The design of a liver-selective form of interleukin-10

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Chapter - 7

Modification of IL-10 with M6P yields a liver-selective cytokine with antifibrotic activities in rats

submitted

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Abstract

IL-10 is a promising new drug to treat fibrotic diseases, because it exerts anti-inflammatory and antifibrotic activities. However, the use of IL-10 in antifibrotic therapies is confronted with the undesired systemic immunosuppressive effects, as noted in a flare-up of viral levels in patients with hepatitis C virus. IL-10 also has a short serum half-life with predominant elimination by the kidneys which limits the exposure time of this cytokine to liver cells. Therefore, the aim of this study was to develop a liver-selective form of IL-10 with significant distribution to hepatic stellate cells (HSC). IL-10 was modified with mannose 6-phosphate (M6P) to obtain a compound with affinity for M6P/IGF-II receptor which is highly present on activated HSC. The effects of M6PIL-10 and IL-10 were studied in bile duct ligated rats. Treatment started four days after ligation for 3 consecutive days with M6PIL-10, IL-10, and vehicle (PBS). With gammacamera imaging techniques, we showed a high distribution of radiolabeled-M6PIL-10 to the liver in contrast to radiolabeled-IL-10 which mostly accumulated in the kidney. As compared to the control group, rats treated with M6PIL-10 or IL-10 showed a clear reduction in several parameters associated with inflammatory and fibrotic processes. The average fibrosis-score went from 3.4 (BDL control) to 2.4 by IL-10 and to 2.2 by M6PIL-10. In conclusion, modified IL-10 efficiently reaches the target organ, the liver, with significant antifibrotic effects *in vivo*. This successful strategy to target a potent therapeutic cytokine, IL-10, to the liver offers good perspective opportunities to use M6PIL-10 for the treatment of liver fibrosis.

Introduction

Chronic liver disease is responsible for over 1.4 million deaths annually and in the western world is among the top ten causes of death. Liver fibrosis, which is an outcome of persistent hepatic inflammation, if left unmanaged has serious long-term consequences for patient morbidity and mortality. Various factors such as viruses, toxins, alcohol abuse, autoimmune diseases, chronic biliary stasis, metabolic disorders can elicit the onset of this disease.[1] Persistence of profibrogenic factors will eventually lead to cirrhosis, the end-stage of liver fibrosis. Liver transplantation is currently the best option for patients with cirrhosis.[1-3] However, this treatment carries significant challenges. Many new drugs are being explored nowadays. So far, however, no drugs are approved as antifibrotic agents in humans for safety reasons.[4] Such therapies need to be efficient, safe and without long-term adverse effects within the liver or in other organs.

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine released during diseases and is able to attenuate the severity of inflammatory diseases by downregulating the release of many proinflammatory mediators.[5-9] It has attracted much attention since several studies also demonstrated direct antifibrotic properties in HSCs.[10-15] These potential effects have encouraged clinical trials with recombinant IL-10 to treat various chronic diseases including liver fibrosis.[16-20] Several studies showing beneficial effects of IL-10 therapies during various diseases in animal models as well as in clinical trials have been reported.[5-9,16-20] Other studies, however, demonstrated only a limited success of therapy with IL-10 or even showed disappointing results.[21-23] A possible reason for the variable efficacy might be due to the low dose of IL-10 which reaches the target sites. Recombinant IL-10 is a low molecular weight and nonglycosylated protein which is rapidly cleared from the circulation through glomerular filtration after iv administration. The plasma half-life is only 2 minutes. The ultimate local IL-10 concentrations at the site of action, therefore, could be too low to result in clear effects. Increasing the dose of systemically administered IL-10 is limited due to its inherent side effects.[16,17] In accordance with this, a recent study reported beneficial effects of long-term IL-10 therapy to treat HCV-associated liver fibrosis in patients but unfortunately this was accompanied by an immunosuppressive

action. This undesired immunosupressive effect during IL-10 therapy is a consequence of the pleiotropic property of this cytokine.[19] A liver-selective delivery of this cytokine is, therefore, absolutely necessary to circumvent such clinical problems and create biological effects within relevant cells.

Previous studies from our laboratory reported HSC-selective uptake of human serum albumin modified with mannose 6-phosphate.[24] This M6P-modified HSA may serve as a HSC-selective carrier for delivery of antifibrotic drugs via M6P/IGF-II receptor. Upregulation of this receptor during liver injury on HSC, the most relevant cell during liver fibrosis, offers an excellent target for receptor-mediated antifibrotic drug delivery. In the present study, we therefore modified IL-10 with M6P to selectively deliver this cytokine to its site of action i.e HSC. To demonstrate the utility of M6P ligand for hepatic homing, we performed biodistribution studies of radiolabeled-M6P-modified IL-10 using a gammacamera technique. Furthermore, we studied the pharmacological activities of this conjugate in rats at an early stage of liver fibrosis and compared this to the effects of unmodified IL-10.

Materials and methods

Animals

Specific pathogen-free male Wistar rats (200 – 250 g), purchased from Harlan (outbred strain, Zeist, The Netherlands), were used in this study. The rats received a standard diet and were housed under standard laboratory conditions with free access to food and water. Each study was performed following the guidelines of the Local Committee for Care and Use of Laboratory Animals.

Conjugation of mannose 6-phosphate (M6P) to IL-10

M6P-modified IL-10 was prepared by reacting activated M6P[24] and IL-10 at a molar ratio of 400:1. The reaction was carried out in 0.1 M of carbonate buffer pH 9.5, at room temperature for 1 h and then continued for 24 h at 4°C. The product was purified from free M6P with a nanosep centrifugal device (10 kDa, Omega, Pall corp., Michigan, USA). The protein content of the product was determined with NanoDrop® (ND-1000 UV-Vis spectrophotometer, NanoDrop Technology, USA) with a mini Bradford method according to the manufacturer's

instructions. Purified product was characterized with Western blotting using a rabbit polyclonal anti-IL-10 antibody (Santa Cruz Biotechnology, CA, USA). The immuno-reaction was visualized with DAB (3.3'-diaminobenzidine, Sigma, St.Louis, USA).

Imaging study for determination of M6PIL-10 distribution with a gammacamera

IL-10 and M6PIL-10 were labeled with Iodine-123 (^{123}I, GE Health) using standard methods as previously described.[25] Prior to each experiment, free ^{123}I was removed by gel filtration using a PD-10 column (Amersham Pharmacia Biotech), by eluting with phosphate buffer (0.2 M, pH 7.4) to obtain a dosing preparation with less than 5% free ^{123}I.

