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Peptide-Modified Albumin Carrier Explored as a Novel Strategy for a Cell-Specific Delivery of Interferon Gamma To Treat Liver Fibrosis

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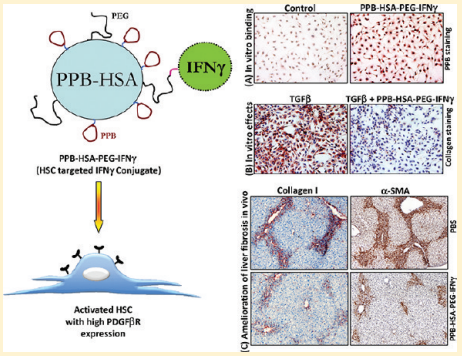
Supporting Information

ABSTRACT: Excessive accumulation of the extracellular matrix proteins primarily produced by activated hepatic stellate cells (HSC) leads to liver fibrosis. To date, no successful therapeutic intervention is available for the treatment of this disease. Platelet derived growth factor beta receptor (PDGFβR) is highly upregulated on disease-inducing activated HSC and thus can be used for delivery of antifibrotic drugs to increase therapeutic efficacy with reduced adverse effects. Interferon gamma (IFNγ) has been recognized as a potent antifibrotic cytokine; however, poor pharmacokinetics and side effects due to frequent administration have limited its clinical use. For HSC-specific delivery, a PDGFβR-specific drug delivery carrier (PPB−HSA) was developed by modifying albumin with PDGFβR-recognizing cyclic peptides. Subsequently, IFNγ was conjugated to PPB−HSA via bifunctional PEG linkers to synthesize PPB−HSA−PEG−IFNγ. *In vitro*, PPB−HSA−PEG−IFNγ retained complete biological activity similar to unmodified IFNγ and showed PDGFβR-specific binding to human HSC and primary culture-activated rat HSC. In TGFβ-stimulated mouse fibroblasts and human HSC, PPB−HSA−PEG−IFNγ induced significant reduction in crucial fibrotic parameters. *In vivo*, the conjugate rapidly accumulated into PDGFβR-expressing HSC in fibrotic livers and activated IFNγ-mediated pstat1α signaling pathway. Furthermore, in a CCl_4-induced acute liver injury model in mice, treatment with HSC-targeted IFNγ strongly ameliorated hepatic fibrogenesis by inducing significant reduction (about 60%; $p < 0.01$) in collagen I and α-SMA expression as well as enhanced fibrolysis (increased MMP/TIMP ratio; $p < 0.05$) while free unmodified IFNγ was ineffective. Furthermore, in contrast to free native IFNγ, the conjugate did not induce macrophage infiltration and IL-1β expression in the liver. In conclusion, these data demonstrate the enhanced antifibrotic efficacy and reduced off-target effects of PPB−HSA−PEG−IFNγ conjugate showing the potential of cell-specific targeting of IFNγ for the treatment of liver fibrosis.



KEYWORDS: platelet derived growth factor β receptor, cyclic peptide, stellate cells targeting, interferon-gamma, liver fibrosis

INTRODUCTION

Liver fibrosis is characterized by the extensive accumulation of extracellular matrix proteins in liver, leading to the formation of hepatic scars.[1,2] To date, no successful clinically approved therapy is available to treat this disease.[3,4] Despite increasing evidence that multiple liver cell types contribute to extracellular matrix components, hepatic stellate cells (HSC) plays a major role in the progression of the disease.[5] During an acute or chronic liver injury, various crucial growth factors and inflammatory cytokines are produced by the infiltrating inflammatory cells, hepatocytes and bile duct epithelial cells leading to the transformation of quiescent hepatic stellate cells into extracellular matrix producing myofibroblasts.[5] Various cytokines like IL-10, IFN-α, IFNγ etc. have been studied *in vitro* and *in vivo* in animal models as prospective therapeutics for liver fibrosis.[6] Among them, interferon gamma (IFNγ) has been shown to have the most potent antifibrotic effects.[7,8]

Interferon gamma is a pleiotropic cytokine produced by activated immune cells (NK, NKT, B cells, T_{H1}, T_C, antigen presenting cells).[9] It displays strict species specificity and interacts with its specific receptor, interferon gamma receptor (IFNγR) that is expressed on nearly all cell types (except mature erythrocytes).[9] IFNγ has been extensively exploited for the treatment of viral, immunological, and malignant diseases due to its antiviral, immunomodulatory and antiproliferative properties.[10] Several studies have described the role of IFNγ in liver fibrosis: it inhibits activation and proliferation of hepatic stellate cells; reduces collagen synthesis and promotes its degradation; abrogates TGFβ response in liver fibrosis[7,11−13] etc. However, short circulation half-life and undesirable systemic side effects have limited its clinical use.[14,15]

Many attempts have been made to prolong the half-life or to enhance the activity of IFNγ by incorporation into liposomes, nanoparticles, elastomers or hydrogels, but none have shown significant improvement.[16,17] Therefore, novel cell-specific delivery strategies are required to improve the therapeutic efficacy

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of IFNγ and to turn it into a biologically active pharmaceutical for the treatment of liver fibrosis. One approach to cell-specific delivery could be based on recognition by the platelet derived growth factor receptor beta (PDGFβR). Several studies have shown that PDGFβR is highly and specifically overexpressed on activated HSC during acute and chronic liver injury.[18,19] Thus, using a cyclic peptide against this receptor (PPB) to target IFNγ specifically to HSC can be a promising approach. Over past years, our group has developed a cyclic peptide C*SRNLIDC* (PPB) that has specific affinity for PDGFβR.[20] Furthermore, PPB has also been exploited as a peptide modified with human serum albumin (HSA) to develop a PDGFβR specific drug-delivery carrier (PPB−HSA) that will impart receptor (target) specificity and increase the half-life of drugs (cytokines). This carrier has been shown to bind specifically to PDGFβR and therefore can be used for the targeting to activated hepatic stellate cells in fibrotic livers.[20] Recently, this carrier has been employed for the delivery of an antitumor drug doxorubicin, to PDGFβR-expressing stromal and tumor cells, in tumors to increase the therapeutic efficacy with reduced adverse effects.[21]

In the present study, we utilized targeted drug-delivery carrier (PPB−HSA) for the delivery of potent antifibrotic cytokine IFNγ to activated hepatic stellate cells for the treatment of liver fibrosis. First, we studied PDGFβR expression in acute liver injury mouse model and in human cirrhotic livers. To examine whether PPB−HSA can be used as a drug carrier, we conjugated IFNγ to PPB−HSA through a bifunctional PEG linker and investigated its binding and antifibrotic effects *in vitro* in different cell types. Subsequently, we evaluated the conjugate for its *in vivo* biodistribution and therapeutic efficacy in acute liver fibrogenesis model in mice.

MATERIALS AND METHODS

Chemicals and Reagents. Murine IFNγ was purchased from Peprotech (Rocky Hill, NJ), and protein LoBind tubes were purchased from Eppendorf AG (Hamburg, Germany). *N*-Succinimidyl-*S*-acetylthioacetate (SATA); hydroxylamine HCl; DMF; EDTA and 3-amino-9-ethylcarbazole (AEC) were procured from Sigma Aldrich (St. Louis, MO). PPB-ATA, an 8 amino acid cyclic peptide (*Cys-Ser-Arg-Asn-Leu-Ile-Asp-Cys*) modified with SATA, was synthesized by Ansynth Service B.V. (Rosendaal, The Netherlands) as described elsewhere.[20,21] All the other chemicals and solvents used in the study were of analytical grade.

Human Liver Specimens. Human liver specimens were obtained from liver transplanted patients suffering from liver cirrhosis and were anonymously provided by the Department of Pathology (University Medical Centre Groningen UMCG, The Netherlands). Control liver tissues were obtained from the unaffected part of the liver from transplanted patients. Necessary approvals were obtained from hospital medical ethics committee.

