-produced cytokines or growth factors and thereby ablating their pathological effects. This notion has been validated in a number of disease models and clinical trials for some cytokine- or growth factor-related disorders (Dalum, et al., 1999; Zagury, et al., 1999; Holmgren, et al., 2006; Le Buanec, et al., 2006; González, et al., 2007; Rad, et al., 2007; Spohn, et al., 2007; Vinageras, et al., 2008; Tissot, et al., 2008; Delavallée, et al., 2008; Spohn, et al., 2008; Tohyama, et al., 2008). In the present study, we prepared two PDGF-B–derived peptide-carrier protein heterocomplexes (kinoids) as PDGF-B vaccines, verified their antigenicity, and tested the suppressive effect of immunization with these two kinoids on CCl_4-induced hepatic fibrosis in mice.

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MATERIALS AND METHODS

Preparation of PDGF-B kinoids. The two antigenic peptides (N-^{23}VFEISRRLIDRTNANF^{38}-C, PDGF-B–VF16; and N-^{72}QVRKIEIVRKKPIFKK^{87}-C, PDGF-B–QK16) were chosen from the human PDGF-B (GenBank: CAA45383.1) according to the literatures in which these fragments were identified to be vital for PDGF-B binding to PDGFRβ (Larochelle, et al., 1989; Engstrom, et al., 1992; LaRochelle, et al., 1992; Brennand, et al., 1997; Patel, et al., 1999). These two fragments are homologous to mouse PDGF-B and have no homology to other known PDGF isoforms. These polypeptides were synthesized on an automated peptide synthesizer (ABI433A; Applied Biosystems, Carlsbad, CA) and subsequently purified by HPLC to reach a purity of 99%. The polypeptide-carrier heterocomplexes, QK16-keyhole limpet hemocyanin (KLH) and VF16-ovalbumin (OVA) were prepared with an EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) cross-linking kit (Imject® Immunogen EDC Kit with KLH and OVA, Pierce, Rockford, IL) according to the manufacturer's instructions. The conjugates were finally desalted by D-Salt™ dextran desalting columns and stored at –20 °C until use.

Animals and experimental protocol. Specific pathogen-free, 6-week-old male BALB/c mice were provided by the Experimental Animal Center, School of Medicine, Xi'an Jiaotong University. All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animal Research according to the institutional guidelines.

For evaluating the antigenicity of the kinoids, 24 mice were assigned to 4 equal groups: KLH, VF16-OVA, QK16-KLH and phosphate-buffered saline (PBS). The mice in the two kinoid groups were given 4 intraperitoneally (i.p.) injections, two weeks apart, of the kinoids (50 μg in 0.2ml). Complete Freund Adjuvant (Sigma, St. Louis, MO) was used for the first immunization, and Incomplete Freund

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Adjuvant (Sigma) was used for all the subsequent immunizations. The mice in the control groups were treated similarly, except that kinoid was replaced by KLH (50 μg in 0.2ml) or PBS. Blood samples were collected from the tail vein for ELISA immediately preceding each immunization. Two weeks after the fourth injection, three mice in each group were euthanized, and blood was collected to isolate the serum for Western blot and the neutralization assay. The remaining mice were maintained, and blood samples were taken every two weeks for ELISA detection of the antibody (Ab) titer. Six months after the first immunization, the mice were euthanized. The liver, lungs, heart and kidneys were harvested, fixed with 10% formalin, sectioned and stained with hematoxylin and eosin (H&E) to evaluate any adverse effects.

Forty BALB/c mice were subjected to the hepatic fibrosis experiment. The mice were assigned to 5 groups: VF16-OVA/CCl_4 (n = 9), QK16-KLH/CCl_4 (n = 9), KLH/CCl_4 group (n=8), CCl_4 (n = 8) and normal control (NC) (n = 6). The mice were maintained and immunized as described in the above experiment except for that the latter two groups were given an equal volume of PBS instead of immunogens. One week after the third immunization, the mice in the former four groups received i.p. injections of CCl_4 (1 ml/kg, dissolved in olive oil to reach a final concentration of 20%) twice a week for six weeks. The mice in the NC group were dosed with an equal volume of olive oil. After 6 weeks of CCl_4 injection, the mice were euthanized (the protocol is illustrated in Figure 2). Blood samples were collected for ELISA detection of anti–PDGF-B Abs. The left lobe of the liver was fixed in 10% formalin for histological examination. Other liver tissue was snap-frozen in liquid nitrogen and stored at –70 °C for hydroxyproline content determination.

ELISA determination of serum anti-PDGF-B Abs. Polystyrene microplates were coated with recombinant human PDGF-BB (R&D Systems, Minneapolis, MN, USA, 20 ng/well). Upon detection,

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the mouse serum was serially diluted (1:2, initiated at 1:200) with 1% BSA/PBS-T (0.02% Tween-20). Goat-anti-mouse IgG (Sigma, 1:5000 diluted with PBS-T) was used as the secondary Ab. A reaction was considered positive if OD $\geq$ 2.1 times of the negative control.

Western blotting. Five micrograms of prokaryotically expressed and purified recombinant human PDGF-B (expressed in *E. coli* with pET-28 a(+) as the expression plasmid, generated by our group) was submitted to a 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with the mouse serum that was diluted at 1:1000 at 4 ℃ overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG for an hour. The bands were developed using an enhanced chemiluminescence (ECL) reagent for 5 minutes.

For detection of the α-SMA expression in the liver tissues by Western blotting, the liver tissues were homogenized in Radio-Immunoprecipitation Assay (RIPA) lysis buffer and 100 μg of the total protein was applied to 12% SDS-PAGE. A mouse anti–α-smooth muscle actin (α-SMA) monoclonal antibody (Labvision, MS-113-P0) was used as the primary antibody. The expression of β-actin was used as the internal control.

Neutralization test. Growth assays with NIH3T3 cells were performed by measuring bromodeoxyuridine (BrdU) incorporation (BrdU Cell Proliferation Assay kit; Calbiochem, San Diego, CA). NIH3T3 cells were plated on 96-well plates (3×10^3/well). The cells were starved in serum-free Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Carlsbad, CA, USA) for 48 hours. Then the starvation medium was removed and replaced with fresh medium containing various dilutions of the antiserum. After a thirty-minute incubation period, recombinant human PDGF-BB (3 ng/ml) and BrdU

were added to the medium. Forty-eight hours post-treatment, the cells were washed with PBS, and the genomic DNA was fixed and denatured with the Fixative/Denaturing solution. BrdU incorporation in the cells was detected by an anti-BrdU Ab and quantified by ELISA according to the manufacturer's instructions.

Histology and immunohistochemistry. Five-micron-thick liver sections were processed by both H&E staining and Masson's trichrome staining to assess the architectural alternations and hepatic collagen deposition (fibrosis). The degree of fibrosis was evaluated semi-quantitatively by the Ishak system (Ishak, et al., 1995).