Rats with liver fibrosis (one week after the ligation of bile duct, N = 3) were used to study the distribution of IL-10 or M6PIL-10. Anaesthetized rats placed on a low-energy all-purpose collimator of a gammacamera were iv injected with a tracer amount of radiolabeled-proteins. The radioactivity was dynamically recorded from 0 to 30 minutes with a frame rate of one total body scan per minute.[26]

A study on the pharmacological effects of IL-10 and M6PIL-10 in a rat model of liver fibrosis

To induce liver fibrosis, rats were subjected to ligation of the common bile duct (BDL). In the first week after this bile duct ligation procedure, rats were used to study the pharmacological effects of IL-10 and M6PIL-10.

Experimental design

Bile duct ligated rats were randomly divided into three groups: rats with liver fibrosis receiving only vehicle (PBS, N = 5), rats with liver fibrosis receiving IL-10 (N = 5) and rats with liver fibrosis receiving M6PIL-10 (N = 5). Untreated normal rats (N = 3) served as reference group. Treatments with a bolus iv dose (8 µg/kg/day) of either IL-10 or M6PIL-10 were started at day 4 after BDL, for 3 consecutive days. At day 7 after BDL, animals were sacrificed and samples of blood (heparinized) and various organs were taken. Liver function, immunohistopathological parameters, and mRNA levels of various parameters

were analyzed to study the effects of IL-10 and M6PIL-10 as compared this to vehicle-treated BDL rats.

Assessment of liver function

Indicators of liver damage such as alkaline phosphatase (AP), alanin transaminase (ALT), aspartate transaminase (AST), total bilirubin index (TBI), direct bilirubin index (DBI), and γ-glutamic transpeptidase (GGT) were measured in plasma samples. These enzymes were measured by routine clinical chemistry according to standard procedures.

Immunohistochemical analysis of the livers

Liver samples were snap frozen in isopentane (-80°C) and immunostaining was performed on cryostat sections (5 μm thick). Various parameters i.e. expression of α-SMA, Desmin/GFAP, type III collagen, iNOS, and IL-10 receptor were stained using indirect immunoperoxidase methods according to standard procedures. Briefly, sections were incubated for 1 h with anti-α-SMA (Sigma), and anti-desmin (Cappel, Ohio, USA), anti-GFAP (NewMarkers, CA, USA) IgGs, rabbit anti-iNOS (gift of Dr. H. Moshage, University of Groningen, Netherlands) and anti-IL-10 receptor (Santa Cruz Biotech., USA) IgGs, goat anti-collagen III (SouternBiotech, USA) IgGs, respectively. Prior to 30 minutes incubation with peroxidase-conjugated secondary antibodies (DAKO, Golstrup, Denmark), sections were incubated with 0.1% H_2O_2 in PBS for 20 minutes to block endogenous peroxidase activity. The secondary antibody was visualized with 3-amino-9-ethylcarbazole (AEC, Sigma).

To detect ROS-producing cells in the liver, liver sections were stained with DAB (3,3-diamino benzidine, Sigma, USA) according to Poelstra *et al.*[27] Briefly, sections were washed in 0.1 N Tris-HCl buffer pH 7.6 and then incubated for 30 min at 60°C in 0.1 N Tris-HCl containing 0.5 mg/mL DAB. The number of periductular DAB positive cells was counted in eight different portal areas per liver section.

To assess total collagen deposition, cryosections were also stained with Sirius red according to standard methods.[28]

Quantitative evaluation

To examine effects of treatment with IL-10 or M6PIL-10 on BDL-induced liver fibrosis, stainings for α-SMA, desmin/GFAP and Sirius red on liver sections were analyzed quantitatively with image analyzing software ImageJ (National Institutes of Health, USA). With this software, the positive area of each staining was measured on digital photographs. The positively stained area per picture was related to the total area of the picture and at least 6 pictures (magnification 40x) per liver section were analyzed.

Staging of liver fibrosis

To assess the effect of IL-10 and M6PIL-10 treatments on the fibrotic process, we graded the collagen III staining with a semiquantitative scoring system according to Histologic Activity Index-Knodell.[29]

Analysis of gene expression

The effects of IL-10 and M6PIL-10 treatments on the mRNA levels for TNF-α, ICAM-1, MMP-13, procollagen α1(1) and α1(2), TGFβ-1, α-SMA, TIMP-1, and TIMP-2 were also examined. The mRNA levels for these parameters were normalized to the mRNA levels for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold induction was calculated based on the mRNA levels for GAPDH mRNA in normal livers.

RNA isolation

Total RNA was extracted from tissue samples of the livers, snap frozen in liquid N_2), using a mini column system according to the manufacturer's instruction (Aurum Total RNA kits, Biorad, USA). RNA concentration in all samples was determined with NanoDrop® (ND-1000 UV-Vis spectrophotometer, NanoDrop Technology, USA). Integrity of mRNAs was checked by electrophoresis on a 2% agarose gel and the absence of DNA from the samples was verified by performing a PCR on the mRNA samples using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, table I) while omitting the reverse-transcriptase step.

Table I. Rat oligonucleotide primers used for the analysis of gene expression by quantitative real time-PCR and conventional RT-PCR

Genes	Nucleotide sequences
TNF-α	Upstream: 5'-ATGTGGAACTGGCAGAGGAG-3'
	Downstream: 5'-GGCCATGGAACTGATGAGAG-3'
ICAM-1	Upstream: 5'-AGGTATCCATCCATCCCACA-3'
	Downstream: 5'-GCCACAGTTCTCAAAGCACA-3'
MMP-13	Upstream: 5'-AGGCCTTCAGAAAAGCCTTC-3'
	Downstream: 5'-GAGCTGCTTGTCCAGGTTTC-3'
TIMP-1	Upstream: 5'-GAGAGCCTCTGTGGATATGT-3'
	Downstream: 5'-CAGCCAGCACTATAGGTCTT-3'
TIMP-2	Upstream: 5'-AAGGACCTGACAAGGACATC-3'
	Downstream: 5'-TGCATCTTGCCATCTCCTTC-3'
α-SMA	Upstream: 5´-GACACCAGGGAGTGATGGTT-3´
	Downstream: 5´-GTTAGCAAGGTCGGATGCTC-3´
TGFβ-1	Upstream: 5´-CAACAACGCAATCTATGACAAAA-3´
	Downstream: 5´-AGTAGTTGGTATCCAGGGCTCTC-3´
Procollagen α1(1)	Upstream: 5´-AGCCTGAGCCAGCAGATTGA-3´
	Downstream: 5´-CCAGGTTGCAGCCTTGGTTA-3´
Procollagen α1(2)	Upstream: 5´-CTCTGGTACCGCTGGAGAAG-3´
	Downstream: 5´-ACCAGCAGCTCCACTCTCAC-3´
GAPDH	Upstream: 5´-CGCTGGTGCTGAGTATGTCG-3´
	Downstream: 5´-CTGTGGTCSTGAGCCCTTCC-3´

