We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



8

Modification of Interleukin-10 with Mannose-6-Phosphate Groups Yields a Liver-Specific Cytokine with Antifibrotic Activity in Rats

Heni Rachmawati[1,2], Adriana Mattos[2],
Catharina Reker-Smit[2], Klaas Poelstra[2] and Leonie Beljaars[2]
[1]*Pharmaceutics, Bandung Institute of Technology, Bandung*
[2]*University of Groningen, Dept. of Pharmacokinetics, Toxicology and Targeting,*
[1]*Indonesia*
[2]*The Netherlands*

1. Introduction

Cytokines and other biological compounds are considered as future drugs and they are of particular interest for the treatment of chronic diseases. These endogenous compounds, that normally mediate local cellular communications, are very promising candidates to generate new drugs because of their high potency (pM-nM concentrations) and their fundamental roles in pathological processes. However, the therapeutic application of cytokines is limited, because several problems are encountered with their application *in* vivo (Schooltink and Rose-John 2002; Standiford 2000; Vilcek and Feldmann 2004). For instance, some cytokines are efficiently degraded in plasma by various enzymes and cytokines are rapidly excreted by the kidneys. Consequently their residence time in the body and thus the exposure to the diseased cells is short (plasma half life is often minutes), which does not favour an optimal biological efficacy. Another major problem is the occurrence of side effects. Because cytokine receptors are ubiquitously expressed in all organs, unusual high plasma concentrations of the cytokine can lead to (unwanted) effects in various organs.

To overcome these problems, we use drug targeting techniques to selectively deliver the cytokine to a specific (diseased) cell (Allen and Cullis 2004; Beljaars, Meijer, Poelstra 2002). The challenge is to improve its distribution within the body and direct the cytokine to a cell of interest, while maintaining the biological activity of that particular cytokine after chemical modification. A conventional way to modify proteins is conjugation with poly-ethylene glycol (PEG) (Jevsevar, Kunstelj, Porekar 2010). The attachment of PEG moieties improves the pharmacokinetics. That is, PEG substitution prevents rapid renal elimination which results in compounds with prolonged plasma concentrations, thereby making a reduced number of doses possible. For instance, PEGasys (PEGylated interferon α2a), an example of a PEGylated cytokine that is now commonly used to treat patients infected with viral hepatitis, is dosed once a week while the unmodified interferon is dosed daily. This leads to an improvement in the compliance and quality of life in patients with chronic diseases. The side effects, however, are not diminished after PEGylation.

Our strategy of active drug targeting, in contrast to the abovementioned PEGylation approach, aims to improve pharmacokinetics and efficacy while simultaneously avoiding side-effects by cell-specific delivery of the cytokine to the diseased cell via receptor-mediated interaction. To that end, the cytokine is modified with homing devices that recognize receptors present on the diseased (target) cells. In the past, we designed sugars and receptor-recognizing peptides that interact with hepatic stellate cells (HSC) (Beljaars and others 1999; Beljaars and others 2000; Beljaars and others 2003). These cells play the central role in liver cirrhosis (Bataller and Brenner 2005; Friedman 2010; Schuppan and Afdhal 2008). Our newly designed homing devices displayed affinity for the mannose 6-phosphate/insulin-like growth factor (M6P/IGF) II receptor, platelet derived growth factor (PDGF)-β or collagen type VI receptors, which are all essential during stellate cell functioning in fibrogenesis, and upregulate in the diseased liver.

Currently, it is accepted that liver cirrhosis is a fibrotic disease that is reversible (Iredale 2007). However, to date, no drug is marketed that is able to reverse the fibrotic process in patients (Pinzani, Rombouts, Colagrande 2005). The only treatment that is applied to these patients deals with the treatment of complications and with eradication of the cause (for instance removal of the hepatitis virus in case of HCV-induced cirrhosis). However, fibrosis often progresses to end-stage liver failure leaving a liver transplantation as the only available option. Therefore, worldwide research focuses on the identification of compounds that are able to reverse the disease, but unfortunately many potential interventions fail in clinical trials (Pinzani, Rombouts, Colagrande 2005). We hypothesize that this failure may be due to an inadequate pharmacokinetic profile of the potential drugs or due to the occurrence of side-effects of these drugs preventing the administration of effective doses, which may be solved by applying drug targeting techniques.

The selective delivery of cytokines to the cells that control pathological processes is quite relevant. PEGylation of cytokines like interferon α, TNFα, and IL-2 has provided substantial benefits, but in that approach cytokines are not actively delivered to the site of action. In the present study, we will show an example of this second approach using the cytokine interleukin-10 (IL10). IL10 has potent immunosuppressive and anti-inflammatory effects (Di Marco and others 1999; Khan and others 2002; Kitching and others 2000; Oberholzer, Oberholzer, Moldawer 2002) and also direct antifibrotic properties in HSC (Cuzzocrea and others 2001; Demols and others 2002; Gloor and others 1998; Louis and others 1998; Louis and others 2003; Thompson and others 1998; Wang and others 1998). Several of these studies showed beneficial effects of IL10 therapies in animal models and clinical trials during various diseases. However, other studies demonstrated only a limited effect of IL10 or even showed disappointing results (Chadban and others 1997; Colombel and others 2001; Herfarth and Scholmerich 2002). This variable efficacy might be due to the low concentration of IL10 at the target sites. Recombinant IL10 is a low molecular weight protein that is rapidly cleared from the circulation through glomerular filtration. The plasma half-life of IL10 is only 2 min (Rachmawati and others 2004). The ultimate concentrations at the site of action therefore could be too low to result in clear effects. Dose escalation of systemically administered IL10 leads to adverse effects due to its inherent biological actions (Fedorak and others 2000; Schreiber and others 2000). In accordance with this, clinical studies reported beneficial effects of long-term IL10 therapy to treat HCV-associated liver fibrosis but this was accompanied by an immunosuppressive action, as noted in a flare-up of the viral burden, and low therapeutic efficacy (Nelson and others 2000)(Meijer and others

2001). Liver-selective delivery of this cytokine may prevent these clinical problems and create biological effects within relevant cells.

Upregulation of mannose-6-phosphate/IGF-II receptors during liver injury on HSC (de Bleser and others 1995) offers an excellent target for receptor-mediated antifibrotic drug delivery as shown with albumin substituted with mannose-6-phosphate groups (Beljaars and others 1999). We therefore modified IL10 with the sugar mannose-6-phosphate (M6P) to selectively deliver this cytokine to HSC in fibrotic livers (Rachmawati and others 2007). We showed that after modification of IL10 with mannose 6-phosphate (M6P) a compound was generated that binds to the M6P/IGF-II receptor which is highly present on activated HSC. Our chemically engineered cytokine, M6P-IL10, displayed good pharmacological activity in vitro in freshly isolated HSC. We now performed biodistribution studies of radiolabeled-M6P-modified IL10 using gamma-camera techniques to examine the active delivery of this compound to the diseased tissue. 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 IL10.