Cell Lines. Mouse 3T3 fibroblasts and RAW macrophages were obtained from American Type Culture Collection (ATCC, Rockville, MD). Human hepatic stellate cells (LX2) were kindly provided by Prof. Scott Friedman (Mount Sinai Hospital, New York, NY). RAW macrophages and 3T3 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% FBS and antibiotics (50 U/mL penicillin and 50 ng/mL streptomycin). LX2 were cultured in DMEM-Glutamax (Invitrogen) supplemented with 10% FBS and antibiotics (50 U/mL penicillin and 50 ng/mL streptomycin).

Synthesis of Interferon Gamma Conjugate. All the reactions were performed in low protein binding tubes (LoBind tubes).

PPB−HSA Conjugate. PPB−HSA was synthesized as described previously.[21] Briefly, HSA (1.5 μmol, dissolved in PBS) was reacted with γ-maleimidobutyryloxy-succinimide ester (GMBS, 30 μmol, dissolved in DMF) for 2 h and extensively dialyzed against PBS using 10 kDa cutoff dialysis membrane cassette (Thermo Scientific, Rockford, IL). PPB-ATA (34.5 μmol; dissolved in DMF) was added to the GMBS-modified HSA overnight, dialyzed against PBS and then further dialyzed against ultrapure water. The final product was freeze-dried for long-term storage at −20 °C.

PPB−HSA−PEG Conjugate. 1.333 nmol of PPB−HSA was conjugated to 66.65 nmol of bifunctional PEG linker (Mal-PEG-SCM, 2 kDa, Creative PEGworks, Winston Salem, NC) overnight at 4 °C and purified extensively using 7K Zeba spin desalting columns (Thermo Scientific).

IFNγ−SATA Conjugate. 0.3205 nmol of murine IFNγ (Peprotech, London, U.K.) was reacted with 8.0125 nmol of *N*-succinimidyl-*S*-acetylthioacetate for overnight at 4 °C and purified by dialysis using 7K Zeba spin desalting columns (Thermo Scientific).

PPB−HSA−PEG−IFNγ Conjugate. The purified products (PPB−HSA−PEG and IFNγ−SATA) were reacted at the ratio (1:3) in the presence of deacetylating reagent (0.1 M hydroxylamine, 25 mM EDTA in PBS pH 7.2) for overnight at 4 °C.

The prepared PPB−HSA−PEG−IFNγ conjugate was extensively dialyzed against PBS using 50 kDa dispodialyzers (Harvard Apparatus, Holliston, MA).

Characterization of IFNγ Conjugates by PEG Staining and Western Blotting. PPB−HSA−PEG−IFNγ conjugate was characterized by SDS−PAGE analysis followed by barium iodide PEG staining. Briefly, proteins (250 ng) were subjected to SDS−PAGE (10%) according to standard protocols. After running the gel, PEG staining was performed as follows. The gels were rinsed with water, followed by fixation in perchloric acid (0.1 M) for 15 min. The gels were washed again and treated with barium chloride (5%) for 10 min. Subsequently, the color was developed using tritisol iodine solution (Sigma). The gels were photographed using G-Box (Syngene, Cambridge, U.K.). In addition to PEG staining, Western blotting was performed where the SDS−PAGE gel with separated proteins was transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Mannheim, Germany). The membranes were blocked with TBST (20 mM Tris HCl pH7.6, 154 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk and incubated with either rabbit polyclonal anti-IFNγ antibody (1:2000; Abcam, Cambridge, U.K.) or rabbit polyclonal anti-PPB antibody (1:1000; custom-made, Harlan) overnight. After washings, the blots were incubated with HRP-conjugated goat anti-rabbit antibody (1:1000; DAKO, Glostrup, Denmark) for 1 h. Subsequently, the blots were washed and developed using Western Lightning-ECL reagent (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions.

Assessment of Biological Activity of Targeted IFNγ in Mouse RAW Macrophages. The bioactivity of IFNγ and IFNγ conjugate was assessed by measuring accumulation of nitrite NO_2, a stable nitric oxide (NO) metabolite produced by murine RAW macrophages.[22] Briefly, cells (1×10^5cells/200 μL/well)

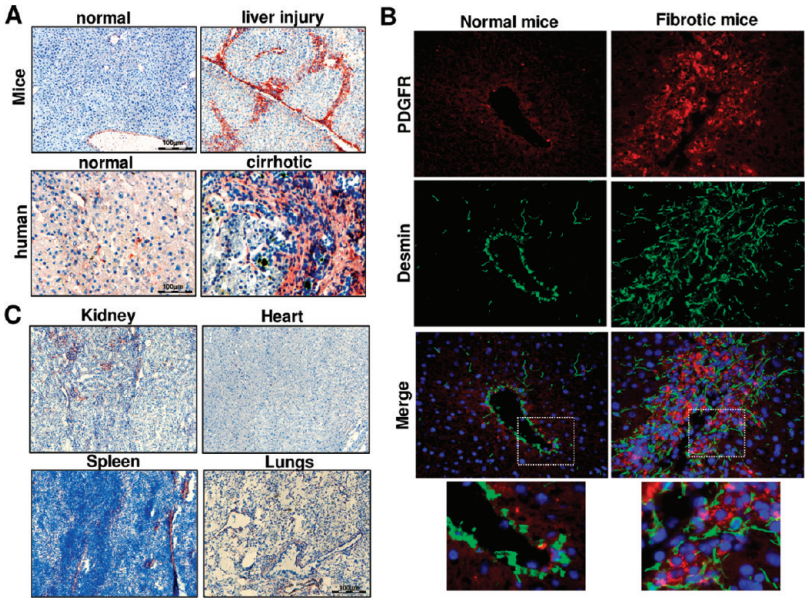

Figure 1. PDGFβ receptor expression on activated HSC in human and mouse fibrotic livers versus normal liver and other organs. (A) Representative microscopic pictures depicting upregulation of PDGFβR in cirrhotic human livers ($n = 3$), and CCl_4 fibrotic mouse livers ($n = 6$) as compared to normal livers. Scale bars, 100 μm. (B) Fluorescent pictures ($\times$100) of liver sections from normal ($n = 6$) and fibrotic mice ($n = 6$) stained for desmin (HSC marker, green), PDGFβR (red) and nuclei (blue) showing colocalization of HSC and PDGFβR. (C) Representative microscopic pictures showing weak expression of PDGFβR in other tissues (kidneys, heart, spleen and lungs). Scale bars, 100 μm.

seeded in 96-well plates were incubated either with medium alone or with different concentrations of PPB−HSA, IFNγ and IFNγ conjugate (5, 10, 20, and 50 ng/mL). After 24 h, the secreted nitrite was measured as absorbance at 550 nm using Griess reagent (1% sulfanilamide; 0.1% naphthylethylendiamine dihydrochloride; 3% H_3PO_4). All the experiments were repeated three times independently in triplicate.

Isolation of Primary Rat Hepatic Stellate Cells. Wistar rats were provided by Harlan (Netherlands) and maintained under *ad libitum* normal diet and 12 h light:12 h dark cycles. HSC were isolated by sequential *in situ* perfusion with collagenase and Pronase, as previously described.[23] Freshly harvested cells were cultured on plastic in DMEM supplemented with 15% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin and 1% L-glutamine. Culture medium of the cells was replaced after every 2 days, and cells were maintained in culture at 37 °C/5% CO_2. Highly activated cells at day 10, characterized for upregulated PFGFβR expression and characteristic phenotypic transformation, were used for the binding studies.

***In Vitro* Binding of the IFNγ Conjugate in Human HSC (LX2 Cells) and Primary Culture-Activated Rat HSC.** Cells (30,000 cells/well) were seeded onto 24-well Falcon culture plates (Becton Dickinson, Heidelberg, Germany) and grown overnight at 37 °C/5% CO_2. Then, cells were incubated with IFNγ, IFNγ conjugate or PPB−HSA−PEG (1 μg/mL). After 2 h of incubation, cells were fixed and immunostainings using anti-PPB and anti-IFNγ IgG were performed. The binding studies were performed at least three times independently.