Immunohistochemistry was performed using the Histostain^{TM}-Plus SP kit. After deparaffinization, rehydration, quenching endogenous peroxidase activity and subsequent blockage with 10% (vol/vol) normal goat serum, the sections were processed by sequential reactions with the primary antibodies (mouse anti–α-SMA monoclonal antibody, 1:800 dilution; rabbit anti-Demin polyclonal antibody, 1:400 dilution; mouse anti-proliferating cell nuclear antigen [PCNA] monoclonal antibody, 1:400 dilution. All from LabVision, Fremont, CA), biotinized secondary antibodies and S-A/HRP. The negative controls were performed by replacing the primary antibodies with pre-immune mouse or rabbit serum.

Computer-assisted semiquantitative analysis was used to evaluate the areas of positive α-SMA and Desmin staining. All images were quantified using Image-ProPlus version 4.5, a commercially available software package from Media Cybernetics (Silver Spring, MD). The imaging of the tissue sections was performed using an automated Image-Pro Plus macro that was calibrated for each microscope objective. The data for both α-SMA and Desmin staining were expressed as the mean percentages of positively stained area over the total tissue section area.

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The parenchymal and mesenchymal cells were blindly counted (1,000 cells analyzed in 10 randomly chosen fields centered on a centrilobular vein at 400× enlargement) for PCNA expression, which was represented as the PCNA labeling index (PCNA LI).

Hepatic hydroxyproline content. The total hydroxyproline content in the liver was determined as described previously (Reddy and Enwemeka, 1996) and expressed as μg/mg wet liver weight.

Statistical analysis. The quantitative data are expressed as the mean ± standard error of the mean (SEM). To assess the statistical significance of inter-group differences in the quantitative data, Bonferroni's multiple comparison tests were performed after One-way analysis of variance (ANOVA), followed by Bartlett's test to determine the homology of variance. Nonparametric data were analyzed by the Mann-Whitney *U*-test. $P < 0.05$ is considered to be statistically significant.

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RESULTS

Immunization with the PDGF-B kinoids efficiently elicits specific anti–PDGF-B neutralizing antibodies in mice. The ELISA with recombinant human PDGF-BB–coated plates showed biweekly immunization with the PDGF-B kinoids resulted in the production of anti–PDGF-B antibodies (Figure 1A). The titers of anti–PDGF-B Abs reached 1: 800 – 1600 after three immunizations and 1 : 6400 – 12800 two weeks after the fourth boosting, respectively, in both QK16-KLH– and VF16-OVA–immunized mice.

To further confirm the specificity of the polyclonal Abs produced by immunization with PDGF-B kinoids, we performed Western blotting to test the reactivity of the antisera with the recombinant human PDGF-B. The result revealed that the antiserum from the mice immunized with both of the two PDGF-B kinoids reacted with membrane-bound PDGF-B (Figure 1B). These results clearly indicated that the two PDGF-B kinoids efficiently and similarly elicited the production of anti–PDGF-B antibodies.

NIH3T3 growth assay was performed to validate whether the Abs elicited by immunization with the PDGF-B kinoids could neutralize the bioactivity of mouse PDGF-BB. As shown in Figure 1C, the antisera displayed dose-dependent inhibitory effects on the proliferation of NIH3T3 cells induced by 3 ng/ml of PDGF-BB, indicating that immunization with both QK16-KLH and VF16-OVA could produce neutralizing anti–PDGF-BB Abs in mice.

Additionally, after immunization, the mice showed no behavioral abnormalities. When the immunized mice were euthanized 6 months post-immunization, examination of the vital organs did not reveal any obvious adverse effects (data not shown).

Vaccination against PDGF-B protects mice from CCl_4-induced hepatic fibrosis. Next, we examined

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the protective effect of immunization with the PDGF-B kinoids on CCl_4-induced fibrosis in mice following the protocol illustrated in Figure 2. The results of ELISA showed that the kinetic pattern of the production of anti-PDGF-B Abs in these mice was similar to that in the former experiment, even though the fourth immunization was carried out one week after initiating CCl_4 injection. The results suggest that CCl_4-induced liver injury and fibrosis does not significantly influence the immune response to PDGF-B kinoid immunization in mice.

After 6 weeks of CCl_4 injection, the mice were euthanized and the livers were subjected to pathological examination. Masson's trichrome staining of the liver sections showed that six weeks of repeated injections of CCl_4 induced obvious and uniform fibrosis in the livers of KLH/CCl_4 and CCl_4 mice. The severity of hepatic fibrosis in the two PDGF-B kinoid vaccination groups was obviously milder than in the KLH/CCl_4 and CCl_4 groups (Figure 3A). Semi-quantitative evaluation by the Ishak system[31] followed by statistical analysis indicated that the fibrosis scores of the two PDGF-B kinoid vaccination groups were significantly lower than that in either the KLH/CCl_4 or the CCl_4 groups while there were no notable differences between the former two groups or the latter two groups (Table 1).

The content of hydroxyproline, a specific indicator of fibrosis, was determined in the liver tissues. As the results of the histological grading, the hepatic hydroxyproline contents of the two kinoid-immunization groups were similar and significantly lower than that of either the KLH/CCl_4 or the CCl_4 groups, and those of the latter two groups were similar (Figure 3B). These results collectively demonstrated that vaccination against PDGF-B inhibited CCl_4-induced hepatic fibrosis in mice.

Vaccination against PDGF-B inhibited the proliferation of HSC/MFB in the fibrotic livers. To evaluate the effects of the vaccination with PDGF-B kinoids on the proliferation of mesenchymal cells

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and parenchymal cells, respectively, we performed immunohistochemistry to detect the expression of PCNA, which is an indicator of cell proliferation. The PCNA immunostaining and subsequent semiquantitative analysis showed that the mice in the CCl_4 and KLH/CCl_4 groups displayed markedly increased hepatic mesenchymal PCNA LIs compared with the mice in the NC group, while the hepatic mesenchymal PCNA LIs in the two PDGF-B kinoid immunization groups were significantly lower than that in either the CCl_4 or the KLH/CCl_4 groups (Figure 4A,B). Since HSCs/MFBs are the main component of the mesenchyma in the fibrotic livers, this result essentially indicated that the proliferation of HSCs/myofibroblasts was suppressed by the vaccination. However, although the PCNA LIs in the parenchyma (predominantly hepatocytes) was markedly increased by CCl_4 injection, there was no significant difference among the four CCl_4-injected groups (Figure 4A,C), indicating that the regeneration of the hepatocytes was not influenced by PDGF-B kinoid vaccination.