2. Results

2.1 Synthesis of M6P-IL10

Mannose-6-phosphate-residues were coupled to the amino acid lysine in recombinant human IL10 as described (Rachmawati and others 2007). The product was first characterized by Western Blotting techniques using a rabbit polyclonal anti-IL10 antibody (Santa Cruz Biotechnology, USA). Western Blot analysis of unmodified IL10 yielded two bands corresponding to a molecular weight (MW) of 18.5 and 37 kD (fig.1) representing the monomeric and the homodimeric form of IL10 (Reineke and others 1998). The prepared conjugate M6P-IL10 revealed a shift in these bands: both bands had a higher MW (resp. approximately 20 and 40 kD) than unmodified IL10 indicating covalent binding of M6P to the cytokine.

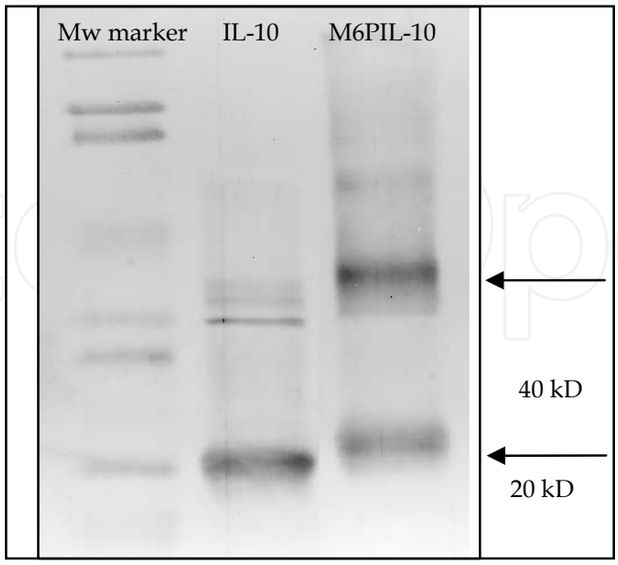


Fig. 1. Western blot analysis of M6P-IL10 and IL10. Note the increase in molecular weight of the monomeric (± 20 kD) and the homodimeric (± 40 kD) forms of M6P-IL10 compared with the monomeric and homodimeric forms of native IL10 (respectively, 18.5 kD and 37 kD).

2.2 Body distribution of IL10 and M6P-IL10

To visualize the body distribution of IL10 and M6P-IL10 with a gamma camera, both proteins were labeled with Iodine-123 (^{123}I). Fibrotic rats were monitored during the course of their disease by subjecting them to gamma camera analysis, just prior to BDL and one, two and three weeks after BDL (respectively, normal, BDL-1, BDL-2, and BDL-3, n=3). Anaesthetized rats were placed on a low-energy all-purpose collimator of a gamma-camera and received an intravenous tracer dose. The results are shown in figure 2. Already two min after iv injection of [^{123}I]M6P-IL10, the gamma-camera detected high levels of radioactivity within the livers (white intensity) and low levels in the kidneys (yellow-red intensity). Hepatic levels of [^{123}I]M6P-IL10 remained high for at least 30 min. The results of the distribution studies were similar in various stages of liver fibrosis (BDL-1, BDL- 2 and BDL-3). In contrast, native [^{123}I]IL10 rapidly accumulated in the kidneys (white intensity) with low uptake in livers (fig.2a), which is in agreement with previous studies (Andersen and others 1999; Rachmawati and others 2004).

Subsequently, we quantitatively measured the distribution of [^{125}I]IL10 and [^{125}I]M6P-IL10 ten minutes after intravenous injection in rats with end-stage liver fibrosis (BDL-3 weeks). Native [^{125}I]-IL10 accumulated in the kidneys in these rats (Fig 2b). Only, 15% and 30% of the dose was found in livers of normal and BDL-3 rats respectively. The rest was dispersed throughout the body or present in the blood. In contrast, [^{125}I]M6P-IL10 accumulated for nearly 60% of the dose within the livers in BDL-3 rats. Uptake in kidneys was only 20%. Differences in blood- , liver- and kidney-concentrations between IL10 and M6P-IL10 were significant ($p<0.05$).

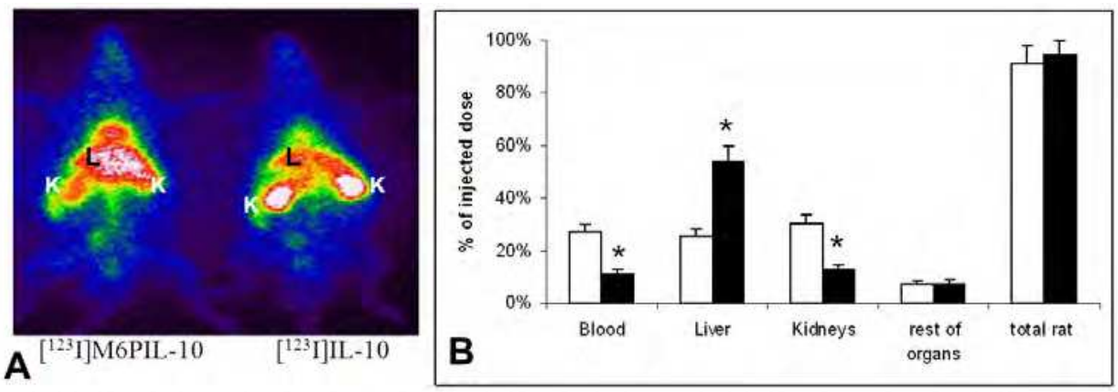


Fig. 2. Organ Distribution of M6P-IL10 and IL10. **A**: Gamma-camera images of [^{123}I]IL10 and [^{123}I]M6P-IL10 distribution in BDL-1 rats. Pictures show an overlay of recordings from t=20 to t=30 minutes after i.v. injection of radiolabeled proteins. The images show a high accumulation of M6P-IL10 in the liver (L) in contrast to IL10, which is mostly distributed to the kidneys (K). **B:** Quantitative measurement of organ uptake of [^{125}I]-labeled IL10 (white bars) and M6P-IL10 (black bars) in BDL-3 rats 10 minutes after i.v. administration of the radiolabeled proteins. N = 3 per group (* $p<0.05$ compared with IL-10 distribution)

2.3 Identification of target receptors

(Modified) IL10 could not be detected within livers by immunohistochemistry, most likely due to the very low dose administered (2-2.5 µg). Therefore, several receptor antagonists were applied to identify the target receptors responsible for the uptake of the proteins in different organs in order to obtain information about the hepatocellular distribution. Rats

were pre-treated with either succinylated human serum albumin (sucHSA) to block the scavenger receptor, or with mannose-6-phosphate-HSA, to block the M6P/IGFII receptor. These receptor antagonists (i.v. dose of 5 mg/kg) were administered 5 min. prior to the i.v. injection of a tracer amount of radiolabeled IL10 or M6P-IL10. Control animals received pre-treatment with unmodified HSA (5 mg/kg).

Kidney accumulation of [^{125}I]IL10 or [^{125}I]M6P-IL10 was not influenced by administration of any of the proteins (fig. 3). Uptake of [^{125}I]IL10 in the livers was also not influenced by any of the proteins, but remained approximately 20% of the dose in all groups. However, liver uptake of M6P-IL10 was reduced from 54 ± 6% in rats receiving only M6P-IL10, to 29± 4% by sucHSA ($p<0.05$) and to 24 ± 8% by M6P-HSA pre-administration ($p<0.05$). Co-administration of both sucHSA and M6P-HSA did not have an additive effect (24 ± 5% liver uptake). Unmodified HSA did not affect liver uptake of M6P-IL10 at all (57 ± 13% liver uptake).