Quantitative real-time PCR (QRT-PCR) and reverse-transcriptase PCR

cDNA was prepared from 1 μg of total RNA in 25 μL with reverse-transcriptase methods according to manufacturer's instructions (Promega Inc., USA). Quantitative real-time PCR was performed with a high-throughput real-time PCR system (ABI 7900HT sequence Detection System, Applied Biosystems, CA, USA). The PCR mixture (20 μL) contained 1.25 μL cDNA, primers (1 μM concentration of each primer, table I), and 2x SyberGreen master mix (Applied Biosystem). An initial denaturing step at 95°C for 10 minutes was followed by 40 cycles of 95°C for 15 seconds, 56°C for 15 seconds, and 72°C (a measuring step) for 40 seconds. Each measurement was performed in three replicates. Data were

analysed with the SDS software 2.1 (Applied Biosystems). The gene expressions were normalized to the signal of the house keeping gene GAPDH.

Conventional RT-PCR was performed to measure the mRNA levels for MMP-13 in the liver. RT-PCR was performed with condition of 5 minutes 95^0C followed by 30 cycles 95^0C for 30 seconds, 56^0C for 30 seconds and 72^oC for 30 seconds, and finally 72^0C for 5 minutes. As a house keeping enzyme, we used GAPDH. GAPDH PCR cycle conditions were 5 minutes 95^0C followed by 26 cycles 95^0C for 30 seconds, 58^0C for 30 seconds and 72^oC for 30 seconds, and finally 72^0C for 5 minutes. PCR products were analyzed on 2% agarose-gels stained with ethidium bromide. The signals from different samples were normalized for the expression of GAPDH and quantified with image analysis software, ImageJ (NIH, USA).

Statistical analysis

Data were presented either as individual values or as mean ± SD. All data were subjected to an unpaired, two-tailed distribution student t-test. Differences were considered significant at $p<0.05$.

Results

Conjugation and characterization of M6PIL-10

Using Western blotting methods, we detected a successful conjugation product of M6P and IL-10. As shown in figure 2 chapter 6, blots of unmodified recombinant human IL-10 yielded two bands corresponding to a molecular weight (MW) of 18.5 kDa and 37 kDa, representing the monomeric and the dimeric form of IL-10.[30] Gel ectrophoresis and blotting of the newly prepared construct did reveal a shift in these bands: both bands had a higher molecular weight of approximately 20 kDa and 40 kDa.

Whole body distribution of IL-10 and M6PIL-10

To test whether the construct had a different body distribution from native IL-10, the distribution of [^{123}I]M6PIL-10 was studied with a gammacamera and compared with the distribution of [^{123}I]IL-10. The results showed that already 2 minutes after iv injection of radiolabeled proteins, high levels of [^{123}I]M6PIL-10

were found in the livers, using a gammacamera, and very low levels were found in the kidneys. Hepatic levels remained high for at least 30 minutes. In contrast, native [^{123}I]IL-10 rapidly accumulated in the kidneys with low accumulation in livers (fig.1), which is in agreement with previous studies.[25,31] These data show a clear shift in distribution of IL-10 from the kidney to the liver after modification of the protein.

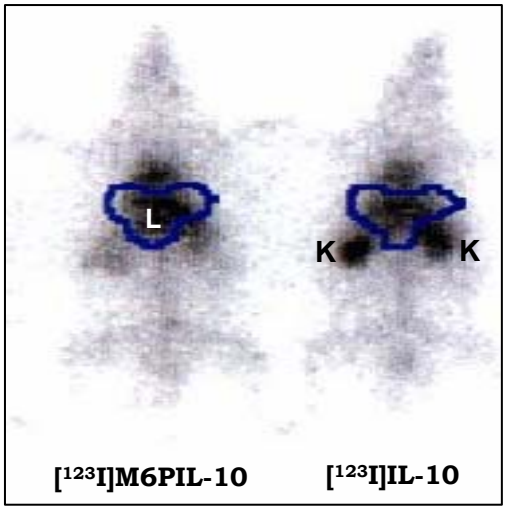


Fig.1. A gammacamera image of [^{123}I]IL-10 and [^{123}I]M6PIL-10 distribution in BDL-1 rats, from 20 – 30 min after iv injection of radiolabeled-proteins. The images show a high accumulation of [^{123}I]M6PIL-10 in the liver (L) in contrast to [^{123}I]IL-10 which is mostly distributed to the kidneys (K).

Effects of IL-10 and M6PIL-10 *in vivo*

To study whether IL-10 and modified IL-10 displayed pharmacological effects within the liver *in vivo*, we treated bile duct ligated (BDL) rats with these proteins. Treatment consisted of a daily iv injection of IL-10 or M6PIL-10 from day 4 to day 6 after bile duct ligation. Control rats received PBS. Parameters reflecting liver function, intrahepatic inflammation and fibrogenesis were analyzed.

Effect of IL-10 and M6PIL-10 on liver function

We measured various plasma levels of markers reflecting liver injury and cholestasis in fibrotic rats 1 week BDL (BDL-1) and in BDL rats receiving treatment with IL-10 or M6PIL-10. The plasma levels of these enzymes in BDL-1 rats receiving IL-10 or M6PIL-10 treatments were not significantly different from BDL rats receiving no treatment (table II).

Table II. Plasma levels of markers reflecting liver injury and cholestasis in fibrotic rats one week (BDL-1), and in BDL-1 rats receiving IL-10 or M6PIL-10 treatment

Parameters	BDL-1 rats (PBS-treated)	IL-10-treated rats	M6PIL-10-treated rats
AP (U/L)	493.8 ± 71.2	476.2 ± 53.8	450.6 ± 16.13
AST (U/L)	350.0 ± 166.6	354.0 ± 118.8	301.0 ± 109.64
ALT (U/L)	92.2 ± 27.44	90.2 ± 24.9	84.0 ± 17.0
TBI (μmol/L)	191.2 ± 43.13	231.6 ± 21.9	194.8 ± 68.6
DBI (μmol/L)	154.0 ± 20.8	175.4 ± 12.4	156.4 ± 39.9
GGT (U/L)	65.0 ± 56.0	74.0 ± 75.95	34.8 ± 23.34

Values represent the mean ± SD of 5 rats per group.