Vaccination with PDGF-B kinoids suppresses activation of HSCs in fibrotic liver. The expression of α-SMA, an indicator of activated HSCs, was assessed immunohistochemically in this study for evaluating the effect of vaccination against PDGF-B on HSC activation during hepatic fibrosis. In the NC mice, the expression of α-SMA was confined to the smooth muscle cells lining the portal and central veins and the large arteries within the liver. Six weeks of CCl_4 injections led to a marked increase in the amount of α-SMA–positive cells distributing in clusters within the fibrous septa (Figure 5A). The computer-assisted semiquantitative analysis revealed that the α-SMA–positive areas in the QK16-KLH/CCl_4 and VF16-OVA/CCl_4 groups were significantly decreased compared with those in either the CCl_4 or the KLH/ CCl_4 groups, while there was no significant difference either between the former two groups or between the latter two groups for the α-SMA–positive areas (Figure 5B). This

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result was further confirmed by Western blotting detection of α-SMA in the liver tissues (Figure 5C).

The expression of Desmin, a marker for intermediately differentiated HSCs (Ballardini, et al., 1988; Cassiman, et al., 2002) was also detected in this study. The result showed that the changes in the expression of desmin was similar to that of α-SMA, except for that the desmin-positive staining was observed at the rim of fibrous setpa and in neighboring hepatocytes in the fibrotic livers (Figures 6A,B).

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DISCUSSION

Vaccination against pathogenic cytokines and growth factors has been verified as a simple, safe and efficient approach for the management of the relevant disorders. In this study, we demonstrated that the two PDGF-B kinoids prepared by cross-linking the PDGF-B–derived polypeptides to the carrier proteins elicited high levels of neutralizing anti-PDGF-B antibodies and displayed marked anti-fibrosis effects on CCl_4-induced hepatic fibrosis in mice. To our knowledge, this is the first study attempting to validate the suppressive effect of vaccination against a profibrogenic cytokine on hepatic fibrosis.

The Abs elicited by both preventive and therapeutic vaccines should have neutralizing abilities, although non-neutralizing Abs might also exert some inhibitory effects by facilitating the clearance of the antigens. In this study, immunization with the kinoids prepared with both PDGF-B–(23-38) (VF16) and PDGF-B-(72-87) (QK-16) elicited high levels of neutralizing anti-PDGF-B Abs. Our results are in agreement with the previously reported results that these two fragments are critical for the binding of PDGF-B to PDGFRβ (Larochelle, et al., 1989; Engstrom, et al., 1992; LaRochelle, et al., 1992; Brennand, et al., 1997; Patel, et al., 1999). Our subsequent results demonstrated that neutralizing PDGF-B by vaccination rendered marked attenuation of CCl_4-induced hepatic fibrosis, in agreement with the previously reported results that inhibition of PDGF-B signaling suppressed hepatic fibrosis (Pinzani, et al., 1994; Wong, et al., 1994; Pinzani, et al., 1996; Borkham-Kamphorst, et al., 2008) and vaccination against pathogenic cytokines could efficiently alleviate the relevant disorders (Dalum, et al., 1999; Zagury, et al., 1999; Holmgren, et al., 2006; Le Buanec, et al., 2006; González, et al., 2007; Rad, et al., 2007; Spohn, et al., 2007; Vinageras, et al., 2008; Tissot, et al., 2008; Delavallée, et al., 2008; Spohn, et al., 2008; Tohyama, et al., 2008).

The inhibitory effect of immunization against PDGF-B on hepatic fibrosis might be due to several

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mechanisms: First of all, neutralization of PDGF-B with anti–PDGF-B Abs is associated with decreased proliferation of HSC/MFB, and therefore decreases the amount of ECM-producing cells. The second cause of this effect might be that the neutralization of PDGF-B weakens the pro-fibrogenic activities of TGF-β1 and connective tissue growth factor (CTGF) because that PDGF-B upregulates the expression of TGF-β1 receptor I and II (Czuwara-Ladykowska, et al., 2001) and connective tissue growth factor (CTGF) (Paradis, et al., 2002). Thirdly, it has been reported that PDGF-B has the ability to directly stimulate the activation of HSCs and the expression of ECM by HSCs/MFBs (Kinnman, et al., 2003; Czochra, et al., 2006; Borkham-Kamphorst, et al., 2004a; Borkham-Kamphorst, et al., 2008), so the neutralization of PDGF-B directly lead to the suppressed of ECM expression. Consistent with this view, our results demonstrated that the vaccination against PDGF-B not only decreased the PCNA LI in the hepatic mesenchyma but also reduced the expression of α-SMA and desmin, the markers for HSC activation, suggesting that PDGF-B possesses an capability of driving the production and deposition of ECM from HSCs/MFBs in addition to the direct mitogenic activity of on HSCs/MFBs. The alleviation degree of CCl_4-induced hepatic fibrosis resulting from the vaccination against PDGF-B is not obviously lower than that resulting from vaccination against TGF-β1 (our unpublished data), suggesting that PDGF-B plays an as important role in hepatic fibrosis as TGF-β1 does.

One of the major concerns about the safety of vaccination against cytokines is that the neutralization of the cytokines by autoantibodies might impair non-targeted healthy tissues because cytokines are highly pleiotropic. This adverse effect was not observed in our current study or in investigations by others (Dalum, et al., 1999; Zagury, et al., 1999; Holmgren, et al., 2006; Le Buanec, et al., 2006; González, et al., 2007; Rad, et al., 2007; Spohn, et al., 2007; Vinageras, et al., 2008; Tissot, et al., 2008; Delavallée, et al., 2008; Spohn, et al., 2008; Tohyama, et al., 2008). The possible explanations might

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include the following: 1) The affinities of the cytokines for their receptors are generally higher than for the autoantibodies raised by immunization. 2) The antigen-Ab reaction is a reversible reaction, and the neutralization cannot be so thorough that it completely eliminates the systemic cytokine. Because of the highly efficient biological activity of cytokines, their physiological functions are not likely to be impaired provided there is a residual level of cytokines. 3) The metabolism of a cytokine in the lesioned tissues is different from that in normal tissues. Cytokines exert biological functions principally in local tissues in an autocrine or paracrine manner. In normal tissues, there is an efficient feedback regulatory mechanism for the production of a cytokine. Removal of the cytokine will trigger the compensatory production of the cytokine, while in lesioned tissues, the production of a cytokine is dysregulated and the cytokine is produced at a constant high rate. Neutralization of the excessively produced pathogenic cytokines does not lead to compensatory overproduction of the cytokine. 4) Pathologic tissues exhibit an abundant stromal lymph flow that facilitates the accumulation of local Abs, while normal tissues demonstrate a negligible lymph turnover flow where the poorly renewed Abs are unlikely to impair the short-distance and instant cytokine reaction occurring within the immunological synapse between tightly associated immune cells. Nevertheless, the safety of cytokine vaccines still need to be investigated carefully in future studies.