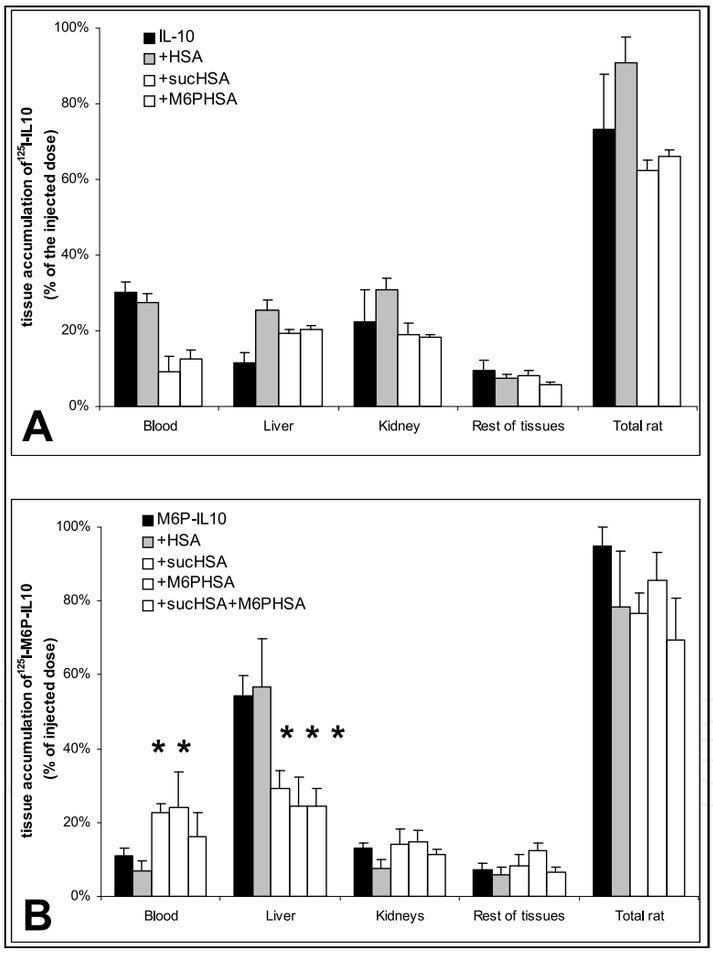


Fig. 3. Organ distribution of [^{125}I]IL10 (fig. **A**) and [^{125}I]M6P-IL10 (fig. **B**) in BDL-3 rats 10 min. after i.v. administration of the radiolabeled proteins. Five min. before administration of these proteins, HSA, sucHSA, M6PHSA or the combination of two proteins was administered to test receptor specificity. Note that sucHSA and M6PHSA influenced liver uptake and blood concentrations of M6P-IL10, whereas IL10 distribution was not affected by any of the proteins. N=4-6 per group. * =$p<0.05$ compared with HSA pre-administration.

2.4 Effects of IL10 and M6P-IL10 in vitro

The in vitro activities of IL10 and M6P-IL10 were studied in culture-activated primary HSC. The presence of IL10 receptors and M6P/IGFII-receptors on these cells was verified by immunohistochemical methods. No effect of IL10 or M6P-IL10 was found on HSC proliferation as assessed by Alamar blue assays (data not shown).

We also examined type I collagen deposition in cultures of HSC treated with IL10 or M6P-IL10 using immunostaining methods. Deposition of type I collagen was clearly detectable in HSC cultures at day-7 (fig 4A) and this staining was reduced in cultures treated for 24 hr with IL10 (Fig 4B) and in cultures treated with M6P-IL10 (fig 4C).

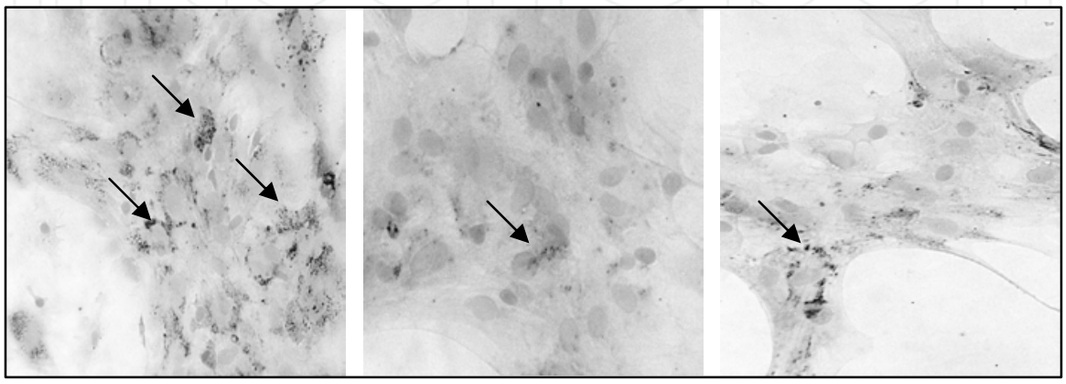


Fig. 4. Type I collagen deposition in cultures of primary isolated rat HSC's as detected with immunostaining methods. Collagen staining (using a goat polyclonal anti-collagen I antibody) is present on HSC's after 7 days in culture (**A**, arrows). Deposition of collagen is attenuated by 24 hr incubation with 12.5 ng/ml IL10 (**B**) or M6P-IL10 (**C**). Original magnification 200x.

2.5 Effects of IL10 and M6P-IL10 *in vivo*

2.5.1 Experimental design

Bile duct ligated rats were randomly divided into three groups: BDL rats received either vehicle (PBS, N = 5), or IL10 (N = 5) or M6P-IL10 (N = 5). Untreated normal rats (N = 3) served as reference group. Animals received a bolus iv dose (8 µg/kg/day) of (modified-) IL10 at day 4, 5 and 6 after BDL. At day 7, animals were sacrificed and samples of blood and various organs were harvested.

2.5.2 Effect of IL10 and M6P-IL10 on liver function

Plasma levels of markers reflecting liver injury and cholestasis in BDL-1 rats receiving IL-10 or M6PIL-10 were not significantly different from untreated BDL rats (table 1).

2.5.3 Effect of IL10 and M6P-IL10 on inflammatory parameters

To study the effects of IL10 and M6P-IL10 on inflammation, staining for reactive oxygen species (ROS)-production and IL10 receptor expression was performed. The number of 3,3'-diaminobenzidine (DAB)-positive cells in the liver was high around necrotic areas and in portal areas. DAB staining reflects ROS production (Poelstra and others 1990) by activated neutrophils, eosinophils and macrophages.

Parameters	PBS-treated rats	IL10-treated rats	M6PIL-10-treated rats
Alkaline Phosphatase (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*
Total bilirubin (μmol/L)	*191.2 ± 43.13*	*231.6 ± 21.9*	*194.8 ± 68.6*
GGT (U/L)	*65.0 ± 56.0*	*74.0 ± 75.95*	*34.8 ± 23.34*

Table 1. Plasma levels of markers reflecting liver injury and cholestasis in BDL-1 rats treated with PBS, IL10 or M6P-IL10. Values represent the mean ± SD of 5 rats per group.