***In Vitro* Effects of the IFNγ Conjugate in Mouse Fibroblasts and Human HSC.** Cells were plated in 24-well (30,000 cells/well) and 12-well (75,000 cells/well) culture plates, grown overnight, and were starved with 0.5% containing medium for 24 h. Starved cells were then incubated with medium alone, PPB−HSA−PEG, IFNγ or PPB−HSA−PEG−IFNγ (equivalent 1 μg/mL) with 5 ng/mL of human recombinant TGFβ1 (Roche) for 48 h. Subsequently, cells (24-well plates) were fixed and stained for collagen I and III (1:100, Southern Biotech, Birmingham, AL). In addition, cells (12-well plates) were lysed with lysis buffer constituted with β-mercaptoethanol (Stratagene, La Jolla, CA) to perform quantitative real time PCR analysis for procollagen 1α1, α-SMA and GAPDH (house-keeping gene) as described below.

For effects on proliferation in mouse 3T3 fibroblasts, cells were starved for 24 h and incubated with medium alone, PPB−HSA−PEG, IFNγ or IFNγ conjugate with 50 ng/mL of human recombinant PDGF (Peprotech) for 48 h. Subsequently, cells were incubated with 0.25 μCi/mL of thymidine ^{3}[H] for 6 h, after which cells were washed thoroughly, fixed with 5% TCA, lysed with 1 mL of 1 M NaOH, mixed with 4 mL of scintillation fluid and measured using scintillation counter. All the experiments were repeated three times independently.

CCl_4-Induced Acute Liver Injury or Fibrogenesis Model in Mice. Normal male balb/c mice (20−22 g) were obtained from Harlan (Zeist, The Netherlands). Animals were kept in cages and received *ad libitum* normal diet, under a 12 h light and 12 h dark cycle. All the experimental protocols for animal studies were approved by the Animal Ethics Committee of the University of Groningen (The Netherlands). Acute liver injury was induced in male C57BL/6 mice by a single intraperitoneal injection of carbon tetrachloride (CCl_4; 1 mL/kg prepared in olive oil) at day 1. At day 2 and day 3, mice ($n = 7$ each group) received intravenous injections of IFNγ, PPB−HSA−PEG, PPB−HSA−PEG−IFNγ (equivalent to 2.5 μg IFNγ/mouse/day) or PBS alone. At day 4, all mice were sacrificed; blood and different organs were collected for further analysis. For *in vivo* biodistribution of the conjugates, CCl_4-treated fibrotic mice ($n = 6$ each group) were intravenously injected with different constructs 10 min prior to sacrifice on day 4 after CCl_4 administration.

***In Vivo* IFNγ (pSTAT1α) Signaling Pathway.** IFNγ signaling pathway was analyzed in acute liver injury mouse model after

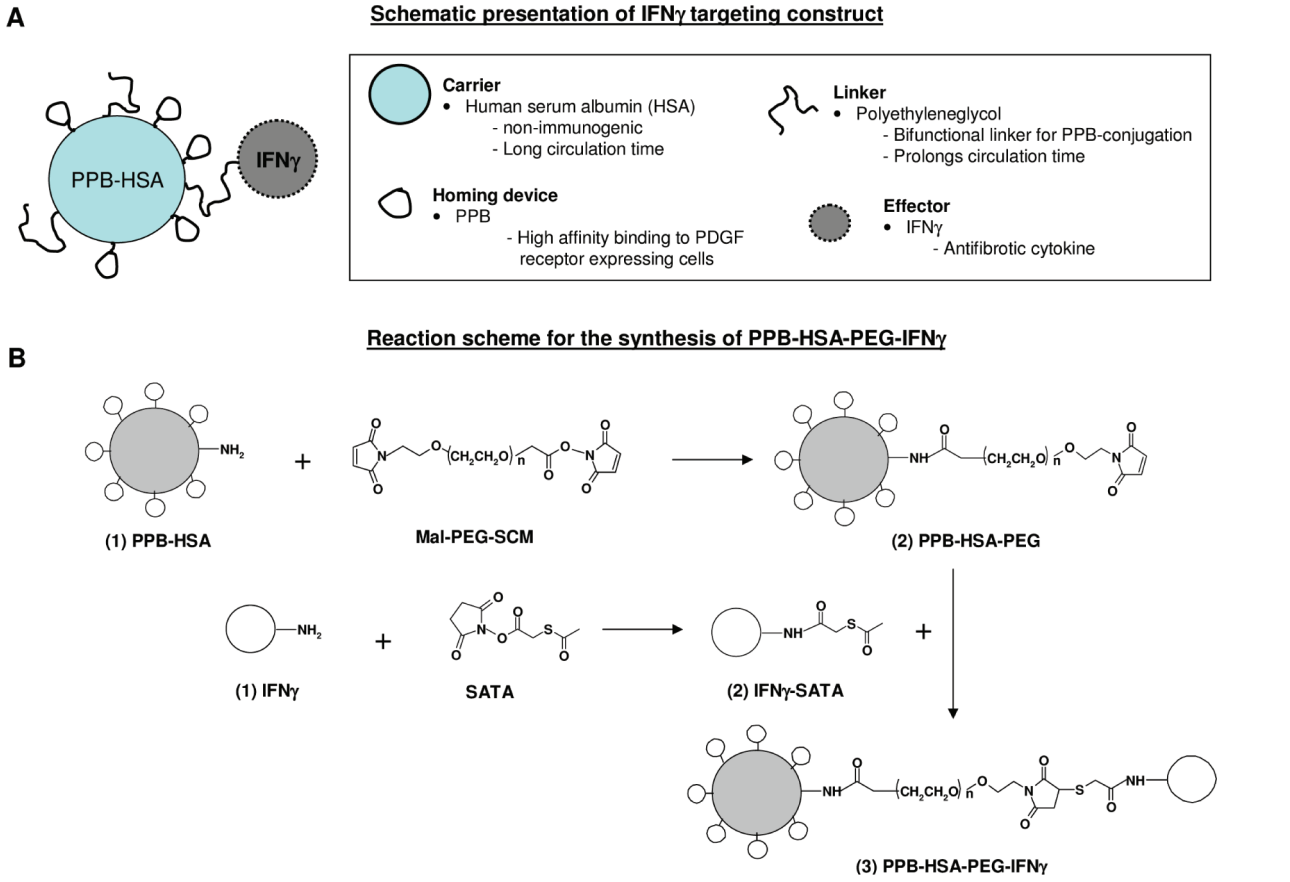

Figure 2. Structure and synthesis of HSC-targeted PPB−HSA−PEG−IFNγ conjugate. (A) Schematic representation of IFNγ targeting conjugate. (B) Reaction scheme for the synthesis of PPB−HSA−PEG−IFNγ conjugate. HSA, human serum albumin; PPB, PDGF receptor recognizing peptide (C*SRNLIDC*); MAL-PEG-SCM, maleimide-PEG-succinimidyl carboxy methyl ester; SATA, *N*-succinimidyl-*S*-acetylthioacetate.

24 h of administration with PBS, PPB−HSA−PEG, IFNγ or PPB−HSA−PEG−IFNγ. Liver tissues samples were homogenized in cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.1% Igepal in 0.5% sodium deoxycholate with 1 tablet of protease inhibitor cocktail and 1 tablet of phosphatase inhibitor (Roche Diagnostics, Mannheim, Germany) in 10 mL) on ice with a tissue homogenizer for 4 min, and the lysates were centrifuged at 12,000 rpm for 1 h at 4 °C. The supernatants were stored at −70 °C until use. 20 μg of protein from each sample was applied on the SDS−PAGE gel (10%) and transferred to PVDF membranes. The membranes were blocked for 1 h in TBST containing 5% nonfat dried milk and were incubated with anti-pSTAT1α antibody (cell signaling, Beverly, MA) or anti-β-actin antibody (Sigma-Aldrich) overnight at 4 °C. Then, the membranes were washed with TBST and incubated for 1 h at room temperature with a secondary horseradish peroxidase coupled IgG antibody (DAKO). The membranes were washed 3 times with TBST, and protein bands were visualized using ECL detection reagent.