Anti-inflammatory effects of IL-10 and M6PIL-10

The effect of treatment with either IL-10 or M6PIL-10 on the hepatic inflammatory responses were examined by staining for iNOS and ROS-producing cells. In addition, IL-10 receptor expression was examined. In BDL-1 rats, iNOS staining was detected in inflammatory cells localized around the portal areas, in occasional groups of hepatocytes in the liver parenchyma and in cells surrounding necrotic areas. The number of ROS-producing cells reflected by DAB-positive cells was high around necrotic areas and in the portal areas in untreated rats. Treatment with either IL-10 or M6PIL-10 strongly attenuated the staining for ROS-production, in particular the staining localized in the portal areas was reduced (fig.2). Quantitative evaluation of DAB-staining, reflecting macrophages and neutrophil influx, by counting the number of positive cells per area showed that rats receiving treatment with IL-10 had a reduction in DAB staining of 35% ± 17% compared to untreated BDL-1 rats ($p<0.05$), whereas the number of DAB-positive cells per area in M6PIL-10-treated rats was reduced by 74% ± 10% compared to untreated rats ($p<0.05$). It can be concluded that both IL-10 and M6PIL-10 exert anti-inflammatory effects within the liver and M6PIL-10 is superior in this respect.

Staining for IL-10 receptor expression on liver section from BDL-1 rats showed positive staining on spindle-shaped cells around the proliferated bile ducts

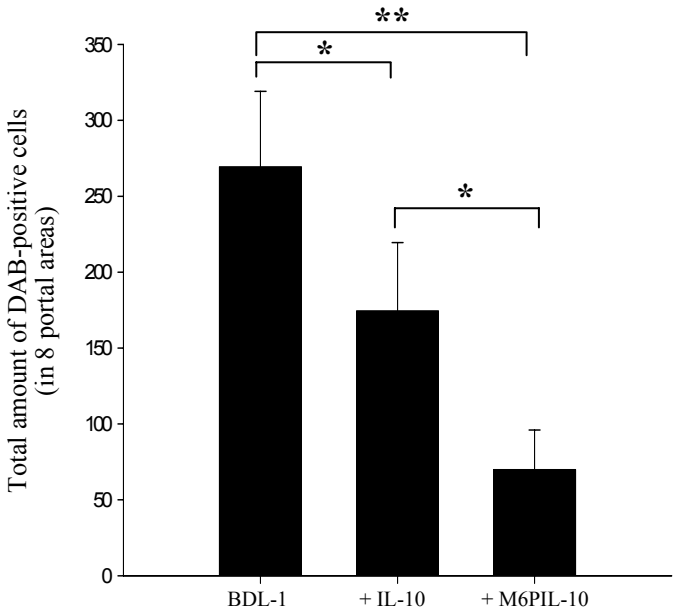


Fig.2. Effect of IL-10 and M6PIL-10 treatment on the number DAB positive cells. Both treatments significantly reduced the number of DAB positive cells in the portal area of the livers.
*p<0.05; **p<0.01.

and around the hepatic arteries. Positive staining for IL-10 receptor was also seen on round-shaped cells in the portal areas. In BDL-1 rats receiving treatment with IL-10, IL-10 receptor expression was reduced, in particular the round-shaped cells in the portal areas were negative (fig.3). In contrast, in BDL-1 rats receiving M6PIL-10, IL-10 receptor expression was still observed although slightly reduced compared to control BDL-1 rats.

Antifibrotic effects of IL-10 and M6PIL-10

Using immunohistochemical techniques, we studied the effects of IL-10 or M6PIL-10 treatment in rats with liver fibrosis. Various parameters associated with fibrosis were examined i.e. α-SMA, desmin/GFAP and collagen deposition.

Effect on α-SMA staining

Alpha smooth muscle actin staining of liver was used to demonstrate activated hepatic stellate cells (HSCs). Staining for α-SMA positive cells in the normal livers was observed in vascular smooth muscle cells around arteries in portal areas (data not shown). One week after BDL, positive staining for α-SMA was highly increased in cells around portal ducts, and in fibrotic septa. IL-10 and M6PIL-10 clearly diminished α-SMA staining. Particularly the staining localized in fibrotic septa and around the proliferated bile ducts was reduced by both cytokines (fig.4A). Quantitative evaluation of this staining using ImageJ software, as presented in figure 4B, shows a significant reduction by 54% ± 17% and 33% ± 19% after treatment with IL-10 or M6PIL-10, respectively ($p<0.05$).