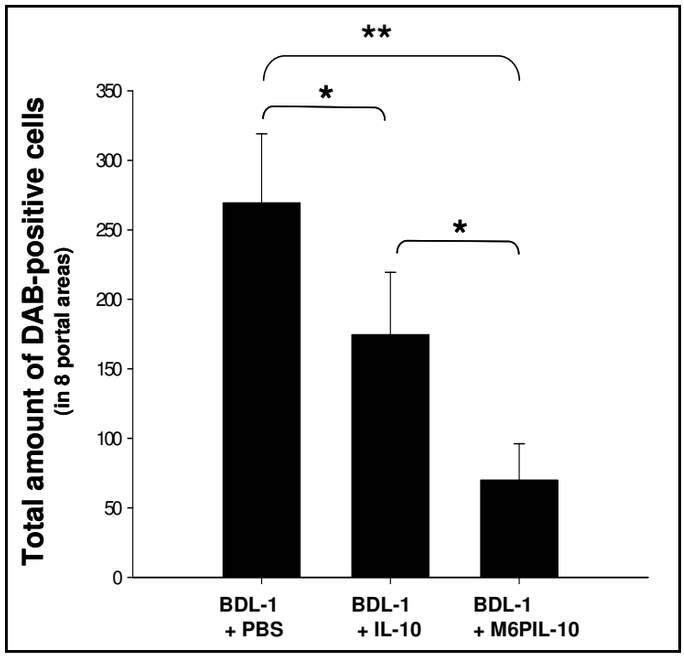


Fig. 5. Effect of IL-10- and M6PIL-10-treatment on the number of DAB-positive cells in livers of BDL-1 rats. Both treatments significantly reduced the number of DAB-positive cells in the portal areas compared with PBS-treated rats. *= $p<0.05$; **=$p<0.01$

IL10 and M6P-IL10 strongly attenuated the staining for DAB (Fig. 5). Quantitative evaluation of this staining by counting the number of positive cells/area showed that DAB staining was reduced by 35% in rats receiving IL10 ($p<0.05$ compared with untreated BDL-1 rats, Fig 5b), whereas the number of DAB-positive cells per area in M6P-IL10-treated rats was reduced by 74% compared with untreated rats ($p<0.05$). Thus, both IL10 and M6P-IL10 exerted anti-inflammatory effects within the liver and M6P-IL10 was superior in this respect.

Staining for IL10 receptors (with anti-IL-10 receptor IgG (Santa Cruz Biotech)) on liver sections of BDL-1 rats revealed occasional positive cells: some cells around the proliferating bile ducts and around hepatic arteries were positive. Based on the localization and the positivity for α-smooth muscle actin or HIS-48, these cells were identified as fibroblasts, HSC and neutrophils. In BDL-1 rats that received IL10, hepatic IL10 receptor expression was

strongly reduced, in particular around the portal areas (fig.6). In contrast, in BDL-1 rats receiving M6P-IL10, IL10 receptor expression was still present.

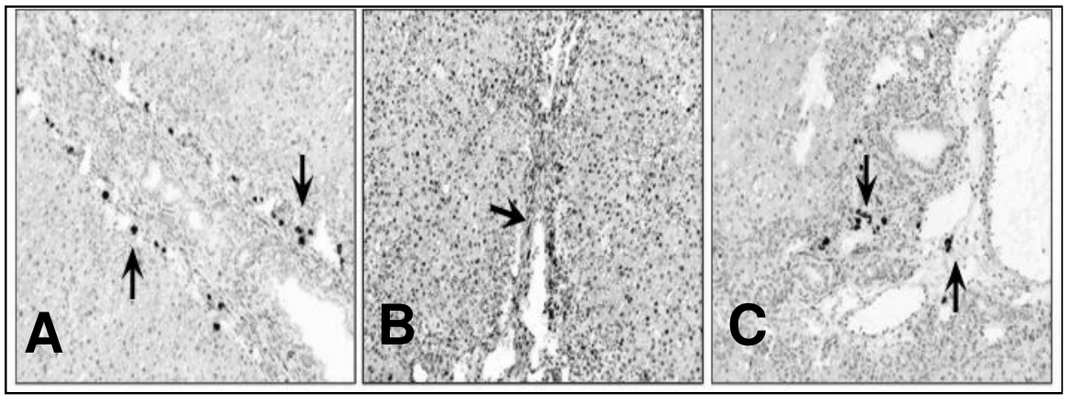


Fig. 6. Intrahepatic staining for IL10 receptor expression in fibrotic livers. In PBS-treated BDL-1 rats, positive cells were found around bile ducts (fig. **A,** arrows). In rats treated with IL10, only occasional IL10 receptor-positive cells were found (fig. **B**) whereas the portal areas contained many IL10 receptor positive cells in BDL-1 rats treated with M6P-IL10 (fig. **C**). Original magnification 100x.

2.5.4 Antifibrotic effects of IL10 and M6P-IL10

Characteristic for fibrosis is deposition of extracellular matrix in tissues. Collagen type I and III are the most important extracellular matrix proteins present in a fibrotic liver. Therefore, we assessed the effect of M6P-IL10 and IL10 administration on the deposition of collagen. First, we evaluated the deposition of fibrous tissue by histochemical staining with Sirius Red (figure 7A). This staining was strongly enhanced in BDL-1 rats compared with normal rats. The portal-to-portal bridging was already apparent in the untreated group at this time point. However, in IL10-treated rats, the portal-portal fibrous bridges were observed in only one out of five rats and bridging was not seen in any of the M6P-IL10-treated rats. The matrix deposition around portal areas was clearly reduced by the treatments compared to untreated group. This reduction was confirmed by immunostaining for collagen type III using goat anti-collagen III IgG (SouternBiotech, USA) antibodies. The strong staining seen in BDL-1 rats was reduced by IL10 and M6P-IL10 (fig. 7B).

With ImageJ software, the effects of IL10 and M6PIL10 on the deposition of fibrous tissue were quantified. In normal livers, 1.0 ± 0.45 % of the total liver area was positive for Sirius Red. This positive area increased to 4.6 ± 1.0% of the livers of BDL-1 rats ($p<0.001$ compared with normal rats, Fig. 7C). In IL10-treated BDL-1 rats the Sirius Red-positive area per liver changed to 3.6 % ± 1.6 (not significant compared with untreated BDL-1 rats). In M6P-IL10-treated BDL-1 rats, Sirius Red-staining changed to 3.4 ± 1.1 % of liver area ($p< 0.05$ compared with untreated BDL-1 rats, Fig. 7C). The effect of IL10 and M6P-IL10 on the fibrotic process was also assessed by staging the lesions via a semiquantitative scoring system; the Histologic Activity Index-Knodell (Ishak and others 1995). Grading of the fibrotic lesions in these livers by the HAI-Knodell's index revealed a reduction of the fibrotic index from 3.2 ± 0.8 in untreated BDL-1 rats to 2.6 ± 0.9 and 2.2 ± 0.45 ($p<0.05$) in rats receiving IL10 and M6P-IL10, respectively (fig. 7D).