Immunohistochemistry and Immunofluorescence. The cells were fixed with acetone:methanol (1:1), dried and stored until immunostaining. Liver and other tissues were cut with a Leica CM 3050 cryostat (Leica Microsystems, Nussloch, Germany) at 4 μm of thickness, dried and fixed with acetone. Cells or tissue sections were rehydrated with PBS and incubated with the primary antibody in appropriate dilution (Table 1 in the Supporting Information) for 1 h at room temperature. Cells or sections were incubated with horseradish peroxidase (HRP) conjugated secondary antibody (DAKO) for 30 min. Following incubation with HRP-conjugated tertiary antibody (DAKO) for 30 min, peroxidase activity was developed with 3-amino-9-ethylcarbazole (Sigma) for 20 min and nuclei were counterstained with hematoxylin (Fluka Chemie, Buchs, Switzerland). Cells or sections were mounted with Kaiser's gelatin (Darmstadt, Germany) and visualized using a light microscope (Olympus UK Ltd., Essex, U.K.). α-SMA staining was performed using M.O.M. kit (Vector Laboratories, Burlingame, CA) as per manufacturer's instructions. For quantitation, 27 microscopic fields at 200× magnification per liver section from each mouse were captured. The stained area in the digital photomicrographs was quantified using automated Cell-D imaging software (Olympus) according to standard procedures and was represented as % positive area per field.

Quantitative Real-Time PCR. Total RNA from cells and liver tissues was isolated using Absolutely RNA Microprep Kit (Stratagene) and RNeasy Mini Kit respectively (Qiagen, Hilden, Germany) according to manufacturer's instructions. The RNA concentration was quantitated by a UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (1.6 μg) was reverse transcribed in a volume of 50 μL using cDNA synthesis kit (Promega, Madison, WI). All primers were purchased from Sigma-Genosys (Haverhill, U.K.). The sequences of primers used in the study are enlisted in Table 2 in the Supporting Information. 20 ng of cDNA was used for

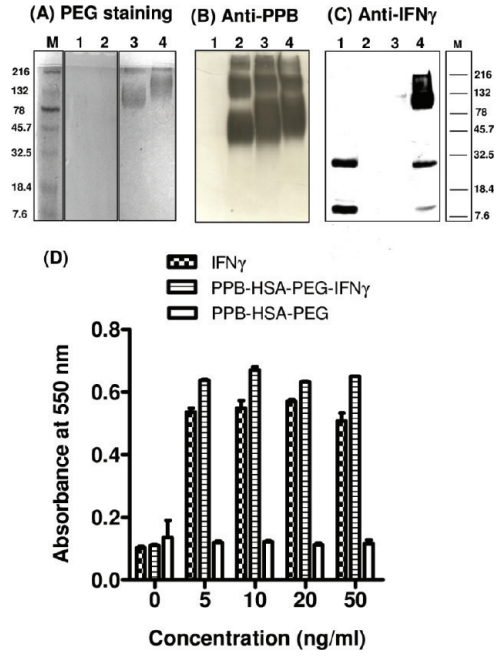

Figure 3. Characterization and *In vitro* assessment of biological activity of PPB−HSA−PEG−IFNγ conjugate. PEG staining (A) and Western blot analysis using an anti-PPB antibody (B) and an anti-IFNγ antibody (C) were used to characterize IFNγ conjugate. M denotes Kaleidoscope prestained molecular weight markers (BioRad, in kDa); lane 1, unmodified IFNγ; lane 2, PPB−HSA conjugate; lane 3, PPB−HSA−PEG conjugate; lane 4, PPB−HSA−PEG−IFNγ conjugate. The shift in the bands of PPB−HSA−PEG−IFNγ conjugate after conjugation and the superimposable pattern after incubation with the anti-IFNγ antibody and PEG staining indicate coupling of IFNγ with PPB−HSA−PEG. (D) Nitrogen oxide (NOx) release in mouse RAW macrophages after incubation with PPB−HSA−PEG, unmodified native IFNγ and IFNγ conjugate in the presence of 100 ng/mL of LPS showing PPB−HSA−PEG−IFNγ retains its biological activity relative to unmodified IFNγ. Data represent the mean ± SEM for three independent experiments performed in triplicate.

quantitative real time PCR analysis. The reactions were performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA) according to manufacturer's instructions and were analyzed by ABI7900HT sequence detection system (Applied Biosystems). Finally, the threshold cycles (Ct) were calculated and relative gene expression was normalized with GAPDH (for mouse) as house-keeping gene.

Statistical Analyses. Data are presented as mean ± standard error of the mean (SEM). Multiple comparisons between different groups were performed by one-way ANOVA with Bonferroni post-test.

RESULTS

Expression of PDGFβ-Receptor on Activated Hepatic Stellate Cells during Fibrosis. We examined the expression of our target, the PDGFβ receptor, in mouse and human fibrotic livers. We found that PDGFβR was highly upregulated in the fibrotic areas (Figure 1A) and specifically colocalized with desmin-positive activated HSC in liver (Figure 1B). Conversely, PDGFβR was almost absent in normal livers (Figure 1A) and expressed to a much lesser extent in other organs such as heart, kidneys, spleen and lungs in the smooth muscle cells around the blood vessels (Figure 1C). The overexpression of PDGFβR on activated HSC in fibrotic livers compared to other tissues and cells indicates that this receptor is an appropriate target for HSC-specific delivery.

Synthesis and Characterization of PPB−HSA−PEG−IFNγ Conjugate. The synthesis scheme of the PPB−HSA−PEG−IFNγ conjugate is illustrated in Figure 2. For the synthesis, PPB−HSA−PEG was generated by covalent modification of PPB−HSA with a 2 kDa PEG bifunctional linker and then IFNγ was covalently coupled to PPB−HSA−PEG using a cross-linker (SATA) (Figure 2B). The synthesis of the conjugate was characterized by PEG staining, Western blot analysis using anti-IFNγ antibody and anti-PPB antibody (Figure 3A−C). As shown in the figure, a clear shift in the molecular weight of PPB−HSA−PEG−IFNγ was visualized in PEG staining, anti-IFNγ and anti-PPB staining corresponding to coupling of 1−3 interferon gamma per PPB−HSA molecule. The band observed in the anti-PPB antibody stained blot (Figure 3A) was in correspondence with anti-IFNγ antibody stained blot (Figure 3B) and PEG staining (Figure 3C). The percentage of conjugation was calculated by analyzing band intensity using ImageJ analysis software indicating presence of 82.5% coupled IFNγ and 17.5% unconjugated free IFNγ (12.2% dimer + 5.3% monomer).

The biological activities of IFNγ conjugate versus IFNγ were studied *in vitro* in mouse RAW macrophages at different concentrations. Both IFNγ and PPB-albumin modified IFNγ conjugate induced a dose-dependent increase in nitric oxide (NO) release in the presence of LPS while no induction in NO was found with equivalent doses of PPB−HSA−PEG (with LPS) (Figure 3D). PPB-carrier modified IFNγ conjugate did not show significant differences in the bioactivity compared to free IFNγ, suggesting that IFNγ conjugate retained complete biological activity following coupling reactions.

Binding of PPB−HSA−PEG−IFNγ Conjugate in Human HSC and Primary Culture-Activated Rat HSC. The binding of the IFNγ conjugate was assessed in human HSC (LX2 cells) and primary culture-activated rat HSC using anti-PPB and anti-IFNγ antibodies. Similar to PPB−HSA−PEG, the targeted mouse-derived IFNγ conjugate showed specific binding to both human HSC (LX2 cells) and primary culture-activated rat HSC, while free mouse IFNγ did not show any binding to both cell types due to species restriction (Figure 4A,B).