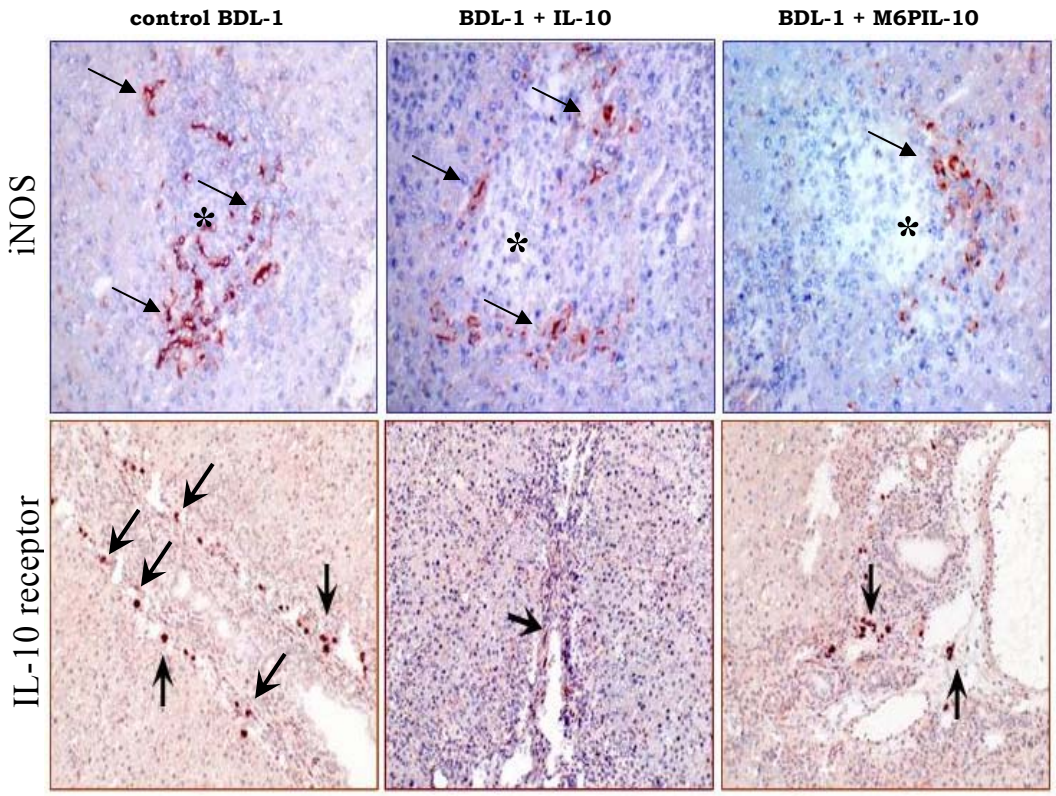
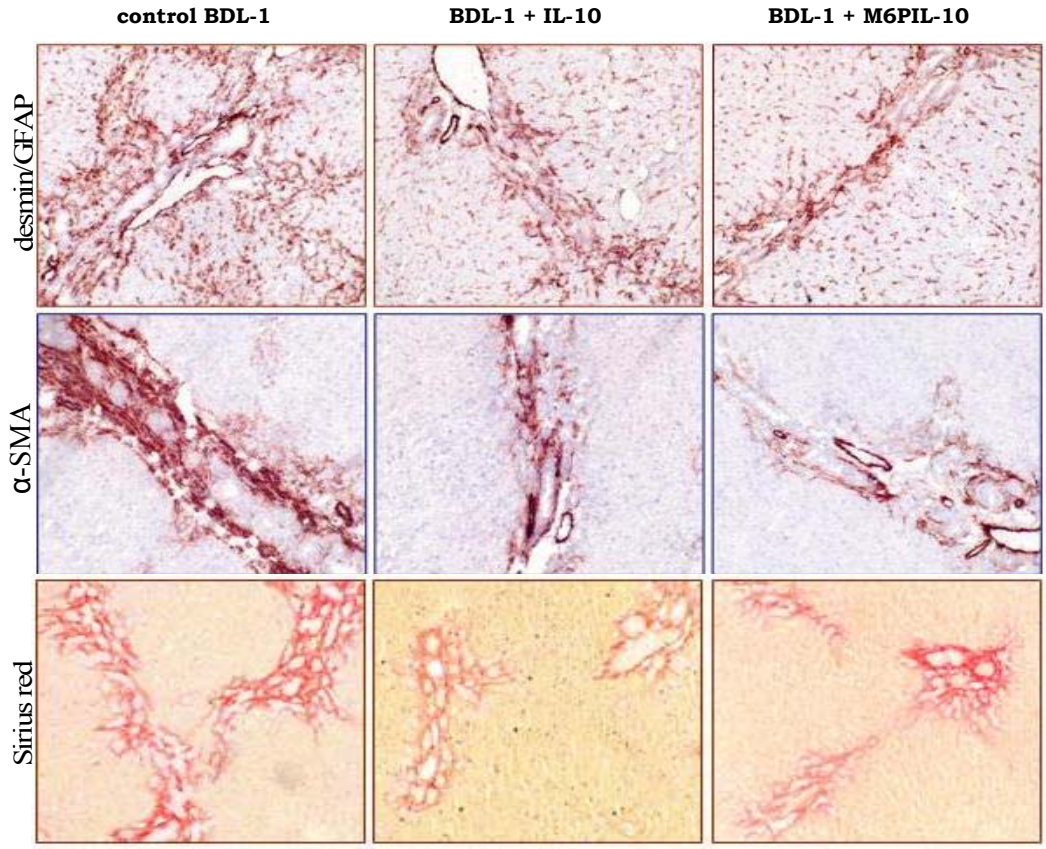


Fig.3. iNOS and IL-10 receptor stainings (arrow) in fibrotic livers. iNOS staining in BDL-1 livers is strongly present around the inflamed area (*) but less in rat livers after treatment with IL-10 or M6PIL-10. Both treatments also reduced IL-10 receptor staining in the portal areas (arrow). Magnification x200 (iNOS) and x100 (IL-10 receptor).

A.



B.

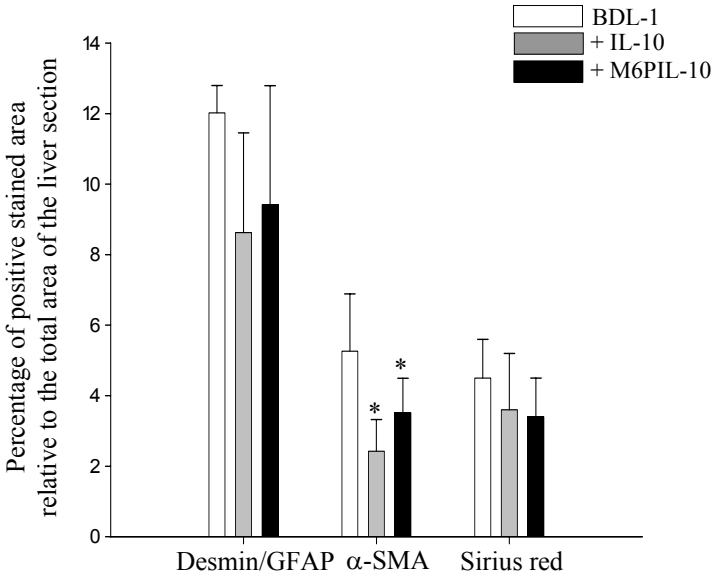


Fig.4. Effect of IL-10 and M6PIL-10 treatments on desmin/GFAP, α-SMA, and Sirius red stainings in fibrotic livers. (**A**) An excess amount of the stainings around the portal area of BDL-1 livers was clearly reduced by the treatments with IL-10 and M6PIL-10. (**B**) A quantitative evaluation of the stainings using ImageJ software shows a significant reduction by IL-10 and M6PIL-10 treatment, only for the α-SMA staining. *p<0.05.

Effect on desmin/GFAP staining

Development of fibrotic lesions was also examined by staining for desmin and glial fibrillary acidic protein (GFAP), both well known markers for HSCs. As shown in figure 4A, desmin/GFAP positive staining was detected in perisinusoidal areas and in fibrotic lesions in the portal areas around the proliferated bile ducts. A clear reduction in positive staining for desmin/GFAP was observed in rats receiving IL-10 or M6PIL-10 (fig.4A). Quantitative evaluation as presented in figure 4B shows a reduction by 28% ± 24% and 22% ± 28% after treatment with IL-10 or M6PIL-10, respectively.