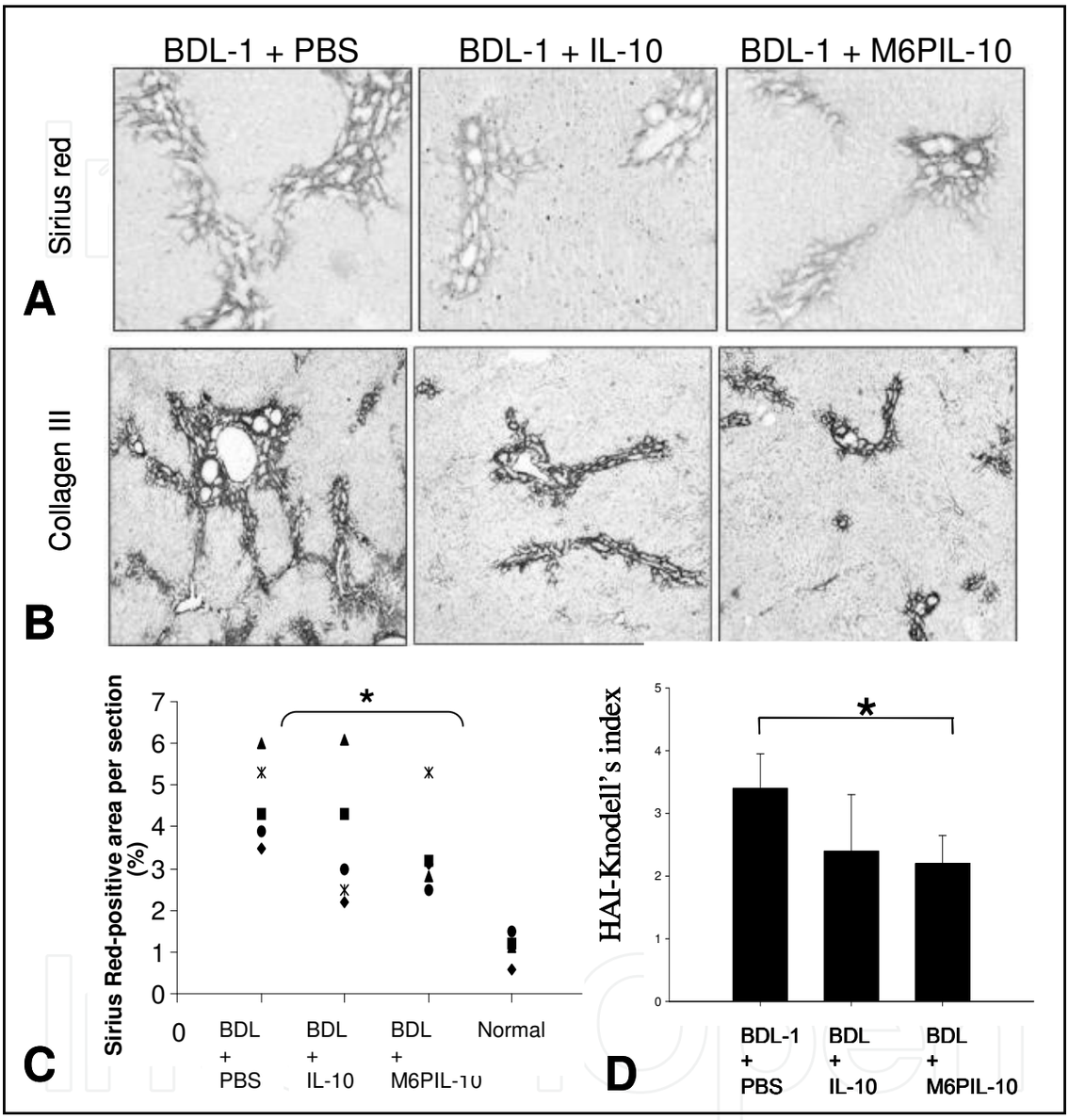


Fig. 7. Collagen deposition in BDL rats after treatment with M6P-IL-10 or IL-10. A. Representative photomicrographs of Sirius Red (fig. **A**) and collagen type III staining (fig. **B**) in livers of BDL-1 rats treated with PBS, IL-10 or M6PIL-10 (magnification 10x4). Figure **C** depicts the quantitative analysis of the Sirius Red stainings in the livers of the different groups as measured by Image J software. The individual values of each rat are shown in the graph. Figure **D** shows the semiquantitative grading of the fibrotic process in BDL-1 rats with the Histological Activity Index-Knodell method. N=5 per group, * = $p<0.05$ compared with PBS-treated BDL rats.

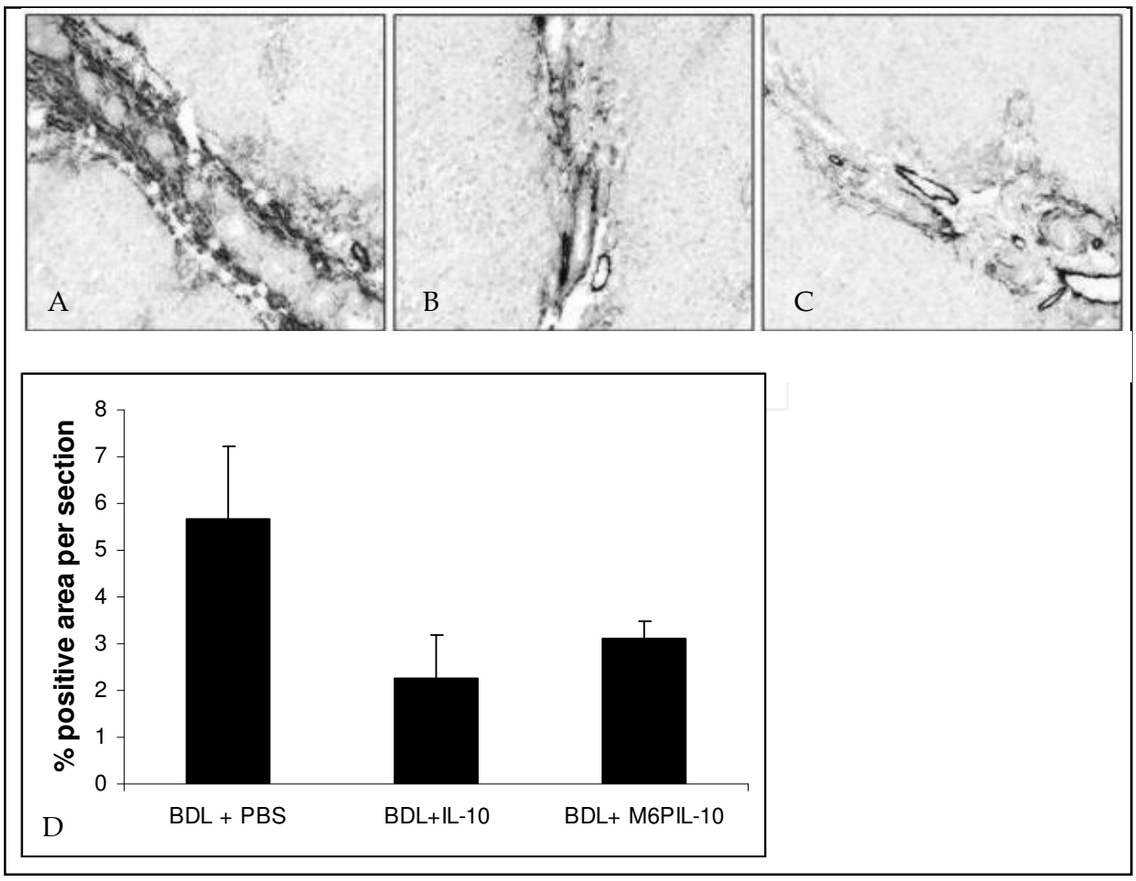


Fig. 8. Representative photomicrographs of immunohistochemical staining for α-smooth muscle actin (αSMA) in livers of BDL-1 rats treated with PBS (fig. **A**) or IL10 (fig. **B**) or M6P-IL10 (fig. **C**). Figure **D** depicts the quantitative analysis of the intrahepatic αSMA staining with Image J software (n=5 animals per group). *=$p<0.05$, Original magnification 100x.