***In Vitro* Antifibrotic Effects of PPB−HSA−PEG−IFNγ Conjugate in Mouse 3T3 Fibroblasts and Human LX2 Hepatic Stellate Cells.** Following binding studies, we investigated antifibrotic effects of PPB-carrier modified IFNγ conjugate in mouse 3T3 and human LX2 cells. In mouse fibroblasts, both unmodified mouse IFNγ and the targeted IFNγ conjugate induced significant reduction ($p < 0.05$) in TGFβ-stimulated collagen expression as shown in Figure 5A−C. Furthermore, both mouse IFNγ and IFNγ conjugate drastically inhibited PDGF-induced cell proliferation ($p < 0.01$) of mouse fibroblasts as assessed by thymidine incorporation assays (Figure 5D). Interestingly, in human HSC (LX2 cells), TGFβ-induced collagen expression, tissue inhibitor of metalloproteinases-1 (TIMP-1) and HSC activation (α-SMA expression) were drastically inhibited following incubation with PPB−HSA-IFNγ conjugate (Figure 6A−C), while free mouse IFNγ did not induce any beneficial effects due to species restriction. These results clearly demonstrate that mouse IFNγ, which is inactive in human cells, can become biologically active in other species when directed

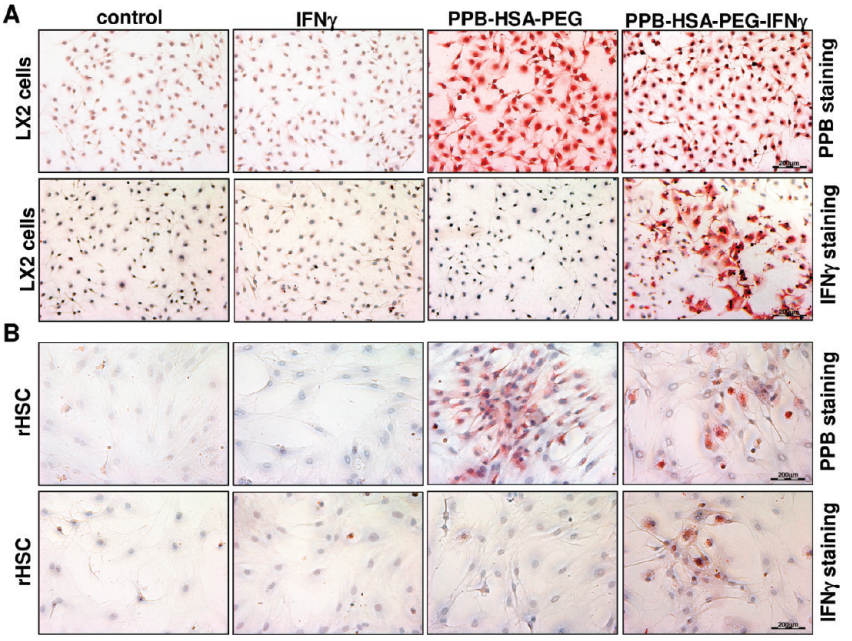

Figure 4. Binding study of IFNγ conjugate in human HSC (LX2 cells) and primary culture-activated rat HSC. (A) Microscopic representation showing binding of PPB−HSA−PEG and PPB−HSA−PEG−IFNγ to human HSC (LX2 cells) as shown by staining with anti-PPB antibody. PPB−HSA−PEG−IFNγ binding to human HSC was further confirmed by staining with anti-IFNγ antibody. Scale bars, 200 μm. (B) Microscopic photographs (scale bars, 200 μm) showing binding of PPB−HSA−PEG and targeted mouse-derived IFNγ conjugate to primary rat HSC (day 10) as shown by anti-PPB staining. Staining with anti-IFNγ antibody further confirms the binding specificity of PPB−HSA−PEG−IFNγ. Mouse IFNγ did not show any binding to human LX2 cells and primary rat HSC due to species differences.

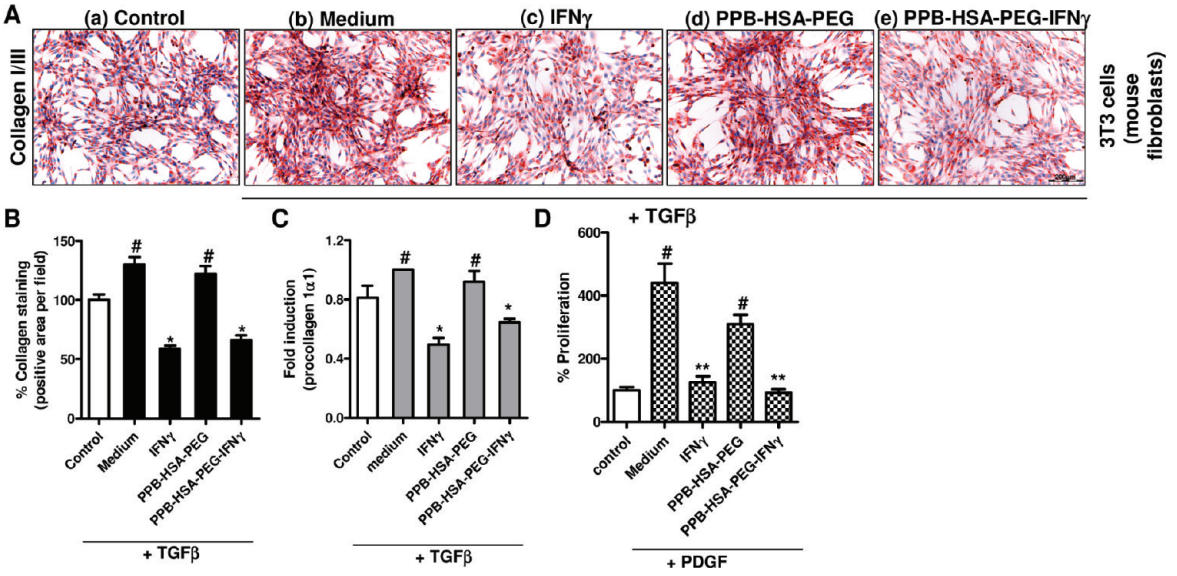

Figure 5. Antifibrotic effects of targeted IFNγ conjugate on mouse 3T3 fibroblasts. (A) Representative microscopic photographs (n = 3) showing collagen staining performed on 3T3 cells, incubated with (a) control (without TGFβ); (b) 5 ng/mL TGFβ; (c) TGFβ (5 ng/mL) + IFNγ (1 μg/mL); (d) TGFβ (5 ng/mL) + PPB−HSA−PEG (1 μg/mL); (e) TGFβ (5 ng/mL) + PPB−HSA−PEG−IFNγ (1 μg/mL). Scale bars, 200 μm. (B) The collagen staining was quantified and expressed in the graph. Data represent the mean ± SEM for at least three independent experiments. (C) Quantitative PCR analysis (n = 3) of procollagen 1α1 (normalized with GAPDH) in 3T3 fibroblasts incubated with medium (alone, without TGFβ), TGFβ (5 ng/mL) or TGFβ (5 ng/mL) in combination with PPB−HSA−PEG, IFNγ or IFNγ-conjugate (1 μg/mL). The groups were normalized to TGFβ-treated cells. (D) Bars represent the cell proliferation (thymidine incorporation) after incubation with PDGFBB (50 ng/mL) in combination with medium (alone, without PDGF), PDGF (50 ng/mL) or PDGF (50 ng/mL) in combination with PPB−HSA−PEG, IFNγ or IFNγ-conjugate (1 μg/mL) in 3T3 fibroblasts. The groups were normalized to control cells (untreated). $^{\#}p < 0.05$ depicts significance versus control; $^{*}p < 0.05$ and $^{**}p < 0.01$ denotes significance versus TGFβ-treated or PDGF-treated cells (n = 3).

through PDGFβR. In both cells, PPB−HSA−PEG did not induce any reduction in fibrotic parameters depicting the effects are IFNγ mediated and not exerted due to the blocking of the PDGFβ receptor.