It should be noted that in this preliminary study we immunized the mice before the induction of hepatic fibrosis, thus our results actually reflects the “preventive” rather than “therapeutic” effect of vaccination against PDGF-B on experimental hepatic fibrosis. Since enhanced PDGF-B signaling not only promotes the initiation of hepatic fibrosis but also plays an as well important role in the persistence of hepatic fibrosis, and the humoral immune response in animals, as well as in patients, with hepatic fibrosis is not seriously impaired (Cheong, et al., 2006), we believe that the vaccination against PDGF-B

could produce a “therapeutic” effect on hepatic fibrosis/cirrhosis. Nevertheless, whether vaccination against PDGF-B could inhibit the advancement or further, facilitate the reversal of established hepatic fibrosis/cirrhosis needs to be investigated in future studies.

In conclusion, our study verified that vaccination against PDGF-B with PDGF-B kinoids markedly inhibited CCl_4-induced hepatic fibrosis, suggesting that this approach might be developed into an efficient, safe, simple and convenient therapeutic strategy for managing chronic fibrotic liver diseases. Furthermore, it has been reported that PDGF-B signaling accelerates the carcinogenesis in the fibrotic livers (Maass, et al., 2011). Therefore, vaccination against PDGF-B for chronic liver diseases might have dual benefits of both suppressing fibrosis and preventing carcinogenesis. In addition, since fibrosis of various organs share a rather common underlying pathological mechanism in which multiple pro-fibrogenic factors are involved, combined vaccination against more than one of these pro-fibrotic cytokines should be considered for fibrosis of various organs and tissues.

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Authorship contributions

Participated in research design: Zhi-Ming Hao and Xiao-Bao Fan

Conducted experiments: Xiao-Bao Fan, Zhi-Ming Hao, Shuang Li, Hou-Qiang Su, Hui-Ping Jiang and Hong-Hong Li

Contributed new reagents or analytic tools: Yi-Fei Lv

Performed data analysis: Zhi-Ming Hao and Xiao-Bao Fan

Wrote or contributed to the writing of the manuscript: Zhi-Ming Hao and Shuang Li

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FOOTNOTES

Z.-M.H and X.-B.F contributed equally to this work.

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Figure legends

Figure 1. Immunization with PDGF-B kinoids elicits anti–PDGF-B neutralizing IgG Abs. (A) Male BALB/c mice were biweekly given 4 i.p. injections of QK16-KLH (50 μg), VF16-OVA (50 μg), KLH (50 μg) or an equal volume of PBS. Blood samples were taken before each immunization and biweekly after the 4 immunization for ELISA determination of the anti-PDGF-B Abs. Indirect ELISA with the recombinant human PDGF-BB–coated (20ng/well) plates showed that the immunization with both of the two PDGF-B kinoids elicited the production of high levels and long-term anti–PDGF-B IgG Abs. **(B)** Five micrograms of the recombinant human PDGF-B expressed in *E. coli* was subjected to 10% SDS–PAGE and subsequently transferred to nitrocellulose membrane. The mouse sera (at a dilution of 1:1000) collected 2 weeks after the forth injection of the PDGF-B kinoids, KLH or PBS were used as the primary Abs. **(C)** NIH3T3 cells (3×10^3/well) in 96-well plates were starved in serum-free DMEM for 48 hours, and then cultured with fresh medium containing various dilutions of the mouse sera collected 2 weeks after the forth injection, human PDGF-BB (3 ng/ml) and BrdU for 48 hours. The proliferation of the cells was evaluated by BrdU incorporation with a BrdU Cell Proliferation Assay kit. The experiment was performed in triplicate and repeated for three times, and the results were expressed as the ratio to the PBS control. The error bars indicate standard error of the mean (SEM).

Figure 2. The experimental protocol for validating the inhibitory effects of vaccination with the PDGF-B kinoids on CCl_4-induced hepatic fibrosis in mice.

Figure 3. Vaccination with PDGF-B kinoids suppresses CCl_4-induced hepatic fibrosis in mice. The

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immunization and induction of hepatic fibrosis were performed as illustrated in Figure 2. **(A)** The mouse liver sections were stained with Masson's trichrome staining to evaluate fibrosis. Bars = 200 μm. **(B)** The hydroxyproline content in the mouse liver tissues was determined by a biochemical assay and expressed as μg/mg wet liver weight. Error bars indicate SEM.

Figure 4. Vaccination against PDGF-B suppresses the proliferation of the mesenchymal cells but not influences that of the parenchymal cells in the fibrotic livers. The vaccinations and CCl_4 injections were performed as illustrated in Figure 2. (A) The expression of PCNA was immunohistochemically determined in the liver sections. The scale bar = 50μm. PCNA LI were evaluated for the mesenchyma **(B)** and the parenchyma **(C)**, respectively, after immunohistochemical staining for PCNA. Error bars indicate SEM.

Figure 5. Vaccination with PDGF-B kinoids suppresses the expression of α-SMA in the CCl_4-induced fibrotic mouse livers. The vaccinations and CCl_4 injections were performed as illustrated in Figure 2. **(A)** The mouse liver sections were immunohistochemically stained for α-SMA. **(B)** The expression of α-SMA was semi-quantitatively assessed in the immunostained liver sections by computer-assisted morphometric analysis. Bars in the photographs = 200 μm. Error bars indicate SEM. (C) Western blotting detection of α-SMA in the liver tissues confirmed the immunostaining results.

Figure 6. Vaccination with PDGF-B kinoids suppresses the expression of desmin in the CCl_4-induced fibrotic mouse livers. The vaccinations and CCl_4 injections were performed as illustrated in Figure 2. **(A)** The mouse liver sections were immunohistochemically stained for desmin. **(B)**

The desmin expression was semi-quantitatively assessed by computer-assisted morphometric analysis. Bars in the photographs = 200 μm. Error bars indicate SEM.