Subsequently, we evaluated the in vivo effects of M6P-IL10 and IL10 on α-smooth muscle staining (α-SMA), that reflects the proportion of activated HSC and myofibroblasts. These cells are responsible for the production of collagen. One week after BDL, staining for α-SMA was highly increased around portal ducts and in fibrotic septa compared with normal rats. IL10 and M6P-IL10 clearly diminished this α-SMA staining (fig.8). Quantitative evaluation of this staining using ImageJ software (fig.8B), demonstrated a significant reduction by 54% ± 17% and 33% ± 19% after treatment with IL10 or M6P-IL10, respectively compared to PBS-treated BDL rats ($p<0.05$).

3. Conclusion/discussion

This study demonstrates that chemical modification of a cytokine, in our study IL10 modified with M6P groups, leads to a compound with improved biodistribution and pharmacological activity *in vivo* in a rat model of liver fibrosis. M6P is a homing device with high affinity for the M6P/IGF-II receptor which is upregulated on the cell membrane of HSCs during liver diseases (de Bleser and others 1995). Upregulation of this receptor on HSC during liver fibrosis yields

an excellent target for receptor-mediated drug delivery. The delivery of antifibrotic compounds to the major pathogenic cells in the liver by modification with M6P groups is a rational and new approach to treat this chronic disease (Schuppan and Popov 2009).

Chemical modification of a cytokine can influence the biological activity, in particular when essential amino acids necessary for interaction of the cytokine with its receptor are conjugated with homing devices or when the conformation of the protein is changed too much. Therefore, it is essential to test whether the prepared conjugate is pharmacologically active. In our study, we coupled several M6P groups to the lysine amino acids within the protein and some of these lysine-groups are present at the receptor-binding site of IL10 (Reineke and others 1998). Attachment of M6P-residues to these groups might therefore affect the biological activities of IL10. Studies on culture-activated primary HSC *in vitro* showed that M6P-IL10 reduced collagen deposition by these cells (fig 4) indicating that IL10-related activities are still intact in the modified cytokine. There was no effect of M6P-IL10 on HSC proliferation but native IL10 also did not affect growth of HSC. Previously, we demonstrated that M6P-IL10 was able to increase collagen degradation (by increasing the MMP13/TIMP ratio) in primary cultures of HSC (Rachmawati and others 2007). Based on its effects on collagen deposition, we conclude that M6P-IL10 is pharmacologically active within the target cell *in vitro*.

The key concept in active drug targeting is that the distribution within the body is confined to the diseased organ/cell-types. This will lead to more optimal effects and less side effects because uptake in other organs is avoided. In pharmacokinetic terms, this means that the Volume of Distribution (Vd) is decreased. The presented results of gamma-camera imaging studies and the biodistribution studies with radiolabeled IL10 and M6P-IL10 indicate a preferential homing of the modified cytokine to the fibrotic liver. The shift in biodistribution of IL10 from the kidney to the liver after coupling of M6P is in accordance with the high liver uptake of M6P modified proteins found in previous studies with HSA as the core protein (Beljaars and others 1999; van Beuge and others 2011). These studies showed uptake of M6PHSA within HSC. The cells responsible for the uptake of M6P-IL10 within the liver could not be directly identified due to the low amount of cytokines administered. Both proteins are only available in the microgram scale and immunohistochemical detection of proteins requires injection of milligrams per rat. Therefore, receptor antagonists were applied to identify the target receptors. These studies indicated that liver uptake of M6P-IL10 was receptor-mediated since the uptake was not attenuated by the control protein HSA whereas sucHSA and M6P-HSA, both ligands for receptors, significantly reduced its uptake. The fact that sucHSA and M6P-HSA both had an effect indicates that M6P-IL10 binds to at least two receptors: the scavenger receptor and the M6P/IGFII receptor, respectively. Involvement of the scavenger receptor, which recognizes strongly anionic compounds, can be explained by the negative charges introduced by phosphate groups (PO_4^{3-}). This was also found in another study in which liposomes were modified with M6P sugars (Adrian and others 2006). The combination of M6PHSA and sucHSA did not completely block the liver uptake which suggests the involvement of yet another receptor, possibly the IL10 receptor which is also present in the liver (fig 6). Based on the expression of M6P/IGF II receptors, scavenger receptors and IL10 receptors, the putative target cells for M6P-IL10 are HSC's, portal fibroblasts, endothelial cells, Kupffer cells and neutrophils within the liver. Antifibrotic effects of IL10 are anticipated in all these cells.

To test whether M6P-IL10 is effective *in vivo*, we now administered (modified-)IL10 to bile duct ligated rats after the initiation of the fibrotic process, i.e. from day 4 till day 7 after BDL. In this time frame, pro-inflammatory activity is high in the liver and fibrosis is initiated (39-43). In addition, M6P/IGF-II receptor expression on HSC is enhanced at day 4 (Greupink and others 2006), which ensures targeting to this receptor at this time point. During the first week after BDL, enhanced IL10 receptor expression was noted (fig 6), also providing a rationale for the start of treatment at day 4.

Treatment with IL-10 or M6P-IL10 had significant effects on the inflammatory activity within the liver. A reduction in the number of infiltrating cells as reflected by DAB-positive cells was noted. These data indicate that M6P-IL10 is pharmacologically active *in vivo*. Based on inflammatory cell influx, its effect may even be superior to native IL10. Of particular interest is the reduction in IL-10 receptor expression after treatment with IL10, but not after treatment with M6P-IL10. The down regulation of the target receptor during treatment is relevant for IL10-based therapies. This may contribute to the lack of effectiveness of such therapies (Chadban and others 1997; Colombel and others 2001; Herfarth and Scholmerich 2002).

Next to the anti-inflammatory effects, we evaluated the effects of M6P-IL10 on fibrogenesis *in vivo*. The target cell of IL10 is the (activated) HSC, the extracellular matrix producing hepatic cell, and therefore antifibrotic effects are primarily anticipated in HSC and the most important feature in this respect is collagen deposition. Our results showed a clear reduction in collagen deposition in these livers after treatment with M6P-IL10 (figure 7). This reduction was established with various methods. The lack of portal-to-portal bridging was evident in nearly all the livers of the cytokine-treated animals. In addition, IL10 and M6P-IL10 also significantly reduced αSMA staining which reflects a reduction in the activation of HSC in these livers. These results indicate that our modified IL10 is pharmacologically active *in vivo*.