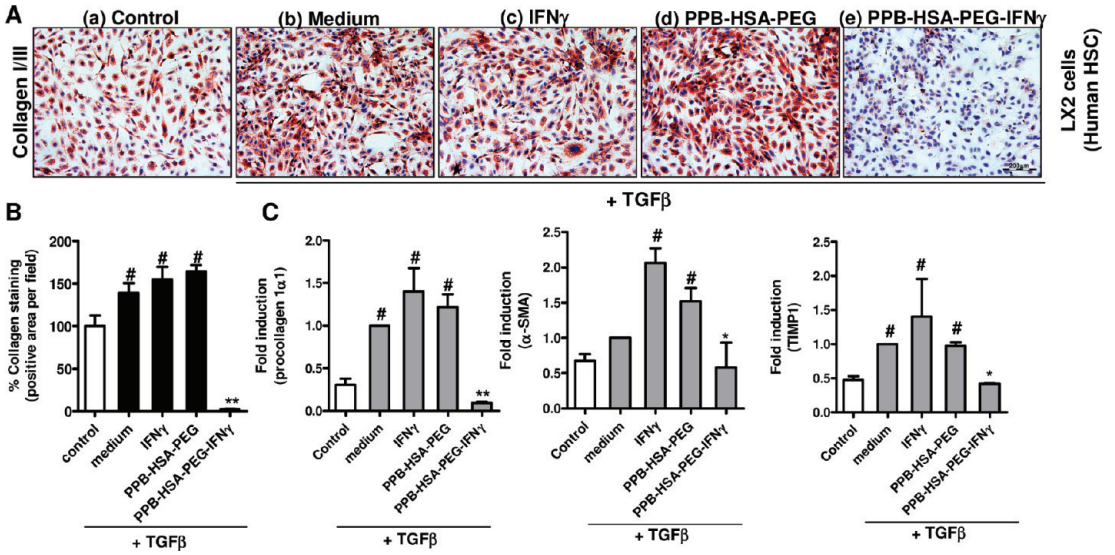

Figure 6. Antifibrotic effects of targeted IFNγ conjugate on Human HSC (LX2 cells). (A) Representative microscopic photographs (n = 3) showing collagen staining performed in LX2 cells, incubated with (a) control (without TGFβ); (b) 5 ng/mL TGFβ; (c) TGFβ (5 ng/mL) + IFNγ (1 μg/mL); (d) TGFβ (5 ng/mL) + PPB−HSA−PEG (1 μg/mL); (e) TGFβ (5 ng/mL) + PPB−HSA−PEG−IFNγ (1 μg/mL). (B) The staining from three different experiments was quantified as depicted in the graph. Data represent the mean ± SEM for at least three independent experiments. Scale bars, 200 μm. (C) Bars represent the quantitative PCR analysis (n = 3) of procollagen 1α1, α-SMA and TIMP1 (normalized with GAPDH) in human LX2 cells incubated with medium alone, TGFβ (5 ng/mL) or TGFβ (5 ng/mL) in combination with PPB−HSA−PEG, IFNγ or IFNγ-conjugate (1 μg/mL). The groups were normalized to TGFβ-treated cells. $^{\#}p < 0.05$ depicts significance versus control or untreated cells; $^{*}p < 0.05$ and $^{**}p < 0.01$ denotes significance versus TGF$\boldsymbol{\beta}$-treated cells (n = 3).

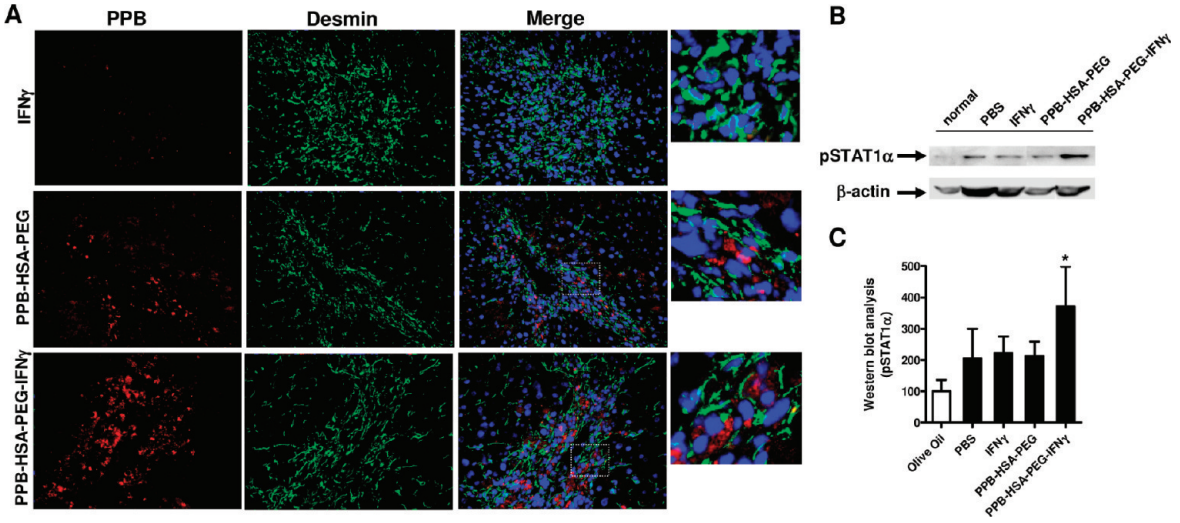

Figure 7. *In vivo* localization of HSC-specific targeted IFNγ conjugate and activation of IFNγ signaling pathway in the acute CCl_4-induced liver injury model in mice. (A) Representative immunofluorescent photographs (n = 6 per group) depicting HSC-specific accumulation of PPB−HSA−PEG and PPB−HSA−PEG−IFNγ in the damaged areas in livers. PPB was visualized with a specific antibody (red); HSC with antidesmin IgG (green) and nuclei by counterstaining with DAPI (blue). 400× magnification and further enlargement are shown in the inserts. (B) The representative bands and (C) quantitative analysis (normalized with β-actin) of the Western blot (n = 7) showing increased activation of pSTAT1α after 24 h of PPB−HSA−PEG−IFNγ administration. $^{*}p < 0.05$ depicts significance versus other treatment groups.

***In Vivo* Localization and Liver Uptake of PPB-Albumin Modified IFNγ Conjugate in an Acute CCl_4-Induced Liver Injury Model in Mice.** The tissue biodistribution of human serum albumin (HSA)-modified PPB has been investigated by our group using radiolabeled and imaging studies showing high distribution of PPB−HSA to fibrotic livers and to PDGFβR-expressing activated hepatic stellate cells.[20] Also in the previous studies,[20,21] we have shown PPB−HSA does not accumulate in other organs which is most likely due to the low density of PDGFβR on normal cells (refer to Figure 1). We, in this study, assessed the accumulation of PPB−HSA−PEG−IFNγ in HSC in liver. *In vivo*, we found that PPB−HSA−PEG and PPB−HSA−PEG−IFNγ conjugate were largely colocalized with desmin-positive hepatic stellate cells in the damaged areas in CCl_4-induced liver injury model, while they were virtually absent in nondamaged areas (Figure 7A). The immunofluorescence staining could not be performed for exogenously