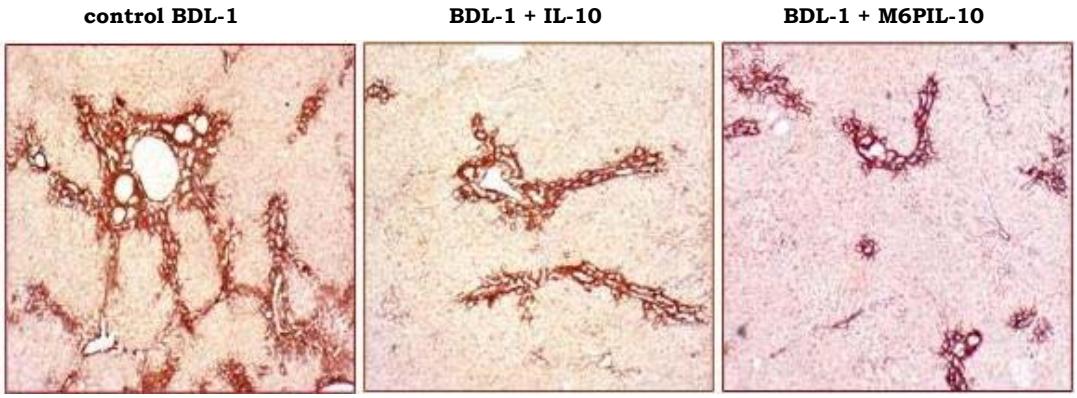
Effect on matrix deposition

Deposition of fibrous tissue was assessed by immunostaining for type III collagen and by histochemical staining with Sirius red. The latter staining reflects total collagen. In normal rat livers, the staining was mainly seen in the portal areas and the arterial walls (data not shown). The staining was strongly enhanced in diseased rats compared to the normal rats. The portal to portal fibrosis bridging was already apparent in the untreated group. In rats treated with IL-10, however the portal-portal and portal-central fibrous bridges were occasionally observed in only one out of five rats and this bridging was not seen in any of the M6PIL-10-treated rats. The matrix deposition around the portal areas was clearly declined by the treatments compared to untreated group (fig.5A).

Quantitative analysis of fibrotic lesions

Using a quantitative imaging analysis technique, we evaluated the effects of treatment with either IL-10 or M6PIL-10 on the deposition of fibrous tissue. The results, as presented in figure 4B and 5B, show that treatment with IL-10 yielded a reduction in Sirius red staining by 20% compared to control BDL-1, while M6PIL-10 treatment reduced the liver area stained for collagen by 23%. In addition, grading of the fibrotic process by the HAI Knodell's index revealed a reduction of the fibrotic index from 3.4 in untreated BDL-1 rats to 2.4 and 2.2 ($p<0.01$) in rats receiving IL-10 and M6PIL-10, respectively (fig.5B).

A.



B.

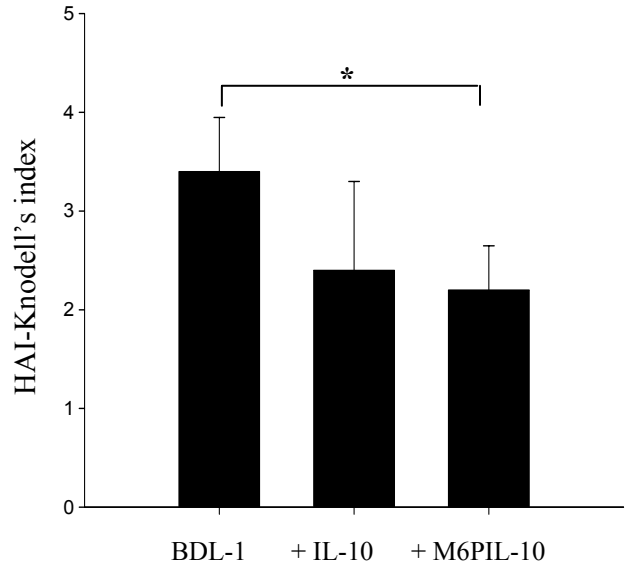


Fig.5. Effect of IL-10 and M6PIL-10 on intrahepatic type III collagen deposition. (**A**) An excess amount of type III collagen deposition with a portal to portal fibrosis and portal-central fibrous bridges were observed in livers at the first week after BDL procedure. Treatment with IL-10 or M6PIL-10 clearly reduced the collagen deposition and the portal-central fibrous bridges. (**B**) A grading of the fibrotic process with a modified Knodell's index revealed a reduction of the fibrotic index from 3.4 to 2.4 by IL-10 and to 2.2. by M6PIL-10 treatments. *p<0.01.

The effects of IL-10 and M6PIL-10 on the mRNA levels for genes associated with the disease

Expression of 9 selected genes, mainly genes associated with the early development of fibrosis in the liver (BDL-1) was examined to assess the effects of IL-10 and its modified form. The mRNA levels were quantified by real time RT-PCR and in the case of MMP-13 by conventional RT-PCR. Compared to non-diseased

livers, the mRNA levels for TNF-α ware the most dramatically increased inflammation associated parameter with 4.5-fold induction (fig.6), followed by mRNA levels for ICAM-1 (4-fold) and mRNA levels for MMP-13 (2-fold). In rats receiving treatment with IL-10, the mRNA levels for TNF-α, ICAM-1, and MMP-13 were not significantly different from untreated BDL-1 rats, only mRNA levels for ICAM-1 were significantly lower in both IL-10 and M6PIL-10 treated rats as compared to control ($p<0.05$).

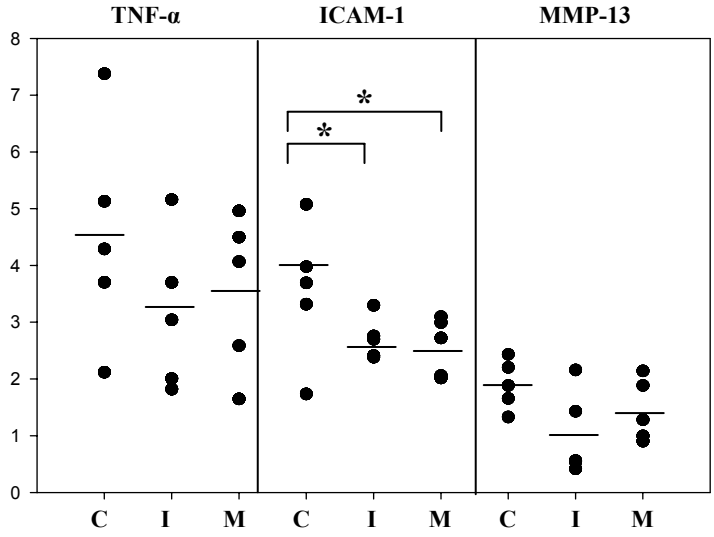


Fig.6. mRNAs expression of inflammatory genes from livers as measured by quantitative real-time PCR techniques. Fold induction as depicted on the y-absis was calculated from mRNA levels of the indicated genes of normal livers. As can be seen in this figure, rats treated with IL-10 (I) or M6PIL-10 (M) had reduced ICAM-1 mRNA levels, compared to BDL-1 rats (C). *$p<0.05$.

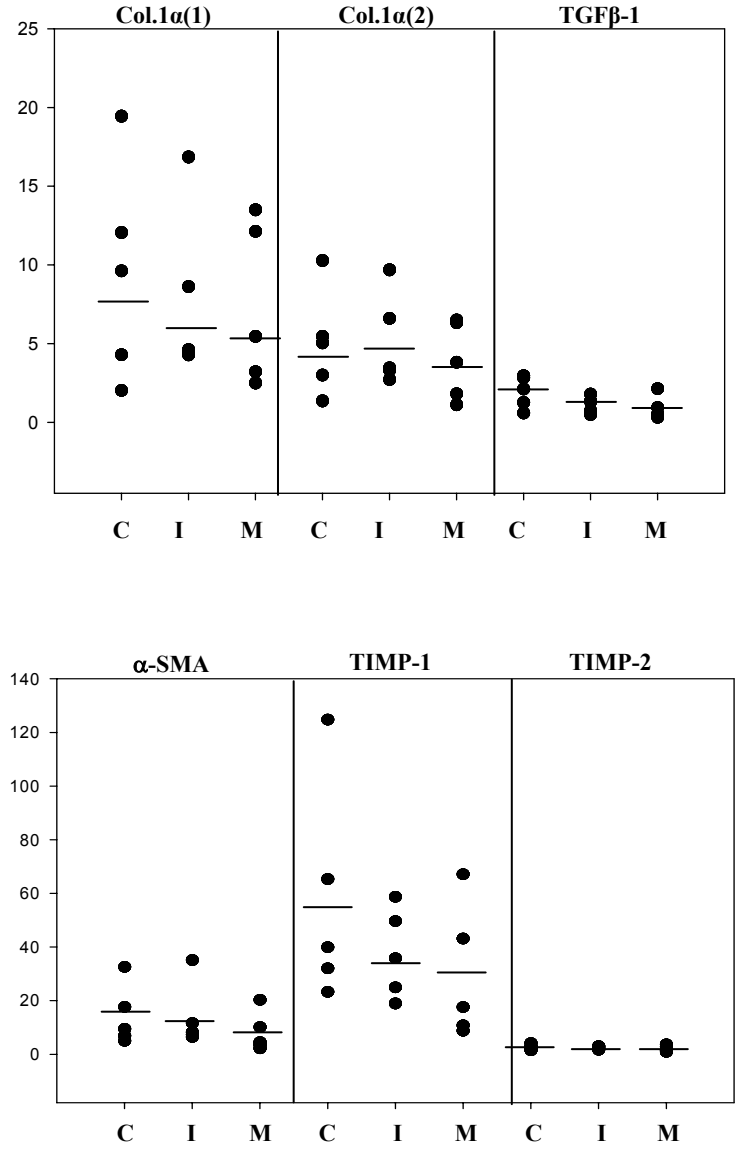


Fig.7. mRNAs expression levels of genes involved in the fibrotic process as measured by quantitative real-time PCR techniques. Fold induction as depicted on the y-absis was calculated from mRNA levels of indicated genes of normal livers. At the first week after BDL-1 (C), the mRNA levels for most relevant genes involved in the fibrotic process were induced, except TIMP-2 gene. No significant effects of IL-10 and M6PIL-10 were found on these parameters.

Examination of mRNA levels for genes associated with the fibrotic process i.e. procollagen α1(1), procollagen α1(2), TGFβ-1, α-SMA, TIMP-1 and TIMP-2,

revealed very strong increases in expression levels for most of these genes. In particular mRNA levels for TIMP-1 were enhanced by a factor 50 compared to normal rats, whereas TIMP-2 mRNA levels were not increased one week after bile duct ligation. Treatment of diseased animals with either IL-10 or M6PIL-10 did not significantly alter the expression levels of any of these genes (fig. 7).

Discussion

This study demonstrates the hepatic targeting and pharmacological effects of a novel liver-specific form of recombinant human IL-10, i.e. M6PIL-10, in a rat model of liver fibrosis. M6P is a specific ligand with a high affinity for the M6P/IGF-II receptor. This M6P/IGF-II receptor is highly upregulated in HSC during liver diseases.[32,33] Upregulation of this receptor on HSC during liver fibrosis yields an excellent target for receptor-mediated antifibrotic drug delivery, since HSC is a major target cell for therapies for liver fibrosis.[34-36] The delivery of antifibrotic drugs via M6P-containing ligands to the most competent cell type in the liver is a rational and new approach to treat this chronic disease.

To test the targeting efficiency of our conjugate, a gammacamera imaging experiment as presented in this study was performed. The result indicates a preferential hepatic homing of modified cytokine. The shift in biodistribution of IL-10 from the kidney to the liver after coupling of M6P is in accordance with the high liver uptake found in earlier studies with HSA as the core protein. These studies showed receptor-mediated uptake of M6PHSA in HSC.[24]

In addition to the examination of the hepatic homing of the conjugate, we addressed the question whether this conjugate is pharmacologically active *in vivo*. Mannose 6-phosphate residues were coupled to the e-amino-groups of lysine within the protein. It is known that lysine-groups are present at the receptor-binding site of IL-10[30] and attachment of M6P residues to these particular lysine groups might therefore affect the biological activities of the construct. However, previous studies have indicated that M6PIL-10 binds to the IL-10 receptor *in vitro* and *in vivo* (publication submitted) and that it is pharmacologically active *in vitro*. To test whether M6PIL-10 has antifibrotic effects in animal with liver fibrosis, we performed the *in vivo* studies described here. In these *in vivo* studies, we scheduled a treatment for 3 consecutive days with either IL-10 or M6PIL-10 (iv injection of 8µg/kg/day) starting at day 4 after the BDL procedure. So, the

treatments were given at an early stage of the disease yet after the initiation of fibrotic process. At 4 days after obstruction, the inflammatory activities and the fibrogenic process are rapidly ongoing.[37] In addition, M6P/IGF-II receptor expression is also enhanced at this time point (data not shown). We found significant effects of IL-10 as well as M6PIL-10 on various parameters associated with progression of the disease. We examined inflammatory parameters such as the number of ROS-producing cells, iNOS expression, IL-10 receptor expression at the protein level by immunostaining methods and TNF-α, ICAM-1 and MMP-13 expression at the mRNA level by RT-PCR techniques. The induction of all these proinflammatory mediators in the first week after the BDL procedure demonstrates that hepatic inflammation is evident at this stage, confirming previous reports.[37-41] The enhanced influx of inflammatory cells was associated with an enhanced intrahepatic staining for ICAM-1 expression (data not shown) and enhanced levels of mRNA for ICAM-1 (fig.7). During the first week after bile duct ligation an upregulation of IL-10 receptor expression was also noted. Upregulation of this receptor during disease may be of significance in view of the antifibrotic effects of IL-10 described in several studies.[15,25,42]

Treatment with either IL-10 or M6PIL-10 had significant effects on the parameters associated with the inflammatory activity within the liver. A significant reduction ($p<0.05$) of the mRNA levels for ICAM-1 induced by IL-10 and M6PIL-10 was seen as well as a significant decrease in the number of DAB-positive cells and iNOS stainings. These data indicate that M6PIL-10 is pharmacologically active *in vivo*. Based on the number of inflammatory cells, its effect may even be superior to native IL-10. This can be explained by the increased hepatic homing of M6PIL-10 as compared to IL-10.

Of particular interest is the reduction in IL-10 receptor expression after treatment with IL-10. The downregulation of the target receptor during treatment was unexpected and is quite relevant for IL-10-based therapies. This effect may contribute to the lack of effectiveness of such therapies. M6PIL-10 also did reduce IL-10 receptor expression, although to a lesser extent than IL-10. This difference can not be explained.

Next to the anti-inflammatory effects of IL-10 and M6PIL-10, we studied the effects of these proteins on fibrogenesis, by examining their effect on various parameters reflecting HSC functioning such as activation, transformation,

proliferation, and scar formation. Immunohistochemical analysis of α-SMA, desmin/GFAP, collagen type III revealed a marked increase of these proteins in BDL rats as compared to normal rats. These proteins were all abundantly expresed around the portal areas indicating the establishment of hepatic fibrosis[35-38] at this time point. Quantitative analysis of these stainings showed a reduced fibrogenesis in groups receiving IL-10 or M6PIL-10, but only α-SMA staining was significantly different compared to control BDL-1 rats.

We also observed enhancement of parameters associated with the fibrotic process at the mRNA level. Induction of the mRNA levels for procollagen α1(1), procollagen α1(2), TGFβ-1, α-SMA, and TIMP-1 was very high. IL-10 nor M6PIL-10 modified mRNA levels for all these parameters. The lack of effectiveness on mRNA levels for various genes was also seen in previous studies from our lab (publication submitted). This may indicate that IL-10 does not affect mRNA levels at all, and exerts its effect only through modulation of post-translational protein levels but it may also be related to the time of sacrifice of the animals. Rats were sacrificed 24 h after the final injection of IL-10 or M6PIL-10, and within this time frame mRNA levels might easily have returned to pre-injection levels.

A reduction in the HAI-Knodell's index, reflecting the severity of fibrosis within the liver, showed that the fibrotic score went from 3.4 to 2.4 and 2.2 by IL- and M6PIL-10, respectively also confirms the antifibrotic effect of both treatments ($p<0.01$ for M6PIL-10).

In summary, bile duct ligation leads to a strong intrahepatic inflammatory response, accompanied by the activation of the fibrogenic process within the first week. IL-10 and M6PIL-10 both significantly reduce some parameters of the inflammatory and the fibrogenic processes within the liver. Both proteins affect the same parameters and the potency of IL-10 and modified IL-10 was near equal in many cases, but the effects of M6PIL-10 on neutrophil activation and the HAI-Knodell index was superior to IL-10 itself. Further dose-response studies are required to establish whether modified IL-10 is superior to IL-10. To our knowledge, this is the first report demonstrating a successful hepatic targeting of a therapeutic cytokine which is pharmacologically active *in vivo* during liver fibrosis.

Several modifications of cytokines have been explored to enhance the residence time in plasma, thereby increasing the pharmacological effects of INF,

IL-2, IL-6 or TNF-α (for a review see Rachmawati *et al*), but this usually refers to passive targeting. Pegylation prevents the rapid renal clearance but no active delivery to the desired site of action is achieved. Only for IL-2, a cell-specific delivery to hepatocytes was achieved, but this cell may not be relevant in many cases. The very profound enhancement in pharmacological effects achieved by passive targeting of some cytokines indicates the benefits of targeting and stresses the importance of exploring a cell-selective delivery of these potent bioactive compounds. As indicated, cell-selective delivery of IL-10 to HSC is quite relevant during the process of fibrogenesis, since this cell type plays a key role in the progression of this disease.[35,36] Also the choice of IL-10 to test the possibility and effects of cell-selective delivery of cytokines is relevant since many reports have shown that endogenous IL-10 plays a pivotal role in the control of inflammatory and fibrogenic processes within the liver.[5,6,9,10,11,13] In fact, these studies have elicited clinical trials with IL-10 in patients with liver fibrosis.[16,17] These studies yielded variable results most likely caused by the very unfavorable pharmacokinetic profile of this molecule,[25] leading to rapid plasma elimination and uptake by tissue other than the liver. The array of biological effects of IL-10 subsequently leads, analogue to many other cytokine-based therapies, to serious adverse effects that block further dose-escalation studies. Although not the subject of the present study, our targeting approach may attenuate the adverse effects of IL-10-based therapies.

In conclusion, a targeting system for IL-10 using a liver specific ligand, M6P, is an attractive approach to deliver therapeutic proteins to treat chronic liver diseases like liver fibrosis. IL-10 conjugated with M6P efficiently reaches the liver and still binds to the IL-10 receptor, reflected by the pharmacological activities of this conjugate *in vivo*. So, this successful strategy to target a potent therapeutic cytokine to the liver may offer a new tool for therapeutic applications of this cytokine for the treatment of liver fibrosis.

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