Although cytokines are interesting compounds which may yield potent new drugs, so far only a relatively few are approved and clinically used. The number is still disappointing low regarding the large number of endogenous cytokines. The main reasons for this are the poor stability and poor pharmacokinetic profile of cytokines. To overcome these pharmacokinetic problems, we apply drug targeting techniques to selectively deliver the cytokine to a specific (diseased) cell. In the current study, we demonstrate the possibilities of this strategy with successful *in vivo* application of a modified IL10. Recently, we also reported on the cell-specific delivery of another very interesting cytokine with antifibrotic activities, that is interferon-gamma (IFNγ) (Bansal and others 2011). This study shows that HSC-targeted IFNγ, in contrast to unmodified IFNγ, blocked liver fibrogenesis in a chronic CCL4 mice model of liver fibrosis, by specifically acting on the key pathogenic cells within the liver. Furthermore, we clearly demonstrated that the targeted IFNγ was devoid of side effects. In addition, others show beneficial effects of a targeted cytokine by means of coupling receptor specific ligands to the cytokine (Curnis and others 2000; Curnis and others 2005; Fournier, Aigner, Schirrmacher 2011; Jazayeri and Carroll 2008; Nissim and others 2004) often focussing on the treatment of tumours. These approaches may lead to a more optimal use of cytokines for therapeutic purposes.

In summary, we demonstrated potent pharmacological effectivity of a novel liver-specific form of the cytokine IL10. After conjugation with M6P, the novel cytokine efficiently accumulates in the liver and attenuates the fibrotic process *in vivo*. Further dose-response

studies are required to examine whether M6P-IL10 is more effective than the native product and exerts less adverse effects. Furthermore, targeting of potentially interesting cytokines to the liver is promising and it may lead to the generation of a therapeutic antifibrotic compound which has not been realized so far.

4. Acknowledgment

We thank Dr. M.N. Lub-de Hooge, Mr. Hans Pol and Mr. Hans ter Veen (Department of Nuclear Medicine, University Medical Center Groningen, The Netherlands) for their radiolabelling of proteins and help during the gamma-camera studies.

5. References

Adrian JE, Poelstra K, Scherphof GL, Molema G, Meijer DK, Reker-Smit C, Morselt HW, Kamps JA. 2006. Interaction of targeted liposomes with primary cultured hepatic stellate cells: Involvement of multiple receptor systems. J Hepatol 44(3):560-7.

Allen TM and Cullis PR. 2004. Drug delivery systems: Entering the mainstream. Science 303(5665):1818-22.

Andersen SR, Lambrecht LJ, Swan SK, Cutler DL, Radwanski E, Affrime MB, Garaud JJ. 1999. Disposition of recombinant human interleukin-10 in subjects with various degrees of renal function. J Clin Pharmacol 39(10):1015-20.

Bansal R, Prakash J, Post E, Beljaars L, Schuppan D, Poelstra K. 2011. Novel engineered targeted interferon-gamma blocks hepatic fibrogenesis in mice. Hepatology 54(2):586-96.

Bataller R and Brenner DA. 2005. Liver fibrosis. J Clin Invest 115(2):209-18.

Beljaars L, Meijer DK, Poelstra K. 2002. Targeting hepatic stellate cells for cell-specific treatment of liver fibrosis. Front Biosci 7:e214-22.

Beljaars L, Weert B, Geerts A, Meijer DK, Poelstra K. 2003. The preferential homing of a platelet derived growth factor receptor-recognizing macromolecule to fibroblast-like cells in fibrotic tissue. Biochem Pharmacol 66(7):1307-17.

Beljaars L, Molema G, Schuppan D, Geerts A, De Bleser PJ, Weert B, Meijer DK, Poelstra K. 2000. Successful targeting to rat hepatic stellate cells using albumin modified with cyclic peptides that recognize the collagen type VI receptor. J Biol Chem 275(17):12743-51.

Beljaars L, Molema G, Weert B, Bonnema H, Olinga P, Groothuis GM, Meijer DK, Poelstra K. 1999. Albumin modified with mannose 6-phosphate: A potential carrier for selective delivery of antifibrotic drugs to rat and human hepatic stellate cells. Hepatology 29(5):1486-93.

Chadban SJ, Tesch GH, Lan HY, Atkins RC, Nikolic-Paterson DJ. 1997. Effect of interleukin-10 treatment on crescentic glomerulonephritis in rats. Kidney Int 51(6):1809-17.

Colombel JF, Rutgeerts P, Malchow H, Jacyna M, Nielsen OH, Rask-Madsen J, Van Deventer S, Ferguson A, Desreumaux P, Forbes A, et al. 2001. Interleukin 10 (tenovil) in the prevention of postoperative recurrence of crohn's disease. Gut 49(1):42-6.

Curnis F, Gasparri A, Sacchi A, Cattaneo A, Magni F, Corti A. 2005. Targeted delivery of IFNgamma to tumor vessels uncouples antitumor from counterregulatory mechanisms. Cancer Res 65(7):2906-13.

Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A, Corti A. 2000. Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nat Biotechnol 18(11):1185-90.

Cuzzocrea S, Mazzon E, Dugo L, Serraino I, Britti D, De Maio M, Caputi AP. 2001. Absence of endogeneous interleukin-10 enhances the evolution of murine type-II collagen-induced arthritis. Eur Cytokine Netw 12(4):568-80.

de Bleser PJ, Jannes P, van Buul-Offers SC, Hoogerbrugge CM, van Schravendijk CF, Niki T, Rogiers V, van den Brande JL, Wisse E, Geerts A. 1995. Insulinlike growth factor-II/mannose 6-phosphate receptor is expressed on CCl4-exposed rat fat-storing cells and facilitates activation of latent transforming growth factor-beta in cocultures with sinusoidal endothelial cells. Hepatology 21(5):1429-37.

Demols A, Van Laethem JL, Quertinmont E, Degraef C, Delhaye M, Geerts A, Deviere J. 2002. Endogenous interleukin-10 modulates fibrosis and regeneration in experimental chronic pancreatitis. Am J Physiol Gastrointest Liver Physiol 282(6):G1105-12.

Di Marco R, Xiang M, Zaccone P, Leonardi C, Franco S, Meroni P, Nicoletti F. 1999. Concanavalin A-induced hepatitis in mice is prevented by interleukin (IL)-10 and exacerbated by endogenous IL-10 deficiency. Autoimmunity 31(2):75-83.

Fedorak RN, Gangl A, Elson CO, Rutgeerts P, Schreiber S, Wild G, Hanauer SB, Kilian A, Cohard M, LeBeaut A, et al. 2000. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active crohn's disease. the interleukin 10 inflammatory bowel disease cooperative study group. Gastroenterology 119(6):1473-82.

Fournier P, Aigner M, Schirrmacher V. 2011. Targeting of IL-2 and GM-CSF immunocytokines to a tumor vaccine leads to increased anti-tumor activity. Int J Oncol 38(6):1719-29.

Friedman SL. 2010. Evolving challenges in hepatic fibrosis. Nat Rev Gastroenterol Hepatol 7(8):425-36.

Gloor B, Todd KE, Lane JS, Rigberg DA, Reber HA. 1998. Mechanism of increased lung injury after acute pancreatitis in IL-10 knockout mice. J Surg Res 80(1):110-4.