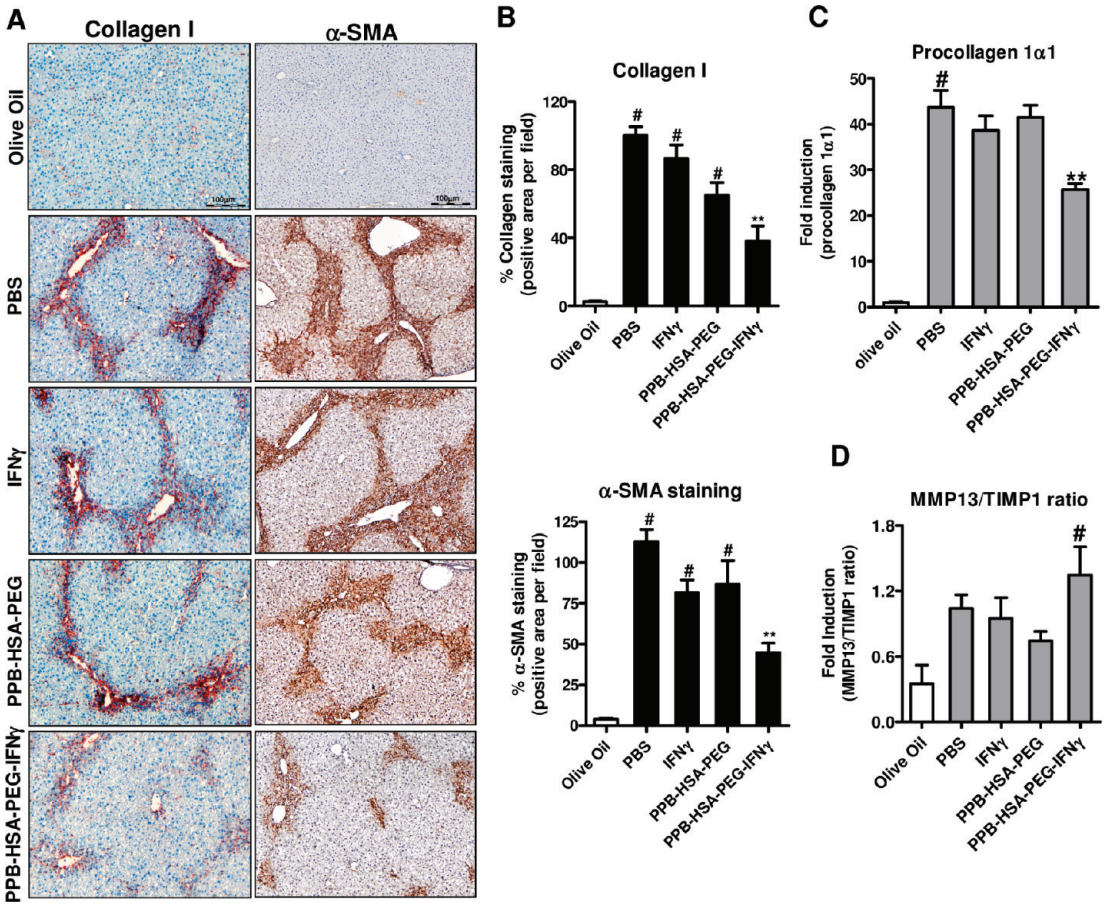

Figure 8. Effects of PPB−HSA−PEG−IFNγ on fibrosis-related parameters in the CCl_4-induced acute liver injury or fibrogenesis model in mice. Representative pictures (A) and quantitative analysis (B) of collagen I and α-SMA-stained liver sections from CCl_4-treated mice (acute model) that received PBS, IFNγ, PPB−HSA−PEG or IFNγ construct PPB−HSA−PEG−IFNγ. Scale bars, 100 μm. The groups were normalized to vehicle group (PBS treated-CCl_4 mice). Quantitative real-time PCR analysis of procollagen 1α1 (C) and MMP13/TIMP-1 (D) in olive oil treated mice (normal) and CCl_4 animals treated with PBS, IFNγ, PPB−HSA−PEG, PPB−HSA−PEG−IFNγ (n = 7 each group). Expression levels were normalized with GAPDH. PPB−HSA−PEG−IFNγ induced a dramatic reduction in collagen I expression ($^*p < 0.01$) and α-SMA expression ($^{**}p < 0.01$). Bars represent mean ± SEM of 7 mice per group. $^{\#}p < 0.05$ depicts significance versus control (olive oil treated) mice; $^{**}p < 0.01$ denotes significance versus PBS-treated CCl_4 mice.

administered IFNγ due to the interference by the endogenous IFNγ.

We also assessed the intracellular activation of pSTAT1α signaling pathway after 24 h administration of IFNγ, PPB−HSA−PEG and PPB−HSA−PEG−IFNγ conjugate in CCl_4-induced liver injury in mice. IFNγ activates and phosphorylates signal transducers and activators of transcription (STAT1) that bind to unique gamma-activated sequence (GAS) regulating IFNγ-responsive genes.[9] We found that the IFNγ conjugate induced significantly increased pSTAT1α activation in comparison to PBS, IFNγ and PPB−HSA−PEG indicating enhanced liver uptake of the conjugate (Figure 7B,C).

Effects of HSC-Targeted PPB−HSA−PEG−IFNγ Conjugate in Acute CCl_4-Induced Liver Injury or Fibrogenesis Model in Mice. A single CCl_4 injection induced acute liver injury in mice, characterized by increased deposition of collagen and increased expression of the HSC activation marker α-SMA compared to control mice (treated with olive oil) (Figure 8). IFNγ and HSC-targeted PPB−HSA−PEG−IFNγ construct were examined for their antifibrotic effects in a CCl_4-induced acute liver fibrogenesis model in mice. After two intravenous injections, only PPB−HSA−PEG−IFNγ construct significantly attenuated collagen I expression (approximately 60% reduction, $p < 0.01$) accompanied by substantial decline (approximately 60%, $p < 0.01$) in α-SMA positive hepatic stellate cells (Figure 8A,B). The reduction in collagen I expression as observed in staining was paralleled by significant reduction (approximately 40% reduction, $p < 0.01$) in the respective transcript levels (Figure 8C). Apart from collagen expression and deposition, the balance between collagen degrading matrix metalloproteinases-13 (MMP-13) and their major endogenous inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP-1), is an important determinant of progression or reversal of fibrosis. PPB−HSA−PEG−IFNγ induced a significant increase in MMP-13/TIMP-1 transcript ratio ($p < 0.05$), suggesting activation of fibrolysis and induction of reversal of fibrogenesis (Figure 8D).

To investigate whether targeting of IFNγ could ameliorate IFNγ-mediated inflammatory response, we studied the interferon gamma mediated effect on macrophage infiltration. Two intravenous injections of IFNγ treatment induced significant increase in macrophage infiltration ($p < 0.05$) as assessed by quantitative PCR analysis for macrophage marker, CD68 and

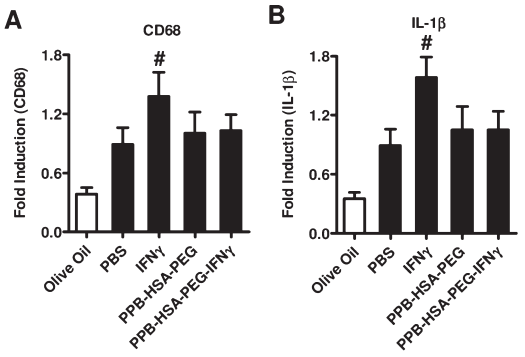

Figure 9. Effect of targeted IFNγ construct on CD68 (macrophage marker) and IL-1β (inflammatory cytokine) mRNA expression in the acute CCl_4-induced liver injury model in mice. Quantitative real-time PCR analysis of, CD68 (A), and IL-1β (B) in olive oil treated mice (normal) and CCl_4 animals treated with PBS, IFNγ, PPB−HSA−PEG, PPB−HSA−PEG−IFNγ (n = 7 each group). Expression levels were normalized with GAPDH. IFNγ induced significant increase in macrophage infiltration (CD68) and inflammatory cytokine (IL-1β) in livers while other treatments did not induce these inflammatory responses. Bars represent mean ± SEM of 7 mice per group. Differences versus control (olive oil treated) mice are presented as $^{\#}P < 0.05$.

increased IL-1β mRNA expression ($p < 0.05$) in livers (Figure 9A,B). Other treatments, importantly HSC-targeted PPB−HSA−PEG−IFNγ, did not induce any change in these parameters, demonstrating absence of off-target effects.

DISCUSSION

The insufficient target specificity or off-target effects of most approaches to treat liver fibrosis have limited their appropriateness for clinical use. In the present study, we have developed a novel targeted conjugate against key disease inducing cells (hepatic stellate cells) in liver fibrosis for the selective delivery of IFNγ. IFNγ is a potent antifibrotic cytokine, which we coupled to PDGFβR-recognizing cyclic peptide-based drug carrier (PPB−HSA) for targeting to activated hepatic stellate cells (HSC), as these cells strongly express PDGFβR during liver fibrosis. The IFNγ conjugate, after chemical conjugation reactions, retained its biological activity and showed specific binding to human HSC and primary culture-activated rat HSC while free mouse IFNγ does not bind to cells due to species specificity. Furthermore, the targeted conjugate showed a strong inhibition of fibrotic parameters *in vitro* in TGFβ-stimulated mouse 3T3 fibroblasts, human HSC and *in vivo* in acute CCl_4-induced liver injury model in mice.

Treatment of liver fibrosis represents a major clinical challenge. Activated hepatic stellate cells and portal fibroblasts (collectively named as HSC) are the key effector cells of liver fibrogenesis.[24] Therefore, therapeutic approaches to silence these activated HSC would be appropriate to inhibit or reverse liver fibrosis. An emerging therapeutic concept is the direct delivery of agents to a specific target cell using cell-specific receptors to increase local drug concentrations while preventing deleterious effects on collateral cells (e.g. hepatocytes) or other organs. Many studies have shown that activated HSC abundantly express PDGFβR,[18,19] which is also confirmed in the present study in human cirrhotic livers and CCl_4-induced fibrotic livers in mice. PDGFβR is normally present in the cells from mesenchymal origin such as vascular smooth muscle cells, fibroblasts, bone marrow and monocytes. However, its expression gets highly upregulated on the key pathogenic cells during various disorders such as atherosclerosis, fibrosis and cancer.[21,25] During liver fibrosis, very high expression of PDGFβR in fibrotic livers compared to other organs or cells as demonstrated here reflects the increased target specificity and reduced off-target effects. We used cyclic peptide (CSRNLIDC) against PDGFβR (PPB) that have shown to be highly specific to the target receptor[20] and therefore employed here to target IFNγ. Human serum albumin (HSA) has been used as a carrier protein to incorporate potent drugs and to introduce a number of peptides on its surface for high receptor binding affinity.[20,21] In addition, albumin has been used as a drug carrier to enhance drug stability and half-life and has been extensively used for the treatment of tumor and other diseases.[26] Recently albinterferon, a fusion protein of albumin and interferon alpha-2b, has been used in phase III clinical trials for the treatment of hepatitis C and showed favorable outcome alternative to pegylated interferon.[27,28] Though attractive, these approaches lack target specificity and therefore might induce adverse effects.

PEGylated formulations of IFNα (PEGIntron and PEGASYS) containing heavy PEG chains (linear 12 kDa and branched 40 kDa PEG respectively) are clinically approved drugs for the treatment of hepatitis C and are known to induce adverse effects due to longer circulation and slow metabolism depending on the PEG size. Therefore, we have used small PEG linker (2 kDa) to couple IFNγ to PPB−HSA in order to provide hydrophilicity, stability, conformational flexibility to the synthesized construct with improved (or equivalent) pharmacokinetics to increase the therapeutic effects while PPB provides target specificity favorable for the prolonged administration to the patients with advanced liver fibrosis. Since cytokines are highly prone to inactivation or loss of biological activity as documented earlier,[29,30] our modified IFNγ with targeted carrier (PPB−HSA) retained its biological activity due to minimal modification and mild chemical conditions, as assessed in murine macrophages using nitric oxide release assay. The target specificity of the conjugate was confirmed by binding to PDGFβR-expressing human HSC (LX2 cells) and rat HSC where mouse-derived free IFNγ did not show binding, in contrast, PDGFβR-targeted IFNγ showed specific binding to both cell types. Moreover, mouse-derived IFNγ was found to be highly effective in mouse 3T3 fibroblasts where it induced reduction in fibrotic parameters but it did not show any effect in human LX2 cells due to species restriction. In contrast, our PPB containing IFNγ construct induced potent antifibrotic effects in both mouse cells and human cells, further demonstrating that the biological activity of PPB−HSA−PEG−IFNγ is mediated through PDGFβ receptor. Previous studies from our group have shown that, using scrambled peptide (CIDNLSRC)[31] or HSA without targeting peptide,[21] the drugs did not induce any beneficial effects explicitly showing the benefit of PPB-mediated targeting. Also in the latter study, the binding of PPB−HSA specifically to PDGFβ receptor positive cells was shown with radioactive studies, while PDGF-receptor negative HepG2 cells did not show binding.

The antifibrotic effects observed with targeted IFNγ are most likely due to the internalization of the construct, which then releases IFNγ or its metabolite intracellularly in the cytoplasm. IFNγ has an extracellular receptor binding sequence and an intracellular receptor binding or nuclear signaling sequence (NLS) that mediates its biological effects.[32] The internally released IFNγ or its metabolite subsequently binds to its intracellular domain of IFNγR, which in turn activates the

JAK-STAT pathway and modulates IFNγ-responsive genes, but this hypothesis needs to be further explored. In this study, we have studied the activation of JAK-STAT pathway *in vivo* and found that the IFNγ construct activated IFNγ-mediated STAT1α signaling to a greater extent than unmodified IFNγ showing increased accumulation in fibrotic livers. Furthermore, the construct (PPB staining) was colocalized with desmin (HSC marker) positive cells depicting specificity of the construct toward PDGFβR-expressing HSC. Following *in vivo* distribution, our HSC-specific construct ameliorated liver fibrogenesis by inducing strong inhibition of collagen deposition and HSC activation. Importantly, the process to reverse the fibrosis that is the increased MMP13/TIMP1 ratio was higher with the targeted IFNγ construct while untargeted IFNγ remained ineffective.

In the literature, a high dose (5 μg/dose/mouse) of IFNγ has been used to induce antifibrotic effects,[33] which might exert more adverse effects. But in this study, we have used a low dose (2.5 μg/dose/mouse) with increased therapeutic efficacy to demonstrate the superiority of targeting. Since IFNγ is an immunomodulatory Th1 cytokine, known to influence macrophages, their main target cells and in-effect therefore native IFNγ[34] induced significant macrophage infiltration and IL-1β (inflammatory cytokine) expression in fibrotic livers following two intravenous administrations. At the low doses, unmodified IFNγ induced its inflammatory effects on macrophages, that are known to express high levels of IFNγ receptors while almost no effect on HSC (with low IFNγR expression). In contrast, our HSC-targeted construct exerted an increased antifibrotic effect due to very high and specific expression of PDGFβR on activated HSC while it did not induce inflammation due to lack of PDGFβR on macrophages or other immune cells.

These data clearly demonstrate the potential benefits of PDGFβR-mediated delivery of IFNγ to the key disease inducing cells involved in the progression of liver fibrosis. This study may therefore provide a novel targeted cytokine-based therapy with increased therapeutic efficacy while off-target effects on healthy tissues can be avoided. Thus, this peptide-albumin carrier based HSC-targeted approach should be explored further for the delivery of therapeutic drugs or cytokines for the treatment of liver fibrosis and other chronic diseases.

■ ASSOCIATED CONTENT

Supporting Information. List of the antibodies and primers used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

α-SMA, alpha smooth muscle actin; CCl_4, carbon tetrachloride; HSA, human serum albumin; HSC, hepatic stellate cells; IFNγ, interferon gamma; MMP-13, matrix metelloproteinases 13; TIMP-1, tissue inhibitor of matrix metalloproteinases; PDGFβR, platelet derived growth factor beta receptor; PEG, polyethylene glycol; PPB, PDGFβ receptor recognizing peptides (C*SRNLIDC*); TGFβ, transforming growth factor beta

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