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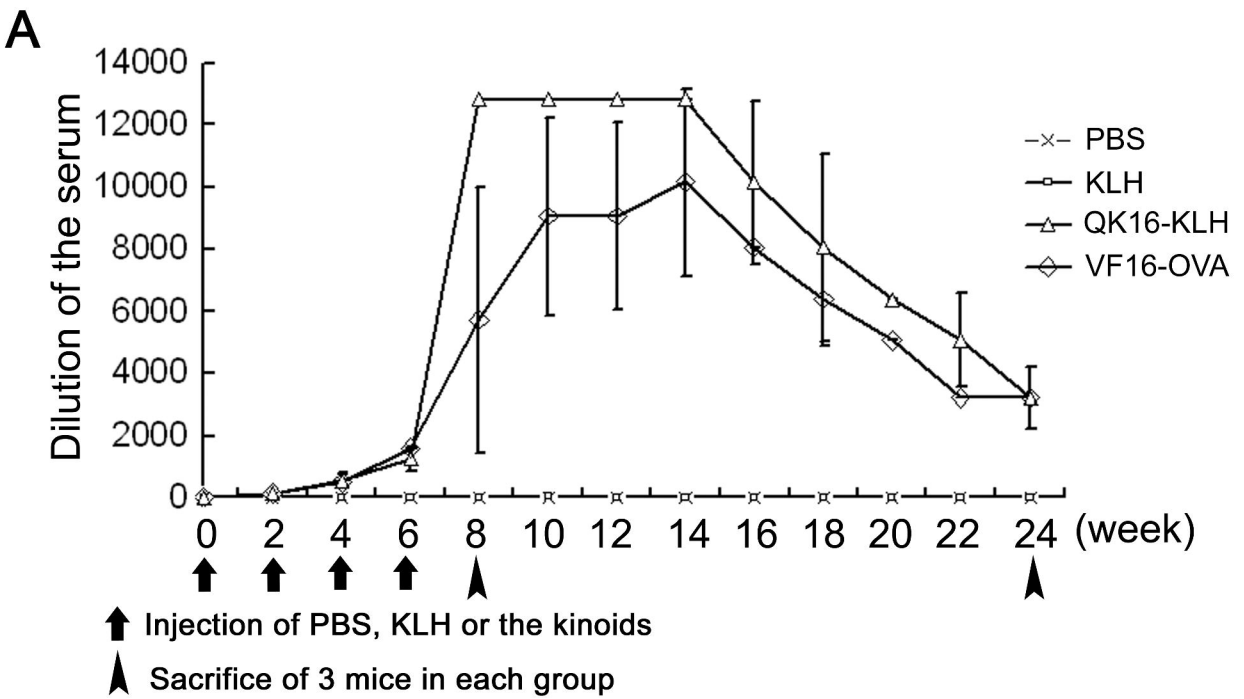
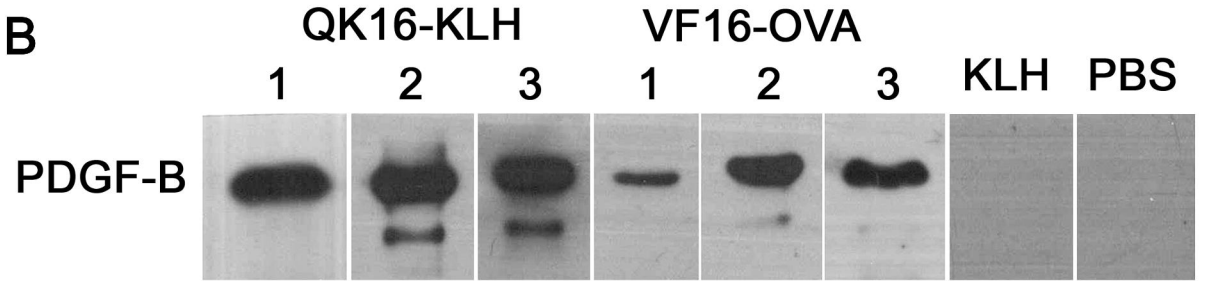
Table 1. Scores of the hepatic fibrosis in various groups

Group	N	Fibrotic score						
		0	1	2	3	4	5	6
NC	6	6	0	0	0	0	0	0
QK16-KLH/ CCl_4*	9	0	2	2	3	1	0	0
VF16-OVA/ CCl_4*	9	0	2	3	2	1	1	0
KLH/ CCl_4	8	0	0	0	3	2	1	1
CCl_4	8	0	0	0	4	1	2	1

* $P < 0.05$ vs either the KLH/CCl_4 group or the CCl_4 group ; $^{\#}$ $P > 0.05$ vs the CCl_4 group. Mann-Whitney *U*-test.

Figure 1


A
Dilution of the serum
14000
12000
10000
8000
6000
4000
2000
0
PBS
KLH
QK16-KLH
VF16-OVA
0 2 4 6 8 10 12 14 16 18 20 22 24 (week)
Injection of PBS, KLH or the kinoids
Sacrifice of 3 mice in each group


B
QK16-KLH
VF16-OVA
1 2 3 1 2 3 KLH PBS
PDGF-B

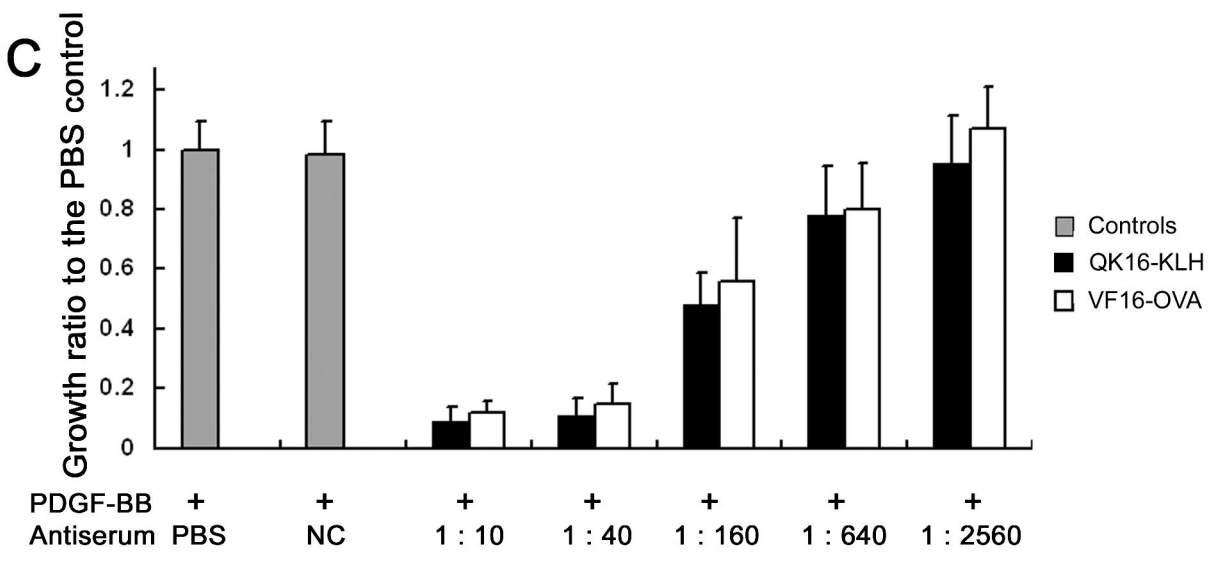

C
Growth ratio to the PBS control
1.2
1
0.8
0.6
0.4
0.2
0
Controls
QK16-KLH
VF16-OVA
PDGF-BB + + + + + + +
Antiserum PBS NC 1 : 10 1 : 40 1 : 160 1 : 640 1 : 2560

Figure 2

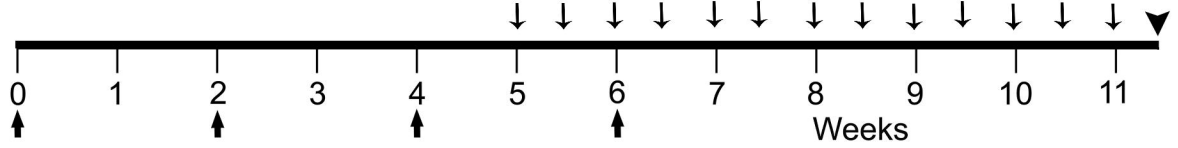
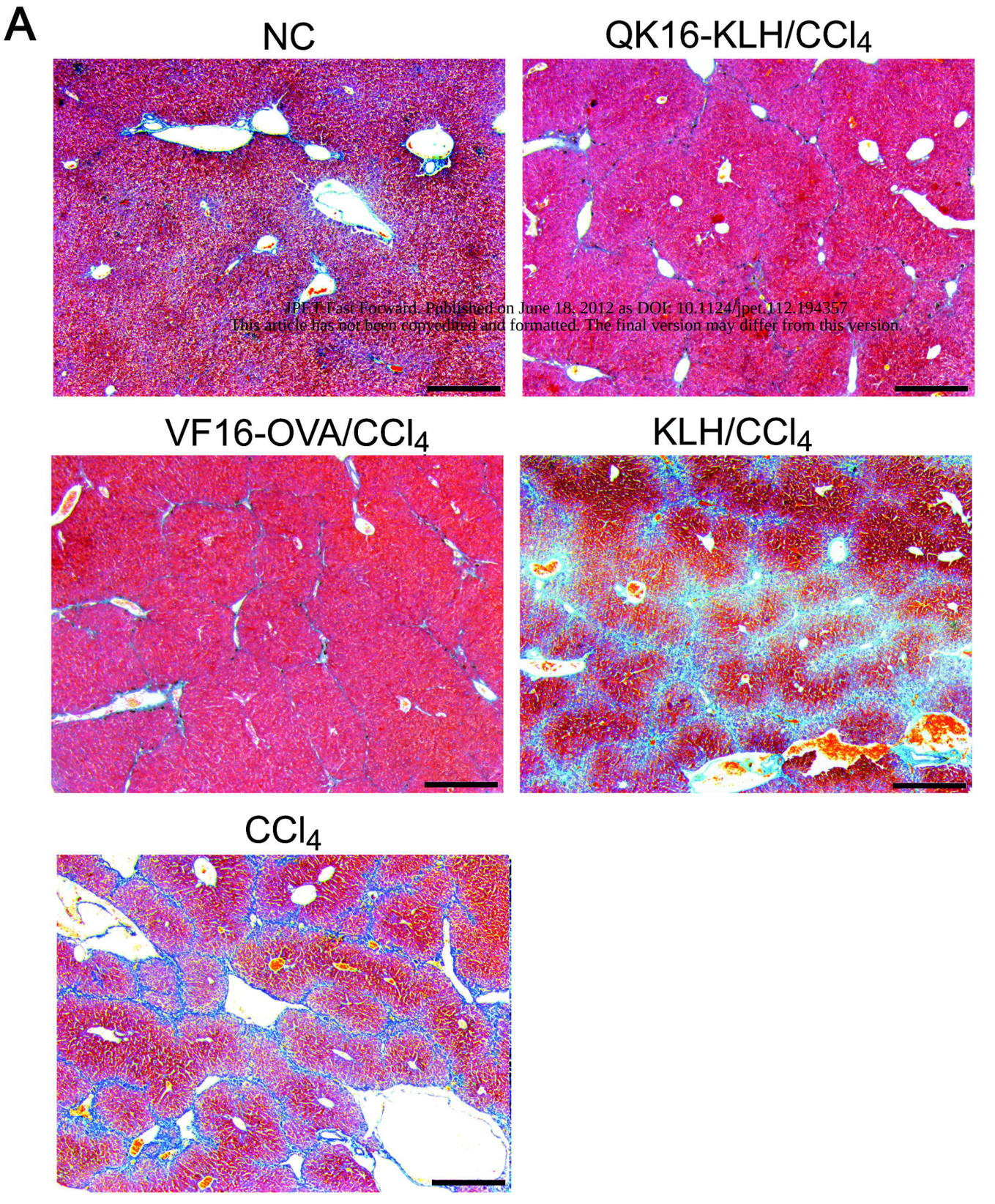

0
1
2
3
4
5
6
7
8
9
10
11
Weeks
i.p. injection with PDGF-B kinoids, KLH or PBS
i.p. injection with CCl_4
Sacrifice

Figure 3



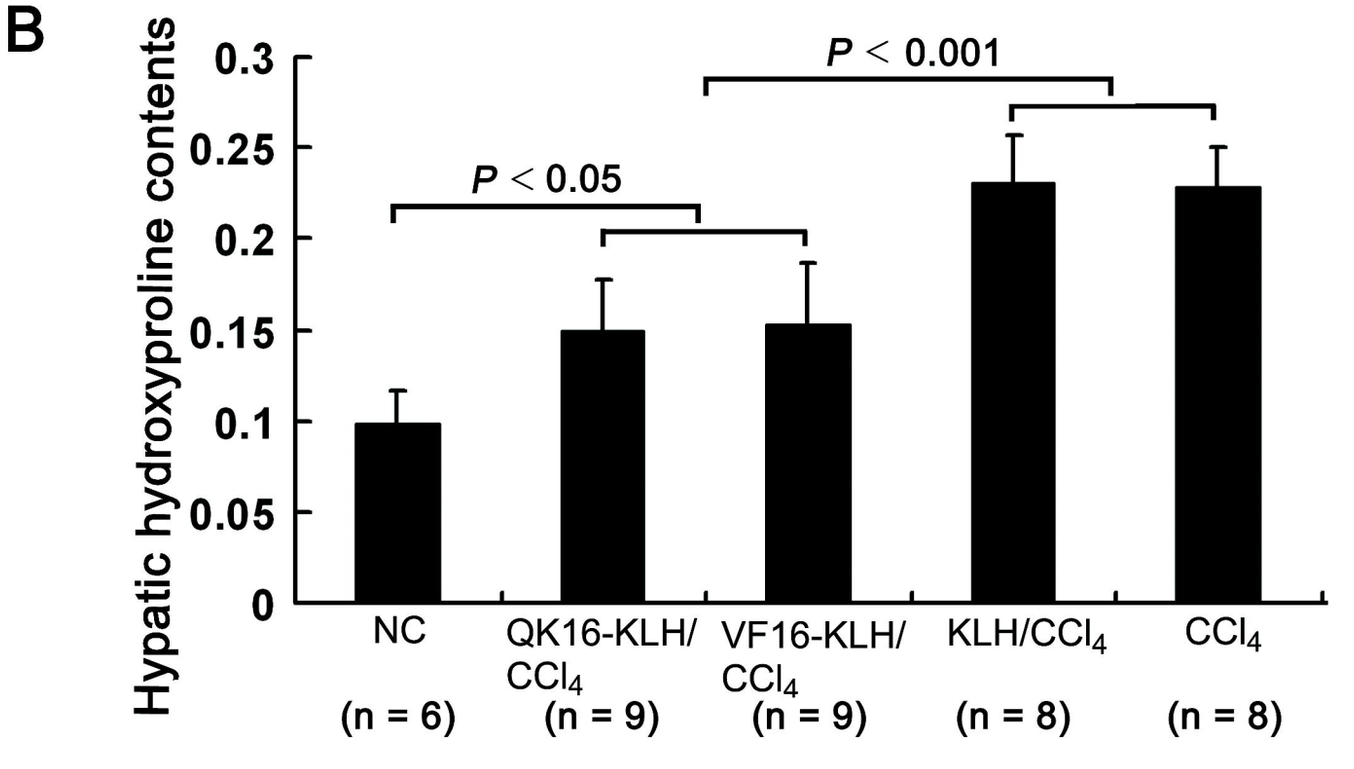


Figure 4

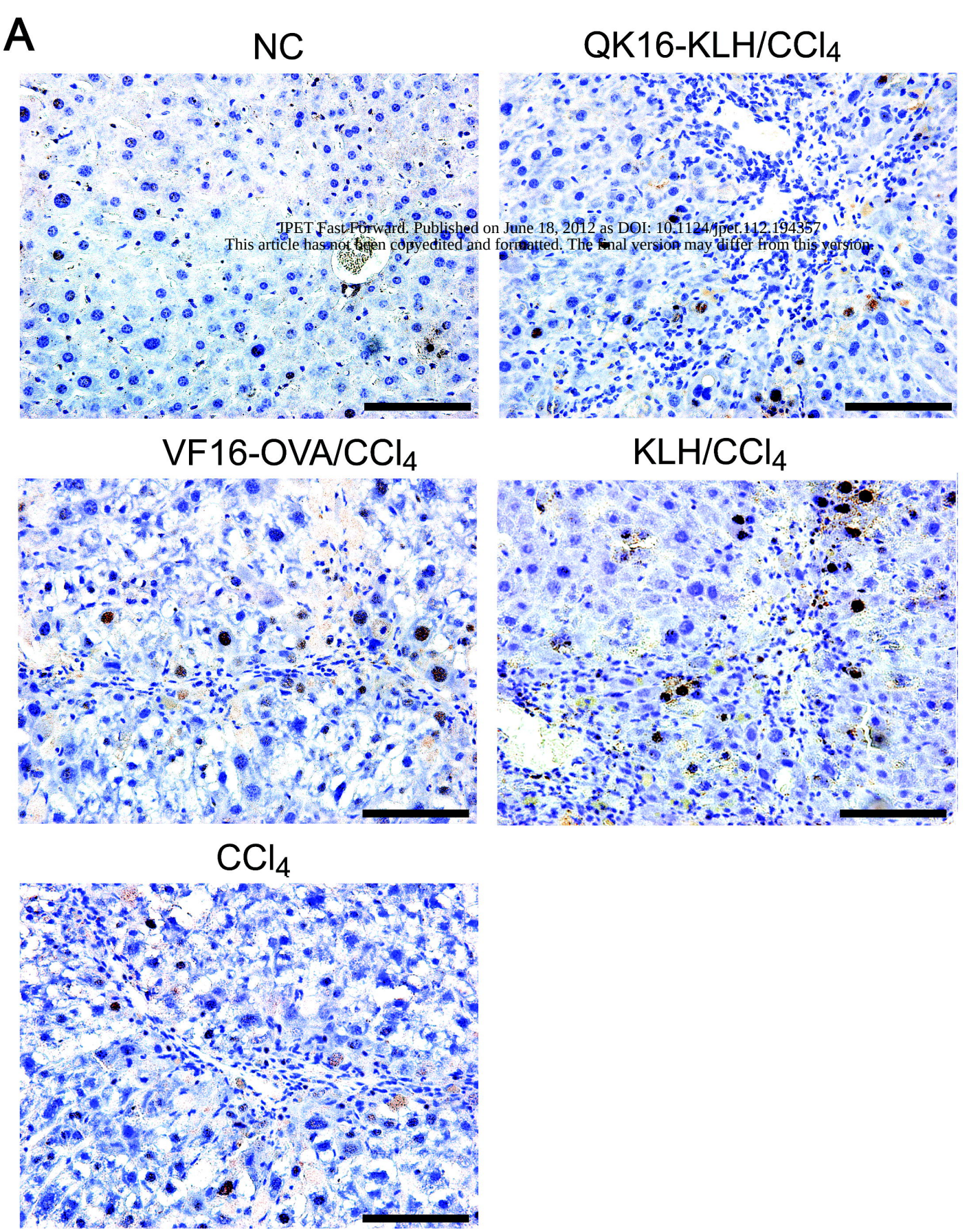
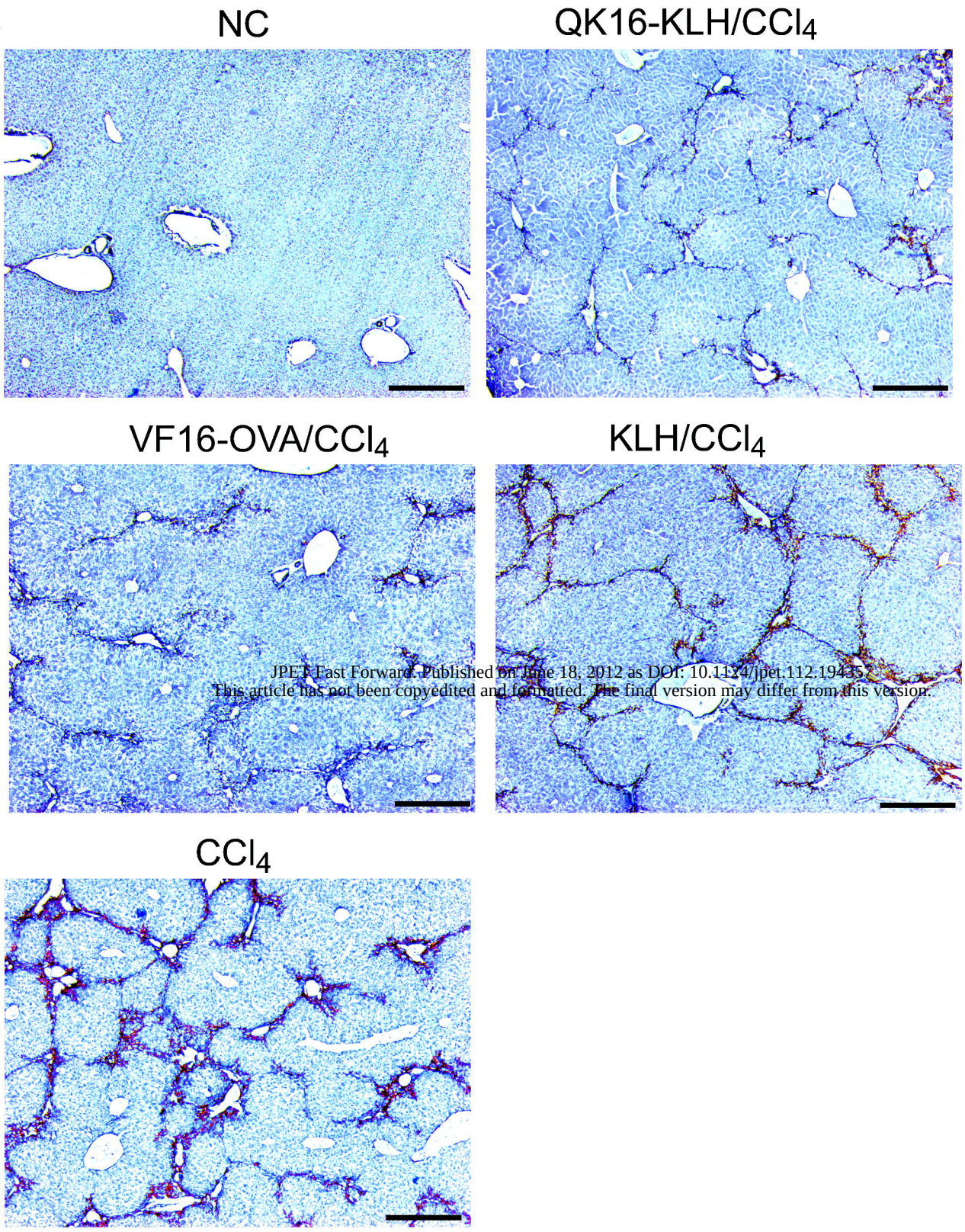


Figure 5

A



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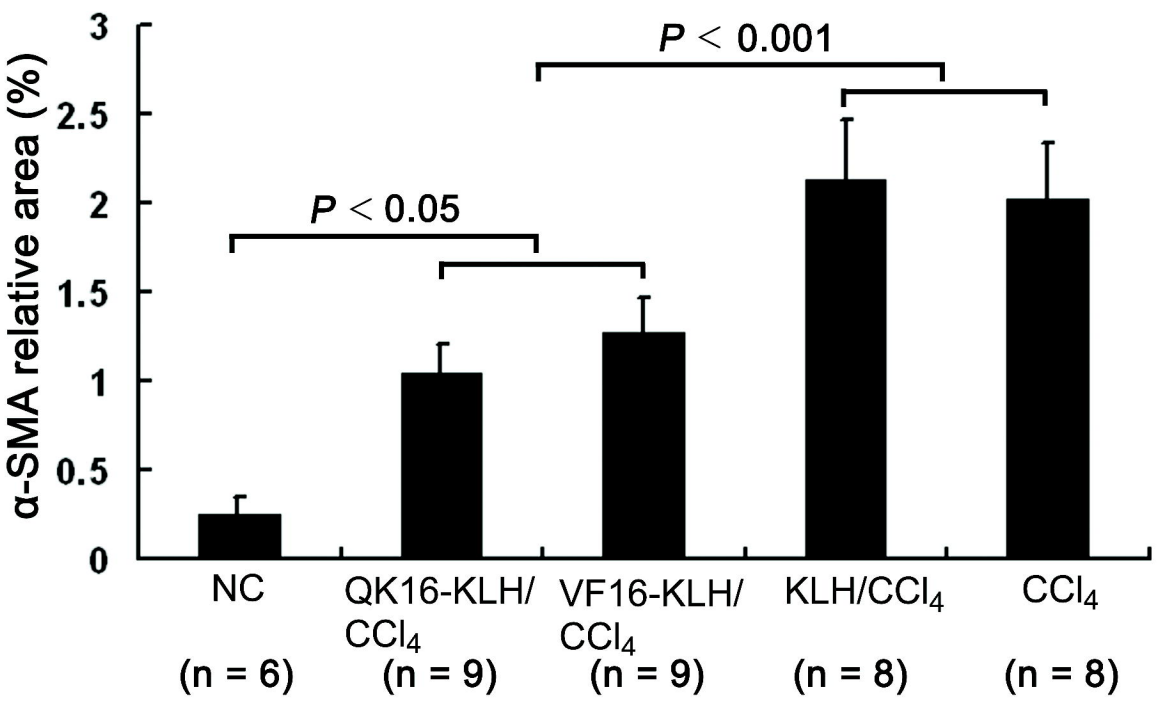

C

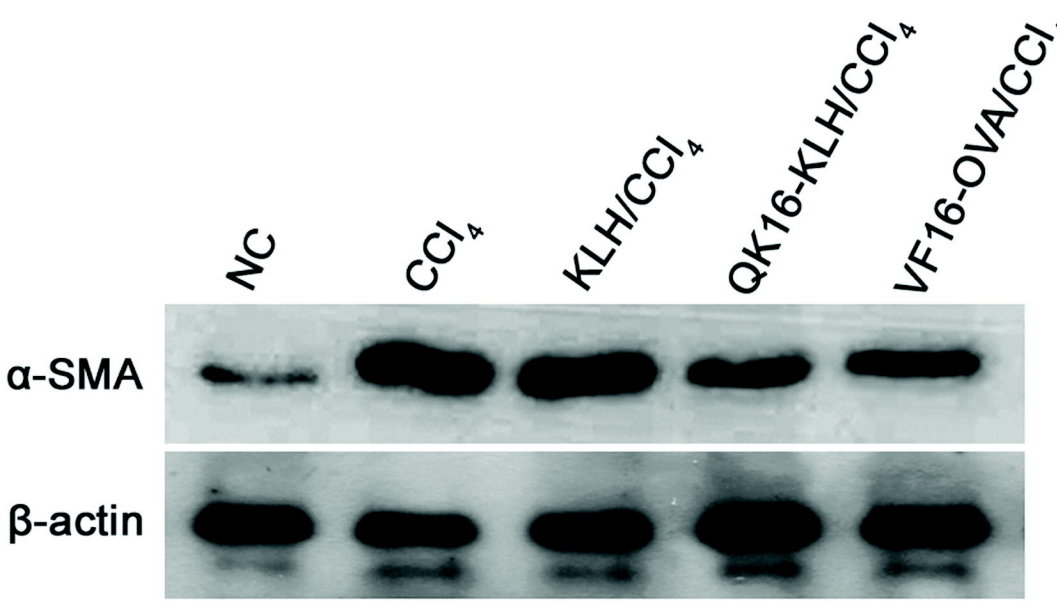

Figure 6