Greupink R, Bakker HI, van Goor H, de Borst MH, Beljaars L, Poelstra K. 2006. Mannose-6-phosphate/insulin-like growth factor-II receptors may represent a target for the selective delivery of mycophenolic acid to fibrogenic cells. Pharm Res 23(8):1827-34.

Herfarth H and Scholmerich J. 2002. IL-10 therapy in crohn's disease: At the crossroads. treatment of crohn's disease with the anti-inflammatory cytokine interleukin 10. Gut 50(2):146-7.

Iredale JP. 2007. Models of liver fibrosis: Exploring the dynamic nature of inflammation and repair in a solid organ. J Clin Invest 117(3):539-48.

Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN. 1995. Histological grading and staging of chronic hepatitis. J Hepatol 22(6):696-9.

Jazayeri JA and Carroll GJ. 2008. Fc-based cytokines : Prospects for engineering superior therapeutics. BioDrugs 22(1):11-26.

Jevsevar S, Kunstelj M, Porekar VG. 2010. PEGylation of therapeutic proteins. Biotechnol J 5(1):113-28.

Khan AQ, Shen Y, Wu ZQ, Wynn TA, Snapper CM. 2002. Endogenous pro- and anti-inflammatory cytokines differentially regulate an in vivo humoral response to streptococcus pneumoniae. Infect Immun 70(2):749-61.

Kitching AR, Tipping PG, Timoshanko JR, Holdsworth SR. 2000. Endogenous interleukin-10 regulates Th1 responses that induce crescentic glomerulonephritis. Kidney Int 57(2):518-25.

Louis H, Le Moine O, Goldman M, Deviere J. 2003. Modulation of liver injury by interleukin-10. Acta Gastroenterol Belg 66(1):7-14.

Louis H, Van Laethem JL, Wu W, Quertinmont E, Degraef C, Van den Berg K, Demols A, Goldman M, Le Moine O, Geerts A, et al. 1998. Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice. Hepatology 28(6):1607-15.

Meijer DK, Beljaars L, Molema G, Poelstra K. 2001. Disease-induced drug targeting using novel peptide-ligand albumins. J Control Release 72(1-3):157-64.

Nelson DR, Lauwers GY, Lau JY, Davis GL. 2000. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: A pilot trial of interferon nonresponders. Gastroenterology 118(4):655-60.

Nissim A, Gofur Y, Vessillier S, Adams G, Chernajovsky Y. 2004. Methods for targeting biologicals to specific disease sites. Trends Mol Med 10(6):269-74.

Oberholzer A, Oberholzer C, Moldawer LL. 2002. Interleukin-10: A complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug. Crit Care Med 30(1 Supp):S58-63.

Pinzani M, Rombouts K, Colagrande S. 2005. Fibrosis in chronic liver diseases: Diagnosis and management. J Hepatol 42 Suppl(1):S22-36.

Poelstra K, Hardonk MJ, Koudstaal J, Bakker WW. 1990. Intraglomerular platelet aggregation and experimental glomerulonephritis. Kidney Int 37(6):1500-8.

Rachmawati H, Reker-Smit C, Lub-de Hooge MN, van Loenen-Weemaes A, Poelstra K, Beljaars L. 2007. Chemical modification of interleukin-10 with mannose 6-phosphate groups yields a liver-selective cytokine. Drug Metab Dispos 35(5): 814-21.

Rachmawati H, Beljaars L, Reker-Smit C, Van Loenen-Weemaes AM, Hagens WI, Meijer DK, Poelstra K. 2004. Pharmacokinetic and biodistribution profile of recombinant human interleukin-10 following intravenous administration in rats with extensive liver fibrosis. Pharm Res 21(11):2072-8.

Reineke U, Sabat R, Volk HD, Schneider-Mergener J. 1998. Mapping of the interleukin-10/interleukin-10 receptor combining site. Protein Sci 7(4):951-60.

Schooltink H and Rose-John S. 2002. Cytokines as therapeutic drugs. J Interferon Cytokine Res 22(5):505-16.

Schreiber S, Fedorak RN, Nielsen OH, Wild G, Williams CN, Nikolaus S, Jacyna M, Lashner BA, Gangl A, Rutgeerts P, et al. 2000. Safety and efficacy of recombinant human interleukin 10 in chronic active crohn's disease. crohn's disease IL-10 cooperative study group. Gastroenterology 119(6):1461-72.

Schuppan D and Popov Y. 2009. Rationale and targets for antifibrotic therapies. Gastroenterol Clin Biol 33(10-11):949-57.

Schuppan D and Afdhal NH. 2008. Liver cirrhosis. Lancet 371(9615):838-51.

Standiford TJ. 2000. Anti-inflammatory cytokines and cytokine antagonists. Curr Pharm Des 6(6):633-49.

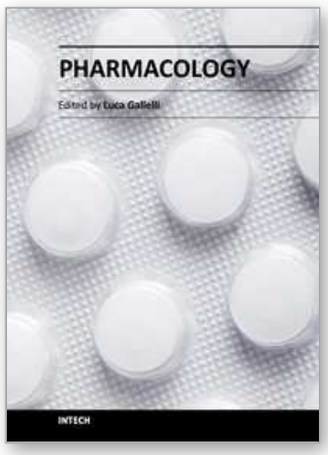
Thompson K, Maltby J, Fallowfield J, McAulay M, Millward-Sadler H, Sheron N. 1998. Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. Hepatology 28(6):1597-606.

van Beuge MM, Prakash J, Lacombe M, Post E, Reker-Smit C, Beljaars L, Poelstra K. 2011. Increased liver uptake and reduced hepatic stellate cell activation with a cell-specific conjugate of the rho-kinase inhibitor Y27632. Pharm Res 28(8):2045-54.

Vilcek J and Feldmann M. 2004. Historical review: Cytokines as therapeutics and targets of therapeutics. Trends Pharmacol Sci 25(4):201-9.

Wang SC, Ohata M, Schrum L, Rippe RA, Tsukamoto H. 1998. Expression of interleukin-10 by in vitro and in vivo activated hepatic stellate c

IntechOpen



Pharmacology
Edited by Dr. Luca Gallelli

ISBN 978-953-51-0222-9
Hard cover, 720 pages
Publisher InTech
Published online 14, March, 2012
Published in print edition March, 2012

The history of pharmacology travels together to history of scientific method and the latest frontiers of pharmacology open a new world in the search of drugs. New technologies and continuing progress in the field of pharmacology has also changed radically the way of designing a new drug. In fact, modern drug discovery is based on deep knowledge of the disease and of both cellular and molecular mechanisms involved in its development. The purpose of this book was to give a new idea from the beginning of the pharmacology, starting from pharmacodynamic and reaching the new field of pharmacogenetic and ethnopharmacology.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Heni Rachmawati, Adriana Mattos, Catharina Reker-Smit, Klaas Poelstra and Leonie Beljaars (2012). Modification of Interleukin-10 with Mannose-6-Phosphate Groups Yields a Liver-Specific Cytokine with Antifibrotic Activity in Rats, Pharmacology, Dr. Luca Gallelli (Ed.), ISBN: 978-953-51-0222-9, InTech, Available from: http://www.intechopen.com/books/pharmacology/modification-of-interleukin-10-with-mannose-6-phosphate-groups-yields-a-liver-specific-cytokine-with

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the Creative Commons Attribution 3.0 License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen